COLLATERAL CIRCULATION

HEART, BRAIN, KIDNEY, LIMBS

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edited by

Wolfgang Schaper
Jutta Schaper

SPRINGER SCIENCE+BUSINESS MEDIA, LLC

Library of Congress Cataloging-in-Publication Data

Collateral circulation: heart, brain, kidney, limbs / edited by
Wolfgang Schaper, Jutta Schaper.
p. cm.
Includes index
ISBN 978-1-4613-6351-4 ISBN 978-1-4615-3092-3 (eBook)
DOI 10.1007/978-1-4615-3092-3
1. Collateral circulation. I. Schaper, Wolfgang. II. Schaper, Jutta.
[DNLM: 1. Brain--blood supply. 2. Collateral Circulation--physiology. 3. Coronary Circulation. 4. Extremities--blood supply. 5. Kidney--blood supply. WG 103 C6968]
QP 110.C64C65 1993
599'. 0116--dc20
DNLM/DLC
for Library of Congress 92-49612
CIP

Printed on acid-free paper.

Cover Design by Wolfgang Schaper, Jr.

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Preface

This is the third book on Collateral Circulation that we have edited since 1971. We wrote the first text together with a small group of collaborators from our laboratory. For the second book we felt that the collateral circulation is only a part of the larger topic of the pathophysiology of myocardial perfusion, and we invited other investigators to contribute chapters. The new text is restricted again to the collateral circulation but other vascular provinces are also included. The new text should demonstrate the progress made in intervening years, not only in our special field but it should also show the broadened basis of our thinking: the new biology, molecular and otherwise, teaches us the interconnectedness of phenomena and provides a new context for the term "regulation".

There were premonitory symptoms for this progress in molecular biology even in 1971 when the first book took shape, i.e., we measured DNA and RNA and wondered how forces exerted on the cell membrane were transmitted to the nucleus. Growth factors and growth inhibitors were already in the air when we wrote the second book, but the amount of biological material necessary to isolate and purify a growth factor were discouraging. What we will show in several chapters of this book is how molecular biological methods proved to be useful tools in the study of complex adaptations in complex tissues. Now, as this manuscript goes to the printer, we feel a little disappointed that our investment in molecular biology has not brought more profit — there are still so many more questions to be studied and so few satisfying answers available that one has to fight the urge not to write but rather to continue doing new experiments. Indeed the Hemingway quotation, the motto of our first book,[1] is still true.

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[1] "there is a tendency when you really begin to learn something not to want to write about it but rather keep on learning always..."(Death in the afternoon)

In spite of all the unquestionable progress that had taken place in these years between books, there had also been obvious regress. With some methods the standards set 20 years ago could not be maintained. An example in point is the radioactive tracer microsphere method that we had perfected into a precision instrument but that we cannot use anymore because new legislation drove the expense of waste disposal into astronomical heights. Alternatives are now available but none has the ease, elegance, and precision of the original. Another example is the labelling index measured with radioactive thymidine in complex tissues: almost nobody can afford today the expenses in connection with cadaver disposal. Alternative methods have been tried like monoclonal antibody-based detection of bromdeoxyuridine (BrdU) and cyclin (PCNA), or in situ hybridization with histone H3, but none has the precision, the resolution, the reproducibility and the universality of the thymidine method: PCNA is difficult with endothelial cells, BrdU does not work in the presence of erythrocytes, histone H3 in situ hybridization does not have good spatial resolution.

The previous two books had been very successful, but they are long sold out, and we had decided some years ago to do an entirely new book. Since the field had diversified considerably, we found it only logical to invite all who had been interested in the topic for the last 5-10 years to contribute chapters. A gratifying number of colleagues agreed, and only a few could not accept the invitation due to being already overburdened with other pressing obligations. The absence of chapters by Dean Franklin and Doug Griggs is sadly felt.

Both previous books contained a very significant amount of original data from our lab that had not been published before in peer-reviewed journals. Some have criticized us for that (especially our good friend, the late Mel Marcus), not because we had cheated by slipping un-reviewed data between hard covers, but because one gets too little credit because of the nature of the source. However, we have not encountered what Mel had feared, on the contrary, we were gratified by the number of citations: the first book has become a citation classic and both books together have beeen cited over 700 times.

Some sort of peer review was employed for this book: each chapter was seen at least by two other chapter contributors to minimize overlap and to clarify regions of potential conflict. Bob Bache has reviewed at least five of the contributions and he has served as the North-American coordination center.

Although books do not enjoy the same prestige as original papers they have other advantages and compensations. Books offer more freedom for the expression of hypotheses, views, and opinions. Books can also be more truthful in the description of the development of an experiment. Often, when writing an article for a peer-reviewed

journal, one had the urge, reinforced by the editors, to make the story appear as if the data were the logical conclusion of serendipitous planning and foresight when in reality the result was the interesting but unexpected outcome of an incompletely thought-out experiment disproving the original working hypothesis.

Compared to previous books, this text was differently produced namely by the authors themselves on their personal computers. We enjoyed that in a way children enjoy new toys, and the fascination with computers can certainly lead to intellectual enrichment but it can also become a detractor.

The true purpose of a forword is to thank those who have helped: we would like to thank Gerhard Stämmler who was invaluable with helping us to get acquainted with our MacIntoshes, he disentangled, debugged and got us out of many ruts, scanned other authors figures properly into their texts, extracted mismatching references etc, etc. Our son Wolfgang Jr. having cast the eye of the professional art director on the manuscripts, arranged the text and illustrations in a manner which is aesthetically pleasing. Dr. Mohri, our postdoctoral fellow and guest scientist from Japan helped with the correspondence but could not stay until the completion of the adventure. Erika Gassig did all the many little but very necessary jobs that we others all forgot to do. Several postdoctoral fellows helped at various stages with the experiments but could not stay long enough to see the projects completed. We are especially indebted to Stefan Sack, Margarete Arras, Sven Rohmann and Rob Schott.

Finally we pray that our precious brainchild be well received (and live and be of use for a reasonable lifespan) in the cruel world of science.

Wolfgang and Jutta Schaper

List of Contributors

Luis Araujo, M.D.
Division of Nuclear Medicine, Department of Radiology, Hospital of the University of Pennsylvania,
Philadelphia, PA, U.S.A.

Robert J. Bache, M.D.
Cardiovascular Division, Department of Medicine, University of Minnesota,
420 Delaware Street S.E., Minneapolis, MN 55455, U.S.A.

Colin M. Bloor, M.D.
Department of Pathology 0612, University of California, San Diego, School of Medicine,
La Jolla, CA 92093-6012, U.S.A.

Susan M. Carroll, M.D.
Department of Pathology 0612, University of California, San Diego, School of Medicine,
La Jolla, CA 92093-6012, U.S.A.

William M. Chilian, Ph.D.
Microcirculation Research Institute, Department of Medical Physiology,
Texas A&M University Health Science Center,
College Station, TX 77843, U.S.A.

Michael V. Cohen, M.D.
Department of Physiology, University of South Alabama, College of Medicine,
Mobile, AL 36688, U.S.A.

David V. DeFily, Ph.D.
Microcirculation Research Institute, Department of Medical Physiology,
Texas A&M University Health Science Center,
College Station, TX 77843, U.S.A.

H. Fred Downey, Ph.D.
Department of Physiology, Texas College of Osteopathic Medicine,
3500 Camp Bowie Blvd., Fort Worth, TX 76107-2690, U.S.A.

James M. Downey, Ph.D.
Department of Physiology, University of South Alabama, College of Medicine,
Mobile, AL 36688, U.S.A.

Maria L. Finocchiaro, M.D.
Catholic University Sacro Cuore, Cardiological Institute,
Largo Agostino Gemelli, 8, I-00168 Rome Italy

James T. Gean, Ph.D.
Department of Physiology, Texas College of Osteopathic Medicine,
3500 Camp Bowie, Fort Worth, TX 76107, U.S.A.

David D. Harrison, M.D.
Cardiology Division, Department of Internal Medicine, Emory University
School of Medicine and Atlanta Veterans Administration Hospital,
Atlanta, GA 30322, U.S.A.

Norman K. Hollenberg, M.D., Ph.D.
Departments of Radiology and Medicine, Peter B. Brigham and Women's Hospital
and Harvard Medical School,
75 Francis Street, Boston, MA 02115, U.S.A.

Konstantin-A. Hossmann, M.D., Ph.D.
Max-Planck-Institute for Neurological Research, Department of Experimental Neurology,
Gleueler Strasse 50, D-5000 Cologne 41, F. R. Germany

John C. Longhurst, M.D., Ph.D.
University of California, Davis, Department of Internal Medicine, Division of Cardiovascular Medicine, TB 172
Davis, CA 95616, U.S.A.

Attilio Maseri, M.D., Ph.D.
Catholic University Sacro Cuore, Cardiological Institute,
Largo Agostino Gemelli, 8, I-00168 Rome Italy

Howard Mass, Ph.D.
Department of Physiology, Texas College of Osteopathic Medicine,
3500 Camp Bowie, Fort Worth, TX 76107, U.S.A.

M. Dan McKirnan, M.D.
Department of Pathology 0612, University of California, San Diego, School of Medicine,
La Jolla, CA 92093-6012, U.S.A.

Masahiro Mohri, M.D., Ph.D.
Max-Planck-Institute, Department of Experimental Cardiology,
Benekestrasse 2, D-6350 Bad Nauheim, F. R. Germany

Werner Risau, M.D., Ph.D.
Max-Planck-Institute for Psychiatry,
Am Klopferspitz 18a, D-8033 Martinsried, F. R. Germany

David M. Roth, M.D.
Department of Pathology 0612, University of California, San Diego, School of Medicine,
La Jolla, CA 92093-6012, U.S.A.

Jutta Schaper, M.D., Ph.D.
Max-Planck-Institute, Department of Experimental Cardiology,
Benekestrasse 2, D-6350 Bad Nauheim, F. R. Germany

Wolfgang Schaper, M.D., Ph.D.
Max-Planck-Institute, Department of Experimental Cardiology,
Benekestrasse 2, D-6350 Bad Nauheim, F. R. Germany

Konrad W. Scheel, Ph.D.
Department of Physiology, Texas College of Osteopathic Medicine,
3500 Camp Bowie, Fort Worth, TX 76107, U.S.A.

Werner Schoop, M.D., Ph.D.
Sonnenbergstrasse 6a, D-7800 Freiburg, F. R. Germany

Hari S. Sharma, Ph.D.
Max-Planck-Institute, Department of Experimental Cardiology,
Benekestrasse 2, D-6350 Bad Nauheim, F. R. Germany

J. David Symons, Ph.D.
University of California, Davis, Department of Internal Medicine, Division of Cardiovascular Medicine, TB 172
Davis, CA 95616, U.S.A.

Hitonobu Tomoike, M.D.
The First Department of Internal Medicine, Yamagata University School of Medicine,
2-2-2 Iida-Nishi, Yamagata 990-23, Japan

Ellis F. Unger, M.D.
National Institute of Health, National Heart, Lung, and Blood Institute,
Bethesda, MD 20892, U.S.A.

Dorothee Weihrauch, D.V.M.
Max-Planck-Institute, Department of Experimental Cardiology,
Benekestrasse 2, D-6350 Bad Nauheim, F. R. Germany

Francis C. White
Department of Pathology 0612, University of California, San Diego, School of Medicine,
La Jolla, CA 92093-6012, U.S.A.

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COLLATERAL ARTERIAL GROWTH AND REACTIVITY: LESSONS FROM THE LIMB AND RENAL BLOOD SUPPLY

Norman K. Hollenberg
Departments of Radiology and Medicine; Brigham and Women's Hospital and Harvard Medical School; Boston, Massachusetts, U.S.A

INTRODUCTION

The extraordinary prevalence of coronary atherosclerosis and its contribution to morbidity and to mortality in Western society has focused interest on the growth and function of the coronary collateral arterial tree (1, 2). On the other hand, occlusive arterial disease is not rare in other vascular beds and many — possibly more — important lessons have been learned from the examination of collateral growth and reactivity elsewhere. To what extent lessons learned in one region are applicable to other regions is incompletely delineated, and clearly will be an important element of research in the next decade.

Indeed, the discovery of collateral arterial growth was made in an unusual anatomic area. Over two hundred years ago, John Hunter became surgeon to the British royal family, and one privilege he thereby enjoyed was to hunt in the Royal Deer Park (3). He took advantage of that opportunity not to hunt, but rather to perform an experiment provoked by the extraordinary annual growth of the stag's antlers. To explore the contribution of the arterial supply to that growth he ligated the major nutrient artery on one side. As anticipated, it was cool and growth of the antler on that side was interrupted. The influence, however, was only transient: After a short time the antler became warm, and exuberant growth occurred. He quickly identified the fact that a collateral arterial supply had grown to bypass the gap created by his ligature and went on to demonstrate that the phenomenon occurred elsewhere by assessing events following femoral artery ligation in the dog (3). In the best tradition of clinical research, he exploited the observation to develop a new surgical procedure for limb aneurysm, by gradually ligating the artery above the aneurysm in the hope that collateral development would make amputation unnecessary. It did.

The recognition of a functional role for renal arterial collaterals also occurred early. During the 1930s, the issue of arterial collateral formation had become controversial (1). Goldblatt, et al (4) in their classic study on the effects of renal artery occlusion noted

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

that the hypertensive response tended to wane with time. During a surgical exploration to examine the renal blood supply in their dogs they recognized the extraordinary growth of arterial collaterals, and demonstrated that their interruption would restore the hypertension. That observation has been confirmed, and the contribution of arterial collaterals to restoring kidney function after renal artery occlusion is unambiguous (5).

In this essay, we will explore what has been learned about the growth and reactivity of collateral arteries to the limb and to the kidney. Limitations of space will preclude discussion of the anatomy of the collateral arterial supply in these two regions or their clinical implications.

Collateral arterial growth: the acute response

In a classic study, widely cited as providing evidence for fundamental differences in the collateral circulation in various regions, Eckstein et al (6) compared distal pressure and retrograde flow following occlusion of the femoral, carotid, and coronary arteries in the dog. They found striking differences in the three vascular beds. Distal pressure and flow fell sharply in all three regions, as anticipated, but in minutes to hours distal femoral pressure and flow had recovered to about 40% of baseline, whereas coronary pressure and flow showed little recovery. Responses in the carotid region were intermediate. The same differential was apparent at the end of one week, but over many weeks distal pressure and flow continued to rise and became similar in the three regional beds. The substantial capacity of the available femoral collateral arterial tree to replace flow and pressure delivery lost to complete femoral artery occlusion has been confirmed many times. In our studies, for example, muscle blood flow measured with microspheres remained at 38% of baseline one minute after femoral artery occlusion, and had risen to exceed 50% within an hour in the dog (7).

In the same study, the renal circulation following complete renal artery occlusion was shown to be much more similar to the coronary than the femoral arterial supply: Distal arterial pressure fell to near zero, and blood flow was reduced to about 5% of control. The delivery of 15 micron and 35 micron microspheres was an identical 4% of normal, but ionic rubidium was delivered at a significantly higher rate, of 6.1%. The results suggested that the available blood supply to the kidney was sharply limited, and that many of the vessels that kept the tissue alive were of capillary size.

Clearly, the substantial availability of an alternative pathway for delivery of arterial blood in the limb and carotid circulation represents arteries already available to

open or expand when the primary blood supply is blocked. There has been interest in whether collateral arterial development thereafter represents growth of preformed vessels or vessels that have been newly formed (8), but no definitive answer is available. As pointed out by Liebow, under some circumstances such as the arteries that traverse what was a serous cavity, there can be no doubt about new vessel formation. The same applies to collateral arteries that form to a transplanted kidney with renal artery stenosis. On the other hand, both situations are typically associated with substantial prior inflammation, which could have led to newly formed arteries, which were then available for collateral growth. For the purposes of further presentation, we will assume that all of the sustained growth of collateral arteries reflects the development of preformed vessels, although the alternative would not alter any argument.

Sustained collateral arterial growth: Perhaps the most detailed and painstaking study of chronic collateral arterial growth was performed by Conrad et al (9) on events following femoral artery occlusion in the dog. They made corrosion casts of the hind quarters arterial tree at four time intervals, from 14-to-78 days after superficial femoral artery ligation. Both the size and the number of identifiable, plastic-filled collaterals increased progressively, indeed linearly over the interval because of the substantial collateral growth to eleven weeks: The obvious candidates as the stimulus to vessel growth, pressure drop across the collateral arterial tree and flow velocity within it, were calculated from the dimensional measurements and were shown to fall precipitously over that time interval because of the substantial collateral growth. Although one can disagree with some of the assumptions employed in the calculation — for example, resistance to blood flow in each collateral was calculated according to the Poiseuille equation — the thrust of their conclusion cannot be denied: Collateral growth continued unabated when the apparent stimulus to growth was decreasing rapidly.

Longland (10), who performed a similar study on the limb blood supply in the rabbit, employed a useful terminology. The plexus of curly-cue arteries that span the gap created by arterial occlusion he called "midzone" vessels. The large cognate artery proximal to the midzone he called a "stem vessel". The arteries beyond the midzone that carry blood back to the distal arterial tree be called "re-entrant". Over sixteen weeks in the rabbit, he showed a linear, sustained increase in the size of stem arteries, to about 200% of baseline, and an accelerating growth of midzone arteries between the eighth and the twelfth week, to over 300% of baseline. Not only does growth continue

beyond what the obvious stimuli should allow, there is differential growth in various sections of the arterial tree and an acceleration defies explanation based on local biophysical forces.

Similar systematic studies on the renal arterial tree have not been performed, but there is a progressive growth of collateral arteries that is not complete at six weeks (11), and which may continue up to a year (5).

The above analyses, whether performed on arteriograms or vascular casts, assessed the vascular lumen. Although it has long been recognized that the vascular wall also undergoes growth, with increasing thickness that must reflect hypertrophy and hyperplasia (12), only in the past two decades have attempts been made to quantify the elements contributing to vascular growth and its time course. Schaper et al (1) reviewed recently the time course and elements of vascular remodeling during collateral growth to the heart. This group was also the first to employ tritiated thymidine and radioautography to explore DNA synthesis in growing coronary collateral vessels (13). The anticipated, unambiguous increase in labeling of all elements of the vascular wall were documented. The phenomenon was confirmed for the collateral blood supply to the kidney in dogs studied 18-to-39 days after renal artery stenosis (11).

The time course of increased endothelial cell labeling in the rat proved intriguing (14). One hundred percent of rats showed an increase in labeling within 24 hours of renal artery occlusion, and the increase was maintained for several days. The rate of arterial labeling thereafter fell gradually, so that about 50% of rats showed an increase at 4 weeks. The magnitude of the increase in labeling is extraordinary. Endothelium is normally a very low turnover system, no more than one: one thousand endothelial cells showing labeling after several hours of exposure to tritiated thymidine. At the peak response during collateral arterial growth, there was more than a 50-fold increase in the frequency with which endothelial cells were labeled.

Another characteristic of collateral arteries that has long been recognized has been their circuitous course (12). The cellular hyperplasia and hypertrophy that contributes to increasing wall thickness has, as a byproduct, the consequence of increasing the length of the artery. Since the two ends (stem and re-entrant arteries) are fixed, the consequence is a serpiginous pathway. We undertook (unpublished studies) to ascertain whether vascular remodeling includes the potential for reversal of this serpiginous character, by comparing collateral arteries to the limb which occurred as a consequence of superficial femoral artery ligation in rabbit pups immediately after weaning, at

4 weeks of age, and in rabbits which have achieved almost their complete growth by 16 weeks of age. At one year, the length of the limbs in the pups had increased several fold, which exceeded substantially the gap created by vascular occlusion, and yet the serpiginous course remained. Despite the continued longitudinal growth of the arteries, the forces that organize vascular structure did not correct the circuitous pathway.

Collateral arterial growth: transfer of information

Theories on the stimulus to collateral arterial growth have invoked obvious candidates such as pressure in the collateral arterial lumen and its gradient from proximal to distal, bulk volume flow, and blood flow velocity, related tangential wall force, hypoxia in the ischemic zone and the products of ischemic metabolism, and other poorly defined chemical factors. Evidence reviewed above, from morphology and time course (9, 10) made it unlikely that the biophysical factors played a sustained role in limb collateral arterial growth: growth continued unabated, or even accelerated, throughout the 16 weeks following vascular occlusion. During that interval, prior growth should have reduced the magnitude of the stimulus. In the case of limb collaterals, measurements of oxygen partial tissue pressure suggested that hypoxia was not the stimulus (15). In the rat, not only blood flow but also the metabolic machinery required to support muscle contraction had returned to control values within 7-to-14 days after femoral artery occlusion (16, 17).

Evidence favoring a humoral communication system mediating collateral arterial growth came from an unexpected source (13): Following coronary artery occlusion, increased endothelial thymidine labeling was found not only in the growing collateral arteries, but also in the coronary venous system where the responsible factors must have differed strikingly. We confirmed and extended that observation for the renal circulation where there was an increase not only in renal vein endothelial labeling, but also in the labeling of ureteric epithelial (11, 14, 18). In favor of a common mechanism was the striking correlation between the magnitude of the increase in renal collateral arterial and renal vein endothelial labeling (11). The time course of labeling in the three areas differed, being best sustained in the arterial tree (14). It is difficult to conceive that physical forces within the collateral arterial system could influence the venous system and the ureter through any means other than a chemical communication.

An approach based on endothelial cell incorporation of thymidine provided yet another approach to distinguishing between local biophysical factors in the arterial

lumen or information emanating from the ischemic zone (19). With a drop in distal pressure following arterial occlusion, blood flow is increased simultaneously throughout the length of the small preformed collateral arterial vessels. If biophysical factors related to blood flow were entirely responsible for endothelial cell hyperplasia, a simultaneous increase in endothelial cell labeling would be anticipated throughout the length of the preformed collateral channels. On the other hand, if the information emanated from the ischemic zone, centripetal spread of the endothelial cell hyperplasia in a retrograde direction from the ischemic zone would be expected. The periureteric vascular supply to the kidney following renal artery occlusion served as a convenient model: The anatomic arrangement makes it unlikely that surgery required to occlude the renal artery would damage directly the collateral supply via this route, and the great length of this collateral supply simplified the identification of a gradient of endothelial cell labeling. Endothelial cell labeling rose sharply within 24 hours of arterial occlusion, first evident in the area immediately adjacent to the renal hilum. Thereafter, a progressive, time-related centripetal gradient in endothelial cell thymidine labeling occurred, indicating that the factors responsible for endothelial cell hyperplasia are less related to blood flow in the lumen than to downstream, ischemic events. These phenomena are not limited to growing collateral arteries: An essentially identical centripetal spread of endothelial cell labeling along the spermatic artery was identified when tumor was implanted in the rat testis (20). Indeed, an identical pattern occurred in rats in which the testis was injected with a tumor-free preparation, when inflammation followed a local infection in the testis (20). These observations raise the interesting possibility that the centripetal gradient in labeling, and proliferation, was not specific for any of the inciting stimuli, but rather reflected a response intrinsic to the vessel wall — whenever an increase in demand for blood flow occurred in the tissues subserved by the vessel. The most straight-forward explanation would involve cell-to-cell communication, a phenomenon which has been identified in many systems (21). The structure of the gap junction in endothelial cells is appropriate to this form of mediation in blood vessel walls (22), and evidence has been presented to indicate that cell-to-cell transfer of molecules indeed occurs between endothelial cells in tissue culture (23).

Evidence on the specific chemical nature of such a factor is currently limited. Our interest in this problem was stimulated by the identification of the fact that extracts of kidneys subjected to renal artery stenosis promoted a vascular proliferative response in

the hamster cheek pouch, where only a chemical communication could have provided the link (24). The factor, whatever its nature, was found in the soluble elements of the tissue homogenates, and clearly differed in its tissue distribution from Folkman's tumor angiogenesis factor (25). Our attempt to isolate and characterize this factor was very much delayed by the observation that ureteric epithelium showed increased turnover, which led us to suspect that the factor appeared in an active form in urine: It does not (26). Very high levels were found in lymph draining ischemic kidneys (26). The factor, with a molecular weight exceeding twenty thousand, led to a marked increase in vascularity and endothelial cell labeling with thymidine in the hamster cheek pouch, employed for the assay. This assay system shows little sensitivity to epidermal growth factor, endothelial cell growth supplement, and fibroblast growth factor, but did show a brisk response to platelet-derived growth factor (27).

Schaper et al (1) have reviewed recently evidence for participation of various known growth factors in collateral arterial growth. In each case, the evidence is ambiguous, and leaves important questions unanswered. Is a single factor responsible for growth and proliferation of all of the vascular wall cellular elements? Are the same factors involved throughout the time course of collateral arterial growth? What leads to the coordination of the growth of the various elements of the artery wall? These questions, of course, are not unique to collateral arterial growth, but rather extend to all of modern biology.

Collateral arterial growth: relation to "tissue need"

Perhaps it is not surprising that collateral arterial growth, tissue need, and work capacity have been linked in every thesis on collateral formation. Support of tissue function, after all, is the primary purpose of an arterial supply. Indeed, John Hunter, pointed out above as the first to recognize collateral arterial growth, suggested over two hundred years ago that the force promoting collateral development is "tissue need": The specific term he used, "the stimulus of necessity" (3), was typically vitalist and thus required no further definition. This reasonable concept has often been repeated, need being translated into physiologic and biochemical events related to tissue ischemia.

Evidence reviewed above suggests that tissue hypoxia is reversed quickly after collateral dilatation and early development, and indeed the metabolic machinery required to support limb skeletal muscle work is repaired in several weeks. To assess these relationships directly, we recently studied rats after superficial femoral artery

ligation, employing arteriography to assess collateral arterial growth; direct blood flow measurement to assess basal and peak limb blood flow during acetylcholine-induced vasodilatation; pressure-flow relations; and contractile power of the gastrocnemius muscle during sciatic nerve stimulation at intervals over three months after artery ligation. Basal and peak limb blood flow and muscle contractile power, clearly reduced one week after ligation, were normal by three weeks. Major collateral arterial growth, assessed by objective methods, progressed between three weeks and three months as might have been anticipated from the earlier studies in rabbits (10) and dogs (9). Limb perfusion pressure — blood flow relations were still altered at three weeks, with blunting of the normal autoregulation, but it became more normal by three months after arterial ligation. These observations support earlier studies, which indicate that collateral arterial growth continues after blood flow adequate to maintain work performance has been restored, and must reflect a response to more subtle abnormalities — perhaps reflected in distal pressure delivery (28).

Examination of the relation between work performed by the kidney and renal arterial collateral growth is equally instructive. The major metabolic burden for the kidney does not involve glomerular filtration, which occurs passively, but rather the reabsorption of the enormous amount of valuable metabolic material that is filtered, but required by the body, and so is reabsorbed by the tubules. Failure of reabsorption of the filtered sodium, chloride, and bicarbonate would lead to depletion and rapid death. Thus, renal ischemia — which leads routinely to a reduction in glomerular filtration rate — is associated with a sharp reduction in renal work and oxygen requirements (29). When filtration ceases, as will occur when distal pressure falls below 50 mm Hg, the metabolic requirements fall to minimal levels. Once again, it is difficult to attribute collateral arterial growth to maintenance of renal "work". Indeed, their growth, as distal pressure and filtration are restored, leads to an increase in work.

Platelet-vessel wall interactions: Knowledge of collateral arterial reactivity is limited, in part because of the complexity of studying collateral arterial tone and responsiveness. Fortunately, there has been substantial recent advance in our understanding of the responses of collateral arteries to platelet products, fortunate in that vascular occlusion largely reflects the long-term effects of atherosclerosis, and atherosclerosis leads to platelet activation, aggregation, and the release reaction (30). Even minimal injury to the endothelium results in platelet aggregation at the site of injury (31). Any break in

the continuity of the vessel wall is met with an instant response from platelets, which contact the zone of injury, spread and clump" (30).

Variation in the sensitivity of smooth muscle preparation to the products of the platelet release reaction has been documented widely. Isolated vessels often demonstrate a striking contraction in response to aggregating platelets and their products (32, 33, 34, 35, 36, 37). Ketanserin, the serotonin S-2 receptor antagonist abolishes a substantial portion of the response of the isolated rat caudal artery to products released by aggregating platelets (33). Serotonin, thromboxane A_2, and thromboxane mimetics induce contraction of the canine and porcine coronary artery (32, 36, 38) the rat caudal artery (33, 39), and human digital arteries (40, 41). In all three systems, serotonin potentiated thromboxane-induced contractions, and a thromboxane mimetic amplified responses to serotonin, which raised the intriguing possibility that their mutual amplification plays a role in the vasospasm that may accompany the platelet release reaction (36, 39, 41). With tail bleeding time in rats as an in vivo model of the platelet: vessel wall interaction, the simultaneous administration of both classes of antagonist, to serotonin and to thromboxane, resulted in much more prolongation of bleeding time than when either agent was employed alone (42).

There are occasional, but quantitatively important regional differences, perhaps species related (43), in vascular responsiveness to serotonin and thromboxane A_2. For example, the canine pulmonary artery shows little or no response to thromboxane A_2, whereas serotonin induces a striking response (35). The coronary artery (36, 38) and the basilar artery of the dog, on the other hand are sensitive to both thromboxane A_2 and to serotonin (44, 45).

Pharmacological antagonists have provided an index of the relative contribution to the in vitro response of various mediators released by platelets. In the case of human digital arteries serotonin was responsible for about 50% of the response (40); in the case of the rat caudal artery, about 60% of the response was serotonin mediated (39); in canine pulmonary arteries, by contrast, serotonin accounted for virtually all of the response (35), reflecting the insensitivity of this vascular bed to thromboxane A_2. A constrictor response to aggregating platelets in each system assessed to date seems to have been accounted for by serotonin and thromboxane, but dilator responses may be accounted for by other platelet factors, such as adenine nucleotides (46).

Aggregating platelets and serotonin relax pre-contracted canine coronary arteries only if intact endothelial cells are present, but produced only contraction if these cells

had been removed (47, 48). The contractile response of coronary arteries to a thromboxane mimetic, on the other hand, was not endothelial dependent (48). The serotonin receptors responsible for the release of EDRF and for the contractile response of smooth muscle differ, since ketanserin does not interfere with release of the vasodilator factor from endothelium, but does block the smooth muscle response (48).

Collateral reactivity: A growing body of evidence indicates that collateral vessels and their responsiveness are complex: collateral arteries are not passive conduits, but rather a reactive system. Intrinsic vascular tone, reversible by vasodilators, is evident in the collateral blood supply to the limb of the dog (49, 50).

Acute occlusion of the terminal aorta in the cat resulted in substantially more ischemia of the spinal cord and limb when the occlusion involved thrombus, leading to speculation that thrombus might release vasoactive factors that further reduced blood flow, through an action on the collateral arterial supply (51). An alternative to release by the thrombus of vasoactive factors was thrombus extension beyond its initial size, or embolization, to occlude the potential collateral vessels mechanically. Schaub et al (52) showed that extension of the thrombus, or embolism, could not account for the more substantial impact of thrombus on hindlimb perfusion in the cat. They then evaluated serotonin as a determinant of blood flow following occlusion of the blood supply to the hindlimb (53). Blood flow fell strikingly in response to serotonin three days after aortic ligation. Both the serotonin antagonist, cinanserin, or depletion of platelet serotonin stores with reserpine, sustained collateral circulation to the limb. A reduction in platelet count induced by an antiserum directed against platelets, on the other hand, was not effective in restoring a limb circulation, despite a striking fall in platelet count. A remarkably small number of activated platelets, it appears are required to induce collateral arterial spasm.

Their observations were rapidly confirmed and extended. Serotonin induced striking ischemia in the rat limb from 5 days to 8 weeks after femoral artery ligation (54). Ketanserin, the 5HT-$_2$ receptor antagonist, prevented that response, and blunted the blood flow reduction and tissue damage induced by acute thrombotic obstruction of the aorta in the cat (55). An action of thromboxane A_2 released by platelets was thought unlikely, as ketanserin was effective, and ketanserin does not inhibit the production, the release or the actions of thromboxane A_2.

The application of quantitative arteriography answered a number of additional questions (56). In the normal dog, serotonin induced the anticipated, dose-related reduction in large artery caliber: At the same time, limb blood flow increased. The reduction in large artery caliber was prevented or reversed by ketanserin, but not the blood flow increase (57). The larger was the normal artery, the larger the absolute and relative reduction in arterial lumen induced by serotonin (57).

The small collateral arteries were strikingly more sensitive to serotonin (56), and that response was also reversed by ketanserin. The increase in sensitivity expressed itself as a 10-to-30-fold reduction in the threshold serotonin dose required to induce vasoconstriction in the profunda femoris and the medial and lateral circumflex femoral arteries, the major stem vessels giving rise to the collateral tree. The slope relating serotonin dose to the degree of vasoconstriction, moreover, became much steeper. Calf blood flow, assessed with radioxenon, fell with serotonin infusion in the collateral-dependent limb, and rose as anticipated in the normal limb. Responses of the collateral arterial supply to norepinephrine were not potentiated and prazosin did not influence the response to serotonin. Taken in all, these data indicated that growing collateral arterial vessels display a specific increase in sensitivity to serotonin via the $5HT\text{-}_2$ receptor, and that the potentiated response was sufficient to limit blood flow.

The isolated, perfused hind quarters of rats studied from 5 days to 2 months after vascular occlusion, showed a striking increase in sensitivity of the collateral bed to serotonin, but not for norepinephrine, a thromboxane A_2 mimetic, or angiotensin II (58). The increase in sensitivity was clearly as serotonin specific. The fact that the responses to the thromboxane mimetic were not potentiated suggests that the serotonin-thromboxane A_2 interaction in vivo, described below, may reflect serotonin-induced amplification of the response to thromboxane A_2, as described earlier in vitro (35, 39, 40).

The duration of this special sensitivity of collateral vessels is prolonged. Studies, performed 3 and 5 days after occlusion, suggested that the response occurs early (54, 55). The longest study reported suggested that collateral vessel supersensitivity in the limb continues for at least 8 weeks in the rat (54). Our unpublished data in the rabbit suggests that supersensitivity to serotonin of limb collateral vessels continues for at least 8 months after femoral artery occlusion.

Does serotonin released from platelets account for the entire collateral arterial response when thrombus complicates vascular occlusion? Helenski et al (59) suggested

that thromboxane A_2 might play a role, but that is controversial (55). When platelet activation was induced in vivo by endothelial injury above the origin of the limb collateral arteries in the rabbit, spasm of the collateral vessels occurred routinely (60). Ketanserin in doses too low to influence the response to norepinephrine (30 g/kg) partially reversed the spasm. Thromboxane synthetase inhibition or an antagonist also induced a partial reversal, somewhat less in degree than that induced by ketanserin. When the two classes of agent were combined, a striking reversal of spasm occurred, substantially greater than when either was employed alone.

The mechanism of the supersensitivity of collateral arterial arteries to serotonin is unclear. One possibility involves the vasodilator influence of the endothelium (47). Endothelial cells of rapidly growing collateral arteries show marked changes, including hyperplasia demonstrated by radioautography with tritiated thymidine (14, 18). Perhaps dividing endothelial cells, and their daughter cells for some time after division, lose their ability to release the relaxant factor.

There are no species-related exceptions: exquisite sensitivity of the limb collateral arterial tree to serotonin has been documented in the cat (55), the rat (54), the dog (56) and the rabbit (unpublished observations). Ketanserin dilated limb collaterals in over 50% of human patients with advanced atherosclerosis (61). The unpredictable, but occasionally striking improvement in symptoms of intermittent claudication and limb perfusion in the patient with peripheral vascular disease treated with ketanserin (62) may reflect these findings: Patients differ in the degree to which limb ischemia induce to an influence of activated platelets and release of vasoactive factors acting on the collateral-dependent limb.

The combination of ketanserin superimposed on aspirin produced a more predictable and striking collateral response on angiography (63) consistent with earlier studies in animals, reviewed above. The therapeutic implications are evident.

Collateral arterial growth: where do we stand?

In view of the fact that so much of the evidence presented above has been negative, it seems useful to summarize the current state of knowledge and attempt a synthesis. Certainly organs differ in the availability of a collateral arterial supply at the moment of abrupt artery occlusion — the heart and kidney showing a minimal available collateral arterial tree, and the carotid and femoral area showing a much more substantial available arterial mass. Tissue survival depends on the acute opening of the available

collateral arteries, and the role of the large differential pressure gradient along the coliaterals in their acute opening is impossible to deny. It is also clear that there must be a very large increase in flow velocity through these vessels, and thus in lateral pressure. This factor seems less important in inducing endothelial cell hyperplasia than does information emanating from the ischemic zone, although it contributes to acute vasodilatation. The early brisk hyperplastic process may well be related to the stimulus provided by tissue hypoxia and accumulation of metabolic products, although there is no evidence specifically supporting this thesis has been developed.

It is well, however, to identify the major mystery. In the case of the limb blood supply, and probably in the case of other vascular beds, although they have not been studied in such detail, collateral arterial growth continues unabated for many months, well beyond the probability that any of these measurable factors represent the primary stimulus. To quote an outstanding review by Liebow (8), in attempting to understand the complexity of this subject "... a sense of wonder and frustration still remains". Perhaps identifying the responsible factors for sustained collateral arterial growth will follow a clear documentation of the precise time course over long intervals. Despite the vast literature on this subject, that remains undefined.

Acknowledgements: Personal research described in this essay was supported by a grant from the NIH (5 P01 CA4167-06). Mrs Diana Capone assisted in the preparation of the manuscript.

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2

DEVELOPMENT OF THE VASCULAR SYSTEM OF ORGANS AND TISSUES

Werner Risau
Max-Planck-Institute for Psychiatry, Martinsried, F. R. Germany

INTRODUCTION

The vascular system is a complex network of vessels connecting tissues and organs in the body. Endothelial cells form the inner lining of all blood vessels. These cells have common properties, for example, a nonthrombogenic luminal surface, abluminal basement membrane and von-Willebrand-Factor production. On the other hand, endothelial cells of capillaries are functionally and morphologically heterogeneous which is important for tissue and organ function. On the basis of morphology they have been subdivided into three groups: continuous, fenestrated and discontinuous capillaries (1). It should be noted that a single organ (e.g. brain, kidney) can contain both fenestrated (choroid plexus, glomeruli) and continuous capillaries.

The development of the embryonic circulation and the subsequent morphogenesis of the vascular system in organs and tissues are the focus of this chapter. Research in this field has been mainly concerned with a descriptive analysis of developmental events and very little is known about the molecular mechanisms that govern these processes. Emphasis will be put on those systems in which progress towards the elucidation of mechanisms has been made or which promise to allow these insights in the near future.

ORIGIN OF VASCULAR CELLS AND THE DEVELOPMENT OF THE EMBRYONIC CIRCULATION

The precursor of blood vessels is the so called hemangioblast (2, 3) which differentiates from the mesoderm very early in embryonic development. In the yolk sac, the hemangioblast or blood island gives rise to hemopoietic as well as endothelial cells (Fig. 1). However, it is not clear whether both cell types have a common progenitor cell. Using in vitro systems of mesoderm induction in the frog Xenopus (4, 5) or, more recently, in isolated quail blastodiscs (6) fibroblast growth factors (FGFs) have been shown to induce blood islands (ventral mesoderm). These model systems will allow to address the questions about the origin, precursors and differentiation of endothelial cells. In the embryo proper (area pellucida in the chick or

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

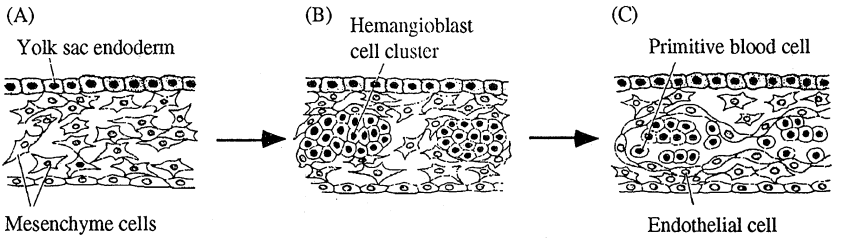

Fig. 1. Formation of blood islands in the mesoderm and vasculogenesis. (After Carlson (7)).

embryonic ectoderm in the mouse) angioblast differentiation is not associated with hemopoiesis. The reason for this difference is unknown.

Angioblasts give rise to blood vessels by fusion and proliferation and this process of the development of blood vessels from in situ differentiating endothelial cells is called vasculogenesis to distinguish it from angiogenesis which is defined as the development of blood vessels from preexisting vessels by sprouting (8, 9, 10, 11). The concept has emerged from the studies by Dieterlen and coworkers (10), that "in ontogeny, vascularization obeys different rules depending on which germ layer the mesoderm is associated with: in mesodermal/ ectodermal rudiments angiogenesis is the rule; in mesodermal/endodermal rudiments vasculogenesis occurs".

Angioblasts have been shown to be highly invasive cells (12). They can migrate as clusters of cells and assemble into blood vessels at different places of the embryo. The process of angiogenesis is also observed early in development. Intersomitic vessels form by sprouting from the dorsal aorta (13) and the sprouting activity of endothelial cells in the yolk sac capillary plexus is very high compared to the area pellucida endothelial cells (14). The molecular mechanisms involved in these processes are elusive.

The cardiovascular system is the first organ system that is laid down during embryonic development. The primitive vascular system of the embryonic body includes, among other vessels, the dorsal aortae, the aortic arches, the cardinal veins, the pulmonary arteries and veins and the endothelial lining of the heart. It forms in anticipation of it being necessary for the further development of a multicellular organism that can no longer be nourished by diffusion. Vasculogenesis without circulation leads to a so called primary plexus of capillaries which subsequently diversify into larger vessels, e.g. dorsal aorta by fusion of capillaries still before the onset of circulation. In addition, vascular channels which originally form in a paired fashion (e.g.

dorsal aortae, heart) later fuse in an anterior to posterior sequence to give rise to a single vessel. In the chick embryo, heart beat starts after two days of embryonic development (Hamburger-Hamilton HH (15) stage 10) and circulation commences shortly thereafter. This leads to dramatic changes in the morphogenesis of the vascular system because some vessels become arterioles and recruit smooth muscle cells, the direction of blood flow changes several times and some vessels regress while others newly form (2, 16, 17). Figure 2 illustrates the embryonic circulation of a 4 week human embryo.

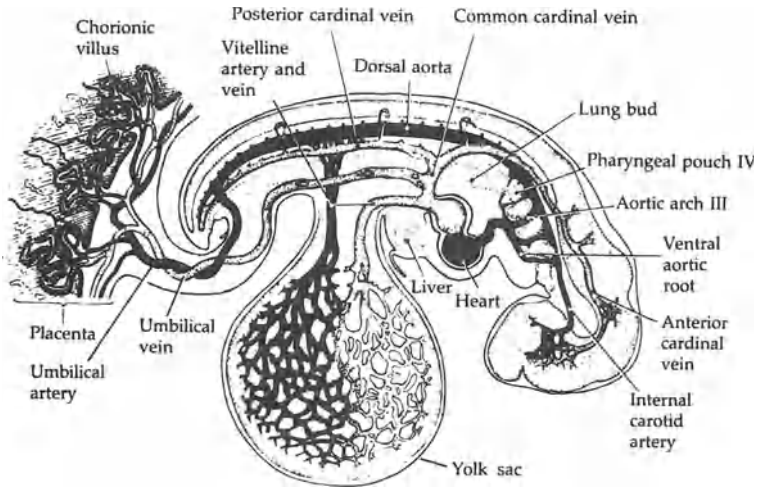

Fig. 2. Circulatory system of a 4-week human embryo. Only the vessels on the right side of the embryo are shown, arteries are in black. (From Carlson (7)).

During the entire embryonic life the vascular system undergoes very rapid and dynamic changes and shows a high degree of plasticity. The mechanisms that are involved in these processes are unkown. It is rather intruiging that the vascular pattern is quite reproducible in a given organ or tissue. This has led to the suggestion that genetic factors might be involved. The development of the primordial aortic arches is likely to be genetically determined. They are organized in the pattern of gills in fishes but in mammals successively regress or are incorporated into other vessels (Fig. 3) (2, 16, 17). Another piece of evidence for genetic control is the regression of the ductus arteriosus (18). However, for the most part the organ and tissue pattern determines the

vascular pattern. One illustrating example is the formation of extra digits in polydactylus mutant chick limb buds. The extra digits are perfectly vascularized like the normal digits (19). Bearing these considerations in mind we may now be interested in the development of the vascular system in specific organs and tissues.

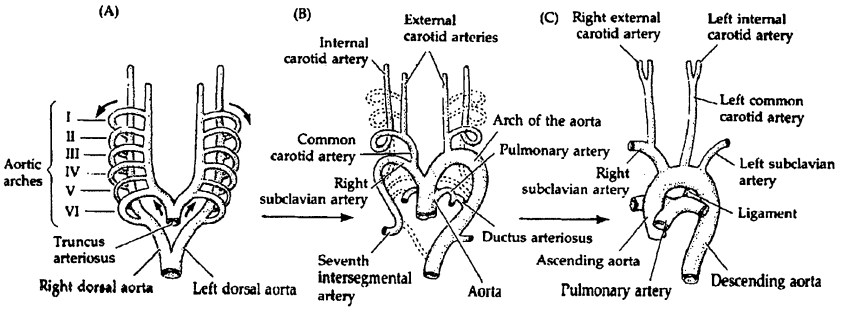

Fig. 3. Regression of aortic arches. The dotted lines indicate degenerating structures. (After Carlson (7)).

Vascularization of the heart

The heart is the first functional organ in the developing embryo. Morphogenesis of the heart is illustrated in figure 4. The presumptive heart cells of a 6-7 hours old avian embryo begin to migrate from lateral sides of the mesoderm and remain in close contact with the endoderm. Heart mesoderm (including endocardium and outer epimyocardium) is probably induced by a local interaction with the underlying endoderm as evidenced by studies in salamander (Ambystoma mexicanum) and cardiac mutant salamanders (20) and also in avian embryos. Recently, polypeptide growth factors of the transforming growth factor-beta (TGF-beta), platelet-derived growth factor (PDGF) andfibroblast growth factor (FGF) families have been reported to play a role in cardiac development (21).

The endocard which is a specialized endothelium that forms in situ has the extraordinary capacity to differentiate into cushion mesenchyme by a localized interaction with factors bound in the heart extracellular matrix (22, 23). This has never been observed for any other endothelium.

After fusion of the endocardial tubes (29 hours of chick development) the unfused openings of the posterior tubes become connected to the vitteline veins which carry nutrient-rich blood from the yolk sac to the heart. The outflow tract (truncus arteriosus)

endothelial cells have been shown to be derived from migrating angioblasts (24). Coronary arteries are present in the 4 to 5 day chick embryo.

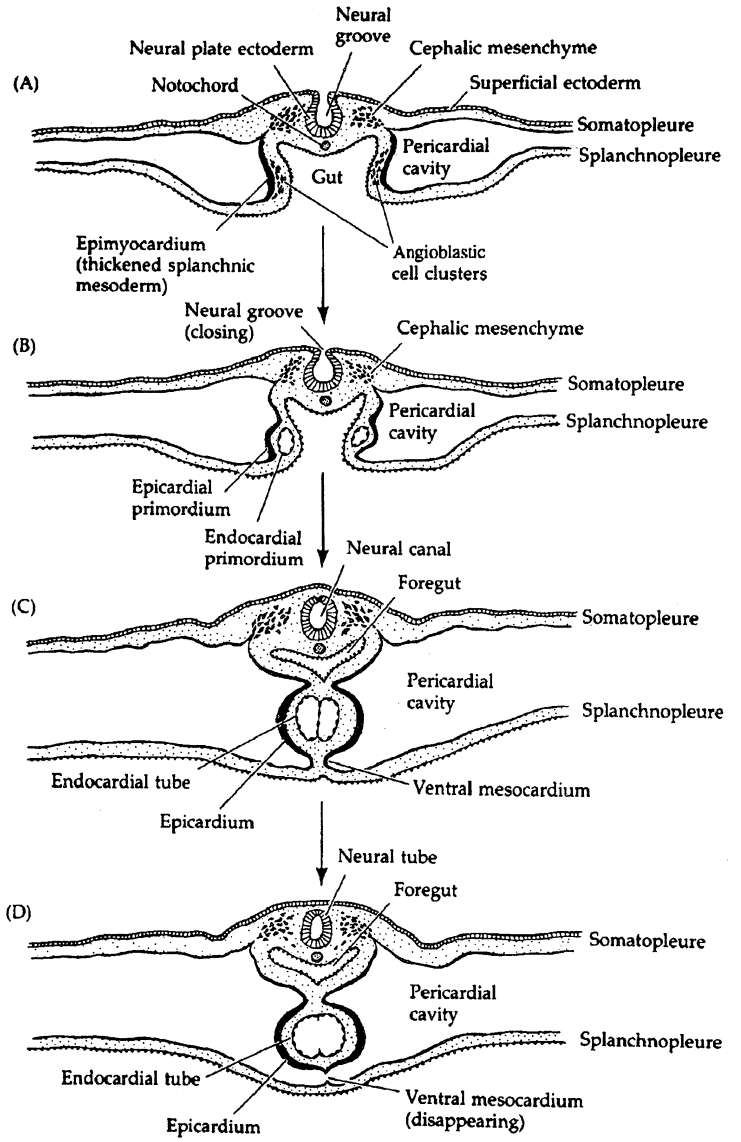

Fig. 4. Formation of the heart. Transverse sections through the heart-forming region of the chick embryo at (A) 25hrs; (B) 26hrs; (C) 28hrs; (D) 29hrs. (After Carlson (7)).

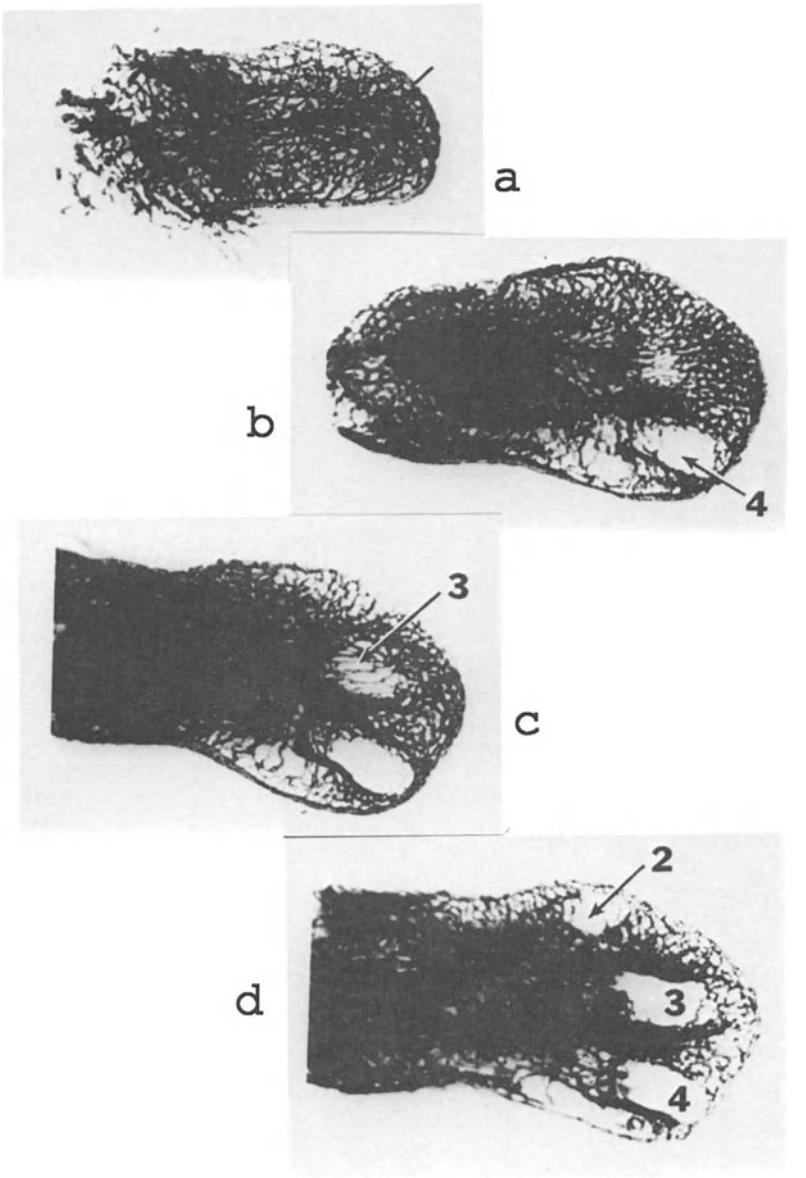

Fig. 5. Vascular network and localized vascular regression in chick limb buds from stages (a) 25 HH; (b) 27 HH; (c) 28 HH; (d) 30 HH. India ink injected, cleared limb buds viewed dorsally. The bar in (a) indicated the avascular region underneath the ectoderm. The numbers correspond to the forming digits where capillaries regress. (From Feinberg et a (30)).

Vascularization of the lung

The pulmonary circulation is established in a three day old chick embryo. The lung is an endodermal tissue that, in agreement with the germ layer concept (see above) is vascularized by vasculogenesis, i.e. endothelial cells differentiate from the local mesoderm. The formation of new lung and possibly other blood vessels has been suggested to occur by a new process of endothelial cell proliferation within existing capillary tubes (25). The specific characteristics of lung endothelium may be influenced by the local environment (26, 27).

Vascularization of the liver

The bilateral vitelline or omphalomesenteric veins enter the embryo at the level of the anterior intestinal portal and join ventral to the gut. Their common stem empties into the sinus venosus of the heart. At the liver anlagen the common trunk is called the ductus venosus. After two days of chick development the liver diverticula (buds) become associated with endothelial cells from the ductus venosus. After three days liver development proceeds rapidly and most of the blood from the vitelline vein is then diverted to the liver which has developed numerous branches from the ductus venosus. During this time the ductus venosus breaks down completely into the network of the hepatic sinusoidal circulation. Therefore, the origin of the vascular bed of the liver is probably one of the more complicated processes in the development of the vascular system. Even the potent method of generating chick-quail chimeric embryos has not been able to address this issue conclusively (28, 29). It appears that as an endodermal organ the liver buds may be able to induce the local differentiation of angioblasts. But, in addition, the concomitant engulfment of the vitteline vein which leads to a splitting up of this large vein to a capillary plexus gives rise to a mixing of endothelial cells originating in situ and from the vitteline vein. The anterior part of the ductus venosus persists and gives rise to the posterior vena cava. A posterior branch of the vitelline vein, the mesenteric vein, gives rise to the stem of the hepatic portal vein.

Vascularization of the limb bud

Before the limb bud grows out of the body wall a capillary plexus has formed at the lateral side of the dorsal aorta which gives rise to multiple vessels one of which will persist as the subclavian or femoral artery. Further angiogenesis occurs concomitantly with limb bud development and eventually forms a homogeneous network of arteries, capillaries and marginal veins (see Fig. 5). It is still debated whether all vessels originate

by angiogenesis or whether also some angioblasts contribute to the vessels in situ (10, 31).

There is a capillary-free space underneath the ectoderm of the limb bud (see Fig. 5a). Hyaluronic acid, an extracellular matrix glycosaminoglycan, may be responsible for this effect (32). The marginal veins beneath the ectoderm are constantly

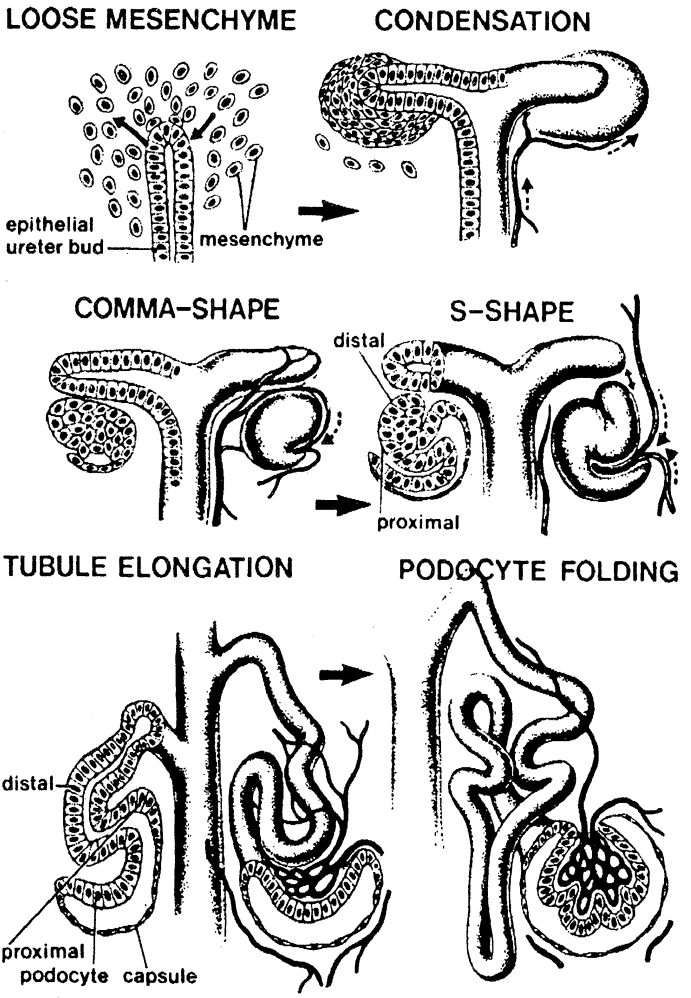

Fig. 6. Development of the kidney showing the branching of the ureter, the conversion of the mesenchyme, and the ingrowth of the vessels. The blood vessels are illustrated as dark tubes on the right side of the drawings. (From Ekblom (40)).

remodeled particularly at the growing tip of the limb bud near the apical ectodermal ridge (33).

Vascularization of cartilage and bone

Cartilage is avascular in embryonic and adult tissues. However, the areas in which cartilage forms are initially vascularized and this vascular network regresses prior to cartilage differentiation. This has been studied in great detail in the digital anlagen of the chick limb bud (Fig. 5) (30, 34, 35). Regression of these vessels is associated with endothelial cell death (30, 35).

When bone differentiates from cartilage capillaries invade (angiogenesis) the forming bone and subsequently give rise to the reticuloendothelial system of the bone marrow displaying discontinuous capillaries.

Vascularization of the kidney

The kidney is vascularized by an angiogenic process. Endothelial cells invade the nephrogenic mesoderm at about day 11 of mouse development. They seem to follow the ureter bud which is an extension of the Wollfian duct and induces the differentiation of kidney epithelial cells from the mesenchyme (36). Figure 6 illustrates that capillary buds invade the S-shaped body and form the glomerular tufts and peritubulary capillaries. Chick-quail transplantation and other experiments have demonstrated that kidney endothelium is of outside origin (37, 38). An angiogenic factor similar to acidic fibroblast growth factor has been purified from embryonic kidney and may be involved in the regulation of kidney differentiation (39).

The capillaries of the kidney are highly heterogeneous. While the glomerular capillaries display undiaphragmed fenestrae, the peritubular capillaries are diaphragmed. Others are continous vessels. The mechanisms that regulate endothelial cell heterogeneity and differentiation are unknown.

Vascularization of the eye

The vascularization of the eye is complicated because for proper vision the cornea and lens must not be vascularized. However, when the lens forms during development it is nourished by the hyaloid vascular system which regresses after birth (41). Some branches of this system, however, persist and may connect up to other structures, e.g. the iris. The retina is vascularized by two systems: one is the choroid beneath the pigment epithelium which is one of the few vascular systems that maintain the

embryonic pattern of a vascular plexus. The other is the internal vascularization of the retina via the optic nerve which is absent in some species, e.g. chick, or only partial, e.g. rabbit. The chick has an additional supply system of the pecten which is localized in the vitreous body. Vessels in the retina and iris, but not in the ciliary body or choroid have continuous capillaries that form a barrier for hydrophilic blood borne molecules (blood-retina barrier).

DEVELOPMENT OF THE BRAIN VASCULAR SYSTEM

A primary vascular plexus surrounds the neural tube very early in embryonic neural development (rat: day 10; chick: day 3). This vascular plexus is derived from migratory angioblasts - the precursors of embryonic blood vessels - that have invaded the head region (42). This is called the external vascularization of the brain which leads to the development of the meningeal arteries and veins (43). From the perineural vascular plexus capillary sprouts invade the neuroectoderm by the process of angiogenesis. The endothelial cells first locally degrade the perineural basement membrane. They then migrate deep into the neuroectoderm and branch in the subependymal layer. This pattern and the subsequent secondary and tertiary invasions are highly reproducible (42, 44). Whereas in other organs which are vascularized by an angiogenic process, e.g. kidney, capillary sprouts are found to form loops this has not been observed during the phase of radial invasion of capillaries into the brain.

Concluding remarks: The development of the vascular system is a fascinating process that involves all aspects of cell biology such as cell proliferation, death, differentiation and structural morphogenesis. It will be exciting to use the tools of modern molecular cell biology to elucidate the mechanisms that regulate these events.

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3

METHODS FOR ASSESSING CORONARY COLLATERAL GROWTH: INSIGHTS INTO MECHANISMS RESPONSIBLE FOR COLLATERAL DEVELOPMENT

David V. DeFily and William M. Chilian
Microcirculation Research Institute, Department of Medical Physiology, Texas A&M University Health Science Center, College Station, Texas, U.S.A.

INTRODUCTION

During occlusion of a coronary artery or in the presence of a severe stenosis, the collateral circulation of the heart is responsible for delivery of oxygen to the myocardium at risk (1, 2, 3, 4). Collateral growth has the potential to dramatically alter the outcome of coronary artery disease. Native collaterals, although present in the normal myocardium, are insufficient in number to provide adequate perfusion to the jeopardized myocardium. In the canine, pre-existing or native collaterals are microvascular, thin-walled conduits, which are composed of an endothelial lining, an internal elastic lamina, and one or two layers of smooth muscles. The numerical density of these vessels is roughly 10 per square centimeter with an average internal diameter of 40 μm (3). The total cross-sectional area of the native collateral circulation is only a fraction of that compared to the normal coronary vasculature, but importantly those vessels undergo remarkable transformation under the appropriate pathological conditions, usually in the setting of myocardial ischemia and/or a coronary stenosis. Well developed collaterals reduce ischemia during angioplasty (5) and also improve the functional recovery of the left ventricle following acute myocardial infarction (6, 7). Despite these important observations showing that coronary collaterals can significantly ameliorate the detrimental effects of coronary occlusion, the mechanisms that are responsible for the induction of collateral growth remain poorly understood. Other contributions in this text examine the latest evidence for the mechanisms involved in collateral growth and development. The purpose of this chapter is to highlight the three commonly used different models of collateral growth and development: ameroid constriction, repetitive occlusion, and microvascular embolization, and how these models can provide insight into the factors responsible for growth and development of coronary collaterals.

Wolfgang Schaper and Jutta Schaper, (eds.), Collateral Circulation.

Ameroid constriction

Thirty five years ago Litvak and colleagues (8) found that implantation of a hygroscopic material (ameroid) around a coronary artery gradually swelled, causing complete coronary occlusion over about eight weeks. Ameroid-induced coronary occlusion is attributed to three factors (3): 1) swelling of the material mechanically compresses the coronary vessel and decreases luminal area; 2) an inflammatory response that causes fibrosis and additional coronary constriction; and 3) formation of platelet plugs or thrombi in the stenosed segment. The ameroid occlusion model causes complete coronary occlusion in 95-100% of experimental animals and infarction is only present in 25-30% of the preparations (3). Associated with collateral growth and development is proliferation of vascular smooth muscle leading to an increased amount of smooth muscle in the vascular wall (3). Native collateral vessels are characterized by only 1-2 layers of vascular smooth muscle, but following full development up to 26 weeks there have been as many as 6 layers of vascular smooth muscle reported in coronary collateral vessels (3).

Collateral growth and development in the ameroid model is profound. Collateral resistance decreases approximately 10-fold and internal diameter of some collateral

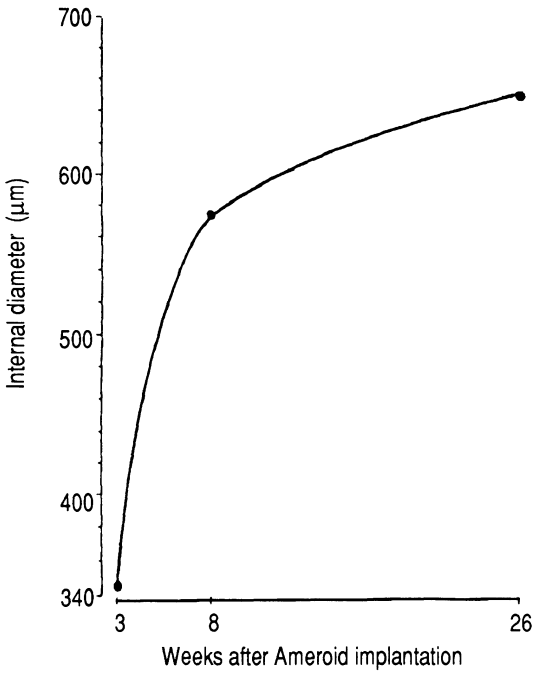

Fig. 1. The increase in internal diameter of coronary collateral vessels following progressive coronary occlusion with an ameroid constrictor. Within 8 weeks of implantation the diameter of coronary collaterals increases almost 15-fold. This is based on an assumed initial diameter of native collateral vessels of 40 μm.

vessels is reported to increase nearly 15-fold (3). Figure 1 illustrates lumenal expansion of collateral vessels during their course of development. A 15-fold increase in the diameter was calculated on the basis of an assumed, original diameter of 40 μm (3). The beneficial result of collateral growth is a tremendous increase in their capacity to provide blood flow, and, thus, oxygen and nutrient delivery to the jeopardized myocardium. Figure 2 illustrates the dramatic increase in collateral blood flow that occurs following the vascular growth due to ameroid implantation (9). Collateral flow was measured in isolated hearts using retrograde flow analysis and is reported as total flow and flow between the circumflex and left anterior descending, septal, and right coronary arteries. Note, collateral flow appears to have been maximized after one month of implantation and remains stable over more extended periods. Most investigators using this model report similar changes in collateral flow or vascular conductance (3, 10, 11).

Ameroid-induced coronary collateral growth may provide insight into the causal mechanisms. Some mechanisms that have been postulated to induce collateral growth because of ameroid-induced constriction have been alterations in tangential wall stress (3), a pressure difference between the collateral-dependent zone and adjacent vascular territories (12), reduced perfusion pressure (13), increased blood flow velocity across collateral vessels (14), and myocardial hypoxia or ischemia (10). Of the previously mentioned factors that are thought to be responsible for coronary collateral growth in the ameroid model, the first four relate to alterations in physical forces, whereas the last one relates to myocardial ischemia. The ameroid model facilitates our

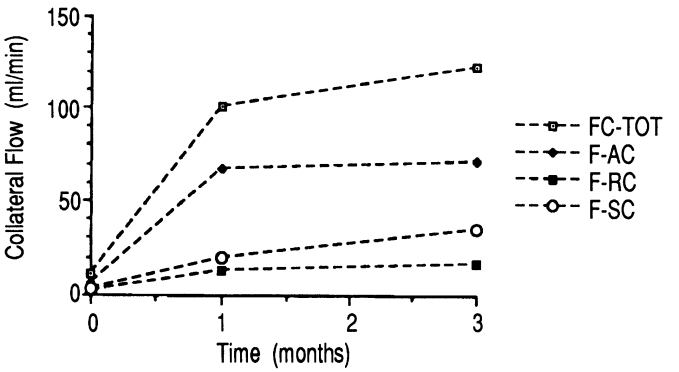

Fig. 2. Collateral development between the major coronary arteries after 1 and 3 months of ameroid implantation. Note the dramatic increase in the total collateral flow (FC-TOT). F-AC, collateral flow from the left anterior descending to circumflex coronary artery; R-RC, collateral flow from the right to circumflex coronary artery; F-SC, collateral flow from the septal to circumflex coronary artery.

understanding of the causal mechanisms involved in collateral growth and development because it can, in part, distinguish between physical forces and myocardial ischemia as mechanisms responsible for producing coronary collateral growth.

To distinguish whether physical forces or myocardial ischemia stimulates vascular growth, Scheel et al (10) implanted ameroid occluders on the circumflex artery of dogs and measured coronary collateral conductance between various vascular territories adjacent to the collateral-dependent zone (Fig. 2) and that between vascular beds in which the pressure difference was absent (Fig. 3). Figure 3 illustrates collateral growth between vascular territories not immediately adjacent to the circumflex artery, i.e., collateral growth between anterior descending and septal arteries and, thus, a lack of a pressure difference or physical forces as induction mechanisms. What is apparent in figure 3 is that there is an increase in collateral-dependent flow between vascular territories that are not immediately adjacent to the ameroid-occluded circumflex artery. This suggests that myocardial ischemia, rather than a pressure difference or other physical forces between adjacent vessels, is the stimulus for collateral growth. However, it is worth noting that the decrease in collateral resistance was greatest between the occluded vessel and adjacent vascular beds, as opposed to between non-involved vascular beds. Specifically, the maximal collateral flow to the ameroid-occluded region is 6-8 times that to non-involved vascular territories.

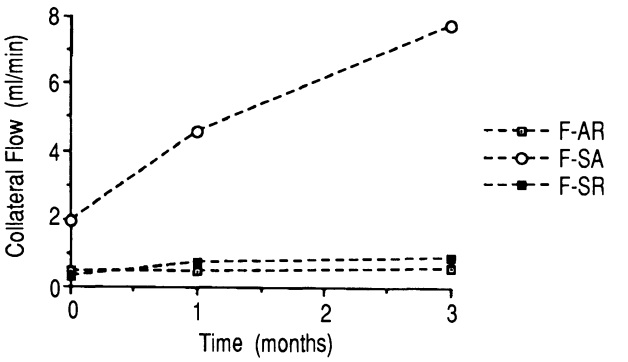

Fig. 3. Collateral flow between coronary arteries in dogs after 1 and 3 months of ameroid implantation. Collateral flows that were studied were between vessels not adjacent to the ameroid occluded circumflex artery. Note that collateral flow increased, especially that between the anterior descending and right coronary arteries. F-AR, collateral flow between the left anterior descending and right coronary artery; F-SA, collateral flow between the septal and left anterior descending; F-SR, collateral flow between the septal and right coronary arteries.

Repetitive occlusion model

Repeated episodes of coronary occlusion of short duration lead to the development of coronary collaterals in the dog and the pony (15, 16, 17). The repetitive occlusions can be performed at hourly intervals for approximately 2 minutes 8-10 times per day (15). After about 40-50 occlusions there is demonstrable evidence for collateral growth as shown by functional recovery of the ischemic segment during the occlusion as well as evidence for collateral-dependent perfusion. After approximately 90 occlusions the collaterals are fully developed and segmental dysfunction does not occur during the circumflex occlusion (Fig. 4). Similar models employing 2 minute occlusions at half-hour intervals have been shown to restore regional segment function, i.e., functional development of collaterals, in dogs within 2-9 days when performed continuously for 24 hours/day (17) or after 420 occlusions in ponies occluded for 8 hours/day (16).

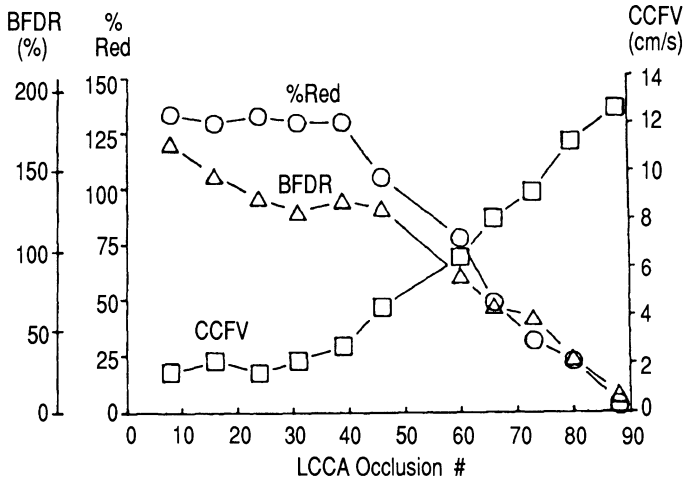

Fig. 4. Direct and indirect indices of collateral perfusion in a dog during development of coronary collaterals using the repetitive occlusion model. Measurements were taken during the last 2-minute occlusion of the day for 12 days. BFDR , blood flow debt repayment following release of occlusion; % Red, percent reduction in collateral dependent segment systolic shortening compared with preocclusion levels; CCFV, collateral flow between the left anterior descending and left circumflex coronary artery.

The mechanisms underlying collateral development in the repetitive occlusion model are conventionally thought to be attributed to myocardial ischemia (16, 17, 18). This is supported by the observation that 15 second occlusions at 4 minute intervals is an insufficient stimulus to produce collateral vessels (19), in contrast to the 2 minute occlusions described above. Although it certainly seems reasonable that the occluded

segment is ischemic and contributes to vascular growth, one cannot unequivocally eliminate the possibility that physical forces contribute, in part, to coronary collateral growth in this model. Within this context, upon release of the coronary occlusion, the previously ischemic segment would certainly be dilated, which would greatly alter the distribution of microvascular resistances and, thus, alter the distribution of microvascular pressures. We have found previously that vasodilation decreases pressures in arterioles, presumably those vessels in which the native collateral arteriole-arterial anastomoses occur (20). If arteriolar pressure is reduced in the ischemic area, but not in the normally perfused area, there would be a pressure gradient driving flow across the native collateral vessel. In the aggregate, one can easily invoke physical forces as a mechanism to facilitate the growth of the coronary collateral vessels in the repetitive occlusion model. Even the lack of collateral growth, from the experiments utilizing 15 second occlusions (19) cannot unequivocally eliminate the possibility of physical forces as a factor involved in collateral growth. Within this context, the 15 second occlusion would produce a less extensive (magnitude and duration) period of hyperemia and would, thus, lessen the pressure differential between the normal and hyperemic segment.

One aspect of the repetitive occlusion model that deserves emphasis regards its applicability towards understanding the development and regression of coronary collateral vessels. Specifically, an investigator has the ability to follow the development of coronary collaterals in conscious animals virtually every one-half hour by indirectly examining collateral flow or by measuring indices of segment function in the occluded segment. Collateral flow can be estimated in conscious animals by measuring the step decrease in left anterior descending flow following the release of the circumflex occluder (21). This analysis assumes that collateral flow to the circumflex vascular territory is supplied exclusively via collaterals from the left anterior descending artery, which is not completely correct and, thus, underestimates collateral flow, but the method does provide an important means of measuring changes in collateral conductance that occur during the course of the repetitive occlusions. In this model, blood flow debt repayment during reactive hyperemia has also been shown to correlate with the regional segmental shortening in the collateral dependent region, i.e. collateral function (17, 18). Studies of the regression of coronary collaterals can also be measured in this preparation, simply by stopping the repetitive occlusions (21, 22). The investigator then can periodically occlude the artery and measure either collateral flow directly as described above, reactive hyperemia, or functional indices (segment shortening) within the occluded region. Increases in reactive hyperemia, or decrements

in either collateral flow or segment function are interpreted as regression of coronary collaterals.

Partial embolization

We developed the partial embolization model a few years ago as an attempt to distinguish whether tissue ischemia or physical forces are the causal mechanism

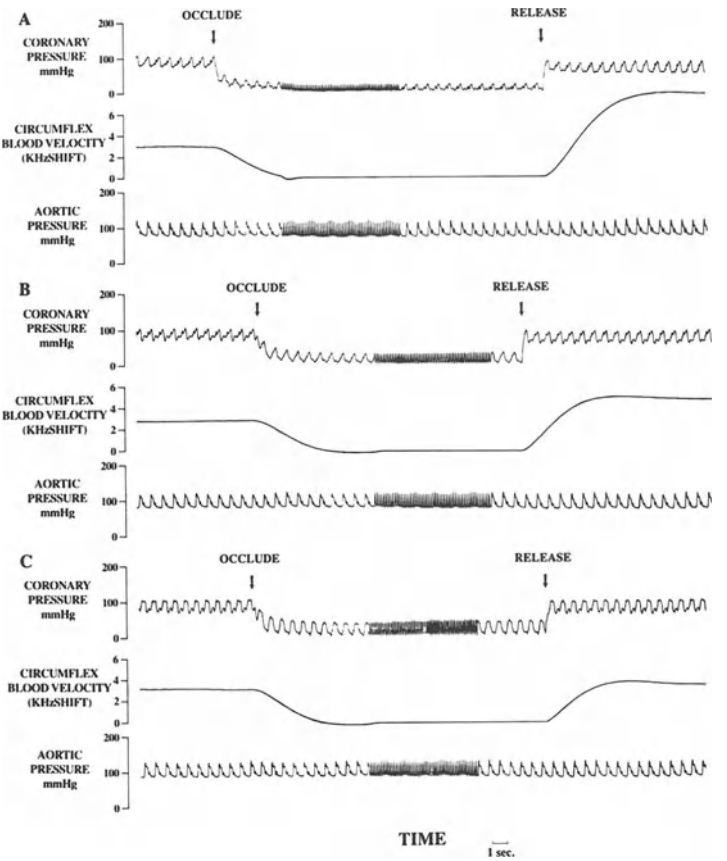

Fig. 5. Coronary reactive hyperemic responses after embolization. A: control responses, in which blood flow increased nearly 3x after 20s occlusion. B: injection of ≈1 x 10^6 microspheres. Note compromised vasodilatory reserve with blood flow increasing about 2x following occlusion. C: injection of ≈1.7 x 10^6 microspheres, in which vasodilatory reserve is nearly abolished, i.e. blood flow increasing only 20% after occlusion. Note that resting blood flow velocity was not changed from control by injection of microspheres, nor was pressure in the circumflex artery affected during perfusion (B and C). After coronary occlusion, peripheral coronary pressure in the circumflex artery did not rise after partial embolization of the distal vasculature.

underlying collateral growth (23). To accomplish this goal, we developed a model of microvascular occlusions produced by partial embolization of the coronary microcirculation with 25 μm microspheres. This procedure allowed us to determine whether myocardial ischemia, without changes in pressure gradients between major coronary arteries, can induce coronary collateral formation. Our method was to inject 25 μm polystyrene microspheres into the circumflex circulation in sufficient numbers to partially embolize the circumflex bed, without altering resting blood flow, but greatly attenuating coronary blood flow reserve (Fig. 5). This figure illustrates coronary reactive hyperemic responses under control conditions (Panel A) and following injections of microspheres (Panels B and C). Injection of approximately 1 million microspheres (Panel B) reduced coronary blood flow vasodilator reserve, i.e., attenuated the reactive hyperemic response following coronary occlusion. Note, that resting blood flow velocity was not changed from control. Following injection of approximately 1.7 million microspheres, coronary reserve was greatly attenuated, but resting flow velocity was not altered. Thus this model produces focal areas of myocardial ischemia produced by the injections of microspheres, but does not alter pressure gradients between coronary vessels, because the baseline resistances are not changed by the experimental interventions.

We were also able to discern the location of the polystyrene microspheres, which were typically adjacent to microvessels, or were found within individual microvessels.

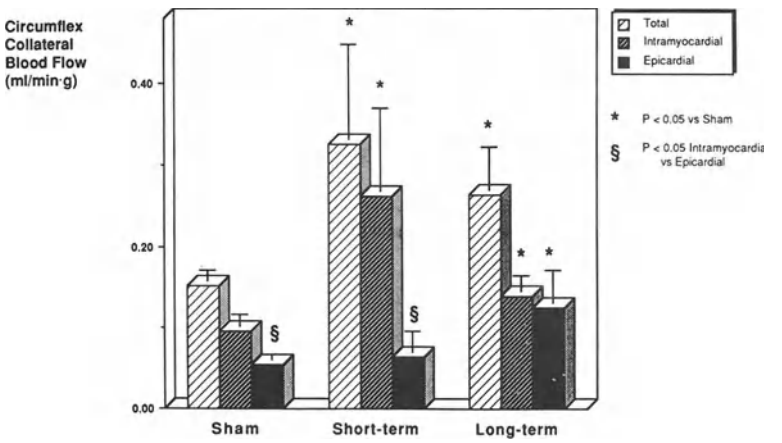

Fig. 6. Collateral blood flow from the right, left anterior descending and septal arteries to the circumflex artery in sham, short-term, and long-term animals. Note that total collateral blood flow was increased in the short-term and long-term embolization groups. Also, in the long-term group, the relationship of intramyocardial to epicardial collateral flow was different than in either the sham or short-term group.

This was possible, because in our preparations following the experimental procedures the heart was perfused with Microfil and the microspheres contained a fluorescent label (fluorescein). Using fluorescence microscopic techniques, we could discern the location of microspheres in the microvasculature and the tissue slices. We also observed small areas of tissue necrosis in areas of the myocardium in which the vasculature was not filled with the Microfil. Because microspheres were observed frequently outside of the lumen of the vessel, we presume that either macrophages or endothelial cells engulfed the spheres and extruded them from the vessel. It is also possible that vessels that were initially embolized by the microspheres necrosed, and there was neoformation of vessels in regions adjacent to these embolized areas.

Collateral flow and vascular resistance were measured 1-3 week (short-term) or 6-8 week (long-term) periods following the embolization procedures. Figure 6 illustrates the collateral-dependent blood flow to the circumflex region which was embolized by the microspheres. This figure illustrates the total collateral of blood flow, in addition to the intramyocardial and epicardial portions. Collateral blood flow was measured using the retrograde perfusion technique in isolated, maximally-dilated hearts (24) and contributions of intramyocardial versus epicardial collateral vessels were ascertained by measuring collateral blood flow before and after cauterization of visible epicardial collateral connections. These data show that intramyocardial collaterals form very quickly (following short-term embolization procedures), whereas the epicardial collaterals form more slowly and are significantly increased from the sham-control in only the long-term group. Also of interest to concepts related to vascular growth in this model were data showing the vascular resistance to antegrade blood flow in maximally-dilated arteries of the different experimental groups (Tab. 1). These data show that even following embolization of the circumflex artery, the minimal resistance was not significantly changed from that of the sham-control group, and comparable to the adjacent left anterior descending artery. These data indicate that in addition to the

Table 1 Vascular resistance to antegrade blood flow in the left anterior descending and circumflex coronary arteries.

Vessel	Sham	Short-Term	Long-Term
LAD	0.26±0.03	0.20±0.03	0.24±0.02
Circumflex	0.18±0.02	0.17±0.01	0.21±0.02

Values are means ± SE in $mmHg \bullet min \bullet ml^{-1}$. LAD, left anterior descending.

embolization procedure inducing collateral growth and development, there may have been angiogenesis of non-collateral vessels in the embolized zone.

In our opinion, the experimental results of the embolization model show that collateral growth can be induced exclusively by myocardial ischemia. We state this with conviction, because a unique feature of this model, in contrast to the ameroid or repetitive occlusion models, is that distal ischemia is produced without altering pressure gradients between major coronary arteries. Thus, the causal factor, be it physical forces or myocardial ischemia, for collateral growth and development in the heart can be directly assessed. One could argue that development of some of the microvascular vessels may have been related to alterations in physical forces in the embolization model. For instance, embolization of a proportion of the 25 μm arterioles would have caused vasodilation of non-occluded parallel channels, in order to maintain baseline coronary blood flow and oxygen delivery. Blood flow and shear stress in these dilated channels would have increased and may have provoked the angiogenic phenomenon that we observed. We emphasize that pressures in the epicardial arteries were not altered by this embolization procedure and the collateral growth in epicardial vessels must have been related to the distal myocardial ischemia, rather than any alterations in a pressure gradient between two adjacent vascular territories. This conclusion is supported by one of the more compelling observations obtained with the ameroid technique; namely, that collateral growth occurred between arteries in which there is no pressure gradient. These observations strongly suggest that ischemia produces a chemical signal that provokes angiogenesis.

The partial embolization model appears to have some advantages and disadvantages for studying coronary collateral growth and development. A disadvantage of this model was that collateral conductance increased to only about two-fold, whereas that occurring in the repetitive occlusion or ameroid models is reported to increase approximately 10- to 15-fold. The reasons for the limited collateral growth in the partial embolization model are not readily clear, but may be related to the extravasation of the microspheres; thus, removing the error signal (ischemia) for collateral growth. An important advantage of this particular model relates to the ability to discern temporal sequences in the development of blood vessels in the myocardium. Specifically, one knows exactly the time at which the intervention was applied to provoke the growth of collateral blood vessels. These are disadvantages of the repetitive occlusion and ameroid models, in that both models appear to suffer from inconsistencies related to the rate of collateral development. Some of these inconsistencies may be caused by biological variation, but also could be related to the rate at which the ameroid

closes causing myocardial ischemia or the myocardial oxygen consumption of the animal in which the repetitive occlusions are performed. Collectively, partial embolization of the coronary microcirculation provides another valuable way to examine mechanisms underlying coronary collateral growth and development.

CONCLUSIONS

Native collaterals are present in the heart, but may not be utilized to provide perfusion under normal conditions. These vessels, however, can open acutely under appropriate experimental conditions, but more importantly, have the ability to dramatically enlarge as to provide enhanced perfusion to jeopardized myocardium. Basic mechanisms that have been thought to be responsible for collateral growth and development have been either alterations in physical forces such as flow velocity across collateral vessels, or myocardial ischemia. Three popular models that have been used to examine mechanisms of collateral growth and development have been models employing ameroid occlusion, repetitive occlusion, or partial embolization of the microcirculation. Each of these models have their inherent strengths and weaknesses, and it is the experimental question that would dictate the particular use. For instance, if one would be interested in examining reactivity of very large collaterals, or perhaps following alterations in reactivity that occur with time, the ameroid model is known to produce changes in diameter of vessels as much as 15-fold. Alternatively, if an investigator is interested in determining molecular and biochemical mechanisms involved in the development of coronary collaterals and needs to know exactly the time of the inducing stimulus, then the partial embolization model would appear to be most satisfactory. On the other hand, if an investigator would like to follow the course of functional collateral development in conscious animals, and perhaps examine regression of coronary collaterals, the repetitive occlusion model is most satisfactory, because not only collateral growth and development be followed daily in conscious animals, but also the inciting stimulus (repetitive occlusions) could be discontinued and mechanisms of regression could then be studied. The use and employment of a particular model is best dictated by the question being asked. Hopefully, questions can be unequivocally answered regarding molecular mechanisms triggering collateral growth, factors that are responsible for halting the growth process, and mechanisms of collateral regression by the use of these models of collateral development.

Acknowledgements: The authors' studies in this area were supported by the following grants from the U.S. Public Health Service: HL32788, HL17669, HL08345.

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4

CORONARY COLLATERAL DEVELOPMENT: CONCEPTS AND HYPOTHESES

Wolfgang Schaper
Max-Planck-Institute, Department of Experimental Cardiology,
Bad Nauheim, F. R. Germany

"It often matters less that a hypothesis is right or wrong than that it is fruitful".
Judah Folkman

Growth as a concept

From the 1930s to the early 1960s it was believed that coronary collaterals were present in only about 4% of human hearts and that they open up passively under the influence of pressure gradients, i.e., in the presence of stenoses or occlusions. There were hints but no evidence that collaterals might develop: Eckstein (1) had shown that peripheral arterial(stump) pressure in some vascular provinces needed some time to rise after acute occlusion. Fulton (2) was the first to show that all normal human hearts had small collaterals and that hearts with coronary heart disease usually had much larger collaterals and that passive enlargement was unlikely merely on the basis of the difference of tissue mass. Fulton had re-discovered an observation by Spalteholz (3) that got lost because the "new" X-ray technique was unable in the 1930s to resolve small but visible arterioles.

Growth as a mechanism of coronary collateral development was introduced by us in 1967 and this was subsequently confirmed for the limbs and for the kidney (see chapter by Dr. Hollenberg).

There is still no conclusive evidence of collateral growth in the brain circulation (see chapter by Dr. Hossmann).

We showed first on the basis of morphometric evidence and later by DNA-labelling that cardiac collaterals grow by division of endothelial cells followed by mitosis of smooth muscle cells and fibroblasts, that the rate of growth was considerable (equalling or even surpassing that of tumors), that the duration of the DNA synthesis phase of the cell cycle (S-phase) is 22 hours for endothelial cells, and that the size of an epicardial canine collateral can reach 50times its original volume (4).

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

This was met with some incredulity at that time (1971), especially since leading textbooks of histology had stated that endothelium and smooth muscle consist of terminally differentiated cells incapable of division. Since the opposition came also from a very respected coronary physiologist, we repeated our experiments a few years later and we showed in another report (5) that cell division was capable to keep pace with a rate of closure of 3 days from the onset of stenosis (externally controlled) to complete closure without infarction.

It was evident for us at that time that epicardial collaterals in the canine model were only partially representative for the situation in the human patient. We therefore performed a DNA-labelling study also in the pig heart that, like the human heart, has a preference for small endomural collaterals. We showed that the enlargement of pig collaterals following major coronary artery stenosis and occlusion is also caused by DNA-synthesis and mitosis of endothelial cells (6). A paucity of smooth muscle in pig collaterals was already noted then as was the frequent occurrence of micro- and macro-infarctions in the porcine heart ameroid model.

Flow as a concept of growth

The earliest concept that explained the growth of collaterals compensating for the consequences of an atherosclerotic occlusion was that of Thoma who stated in 1893 that the velocity of blood flow in an artery determines its size.

Thoma was an embryologist and he formulated his "Law of Histiomechanics" to explain embryonic vasculogenesis (7). Thoma's observation clearly applies to coronary collaterals as the Bland-White-Garland syndrome demonstrates: the left (or the right) coronary artery may originate from the pulmonary artery as a congenital defect which can be survived due to the development of a collateral circulation between a normal and the aberrant coronary artery. After some time the collaterals that now interconnect the arterial- with the low pressure system create a left-to-right-shunt with extremely high blood flow velocities. This leads to enormously enlarged collaterals.

An arterio-venous fistula between the right (or left) coronary artery and the right (or left) atrium leads also to shunts between the high-and low pressure system with very large collaterals. Blood flow through well developed collaterals in these congenital defects is not controlled by the recipient bed but mainly by the pressure gradient between the aorta and the pulmonary artery or the right (or left) atrium. The blood flow velocities and hence the shear stresses in these collaterals are very high (a loud

continuous murmur can be heard) and the size of these collaterals is excessive, surpassing by far those seen in coronary occlusion.

It was mainly Schoop (8) who provided evidence in the early 1960s that Thoma's law is also applicable to developing collaterals in the limb circulation. This had far-reaching implications in that it supported the view that therapy with vasodilators might be helpful in the long run and beyond the acute actions of these drugs and it stimulated pharmacologists to develop drugs with organ specificity, i.e., vasodilators for the coronaries, the brain and the limbs. These pharmocologic endeavors were surprisingly successful, especially for the coronaries where a large number of specific drugs were developed that had very little effect on other circulatory beds (9, 10).

We tried several drugs for their potential to stimulate growth of coronary collaterals. Although the results were encouraging at first, the final result was inconclusive because the models and methods of that time were too crude and the drugs used had effects that interfered with the arterial remodeling (i.e.,they decrease leucocyte stickiness by adenosine potentiation (11).

Shear stress plays an important role today in the physiology of vasomotor control; its molecular mechanisms have been discovered and they have alrady been put to clinical use: increases in shear stress lead to the production of nitrous oxide (NO), its "receptor" is guanylate cyclase and its effect is vasodilation (12, 13). It can be envisaged that a chronically increased shear stress as it exists along preformed collaterals in the presence of stenoses and occlusions may first lead to an upregulation of the NO-synthetase activity but beyond a critical level to a decrease that leads to the other typical consequences of shear stress, i.e., platelet activation and alteration of endothelial cell function resulting in increased expression of adhesion molecules for monocytes, a prerequisite for arterial collateral remodeling.

Wall stress as a concept of growth

Partially due to the failure of coronary vasodilators in the clinical setting (and with it the concept of flow velocity), we advocated the paradigm of tangential wall stress as a molding force in collateral growth (14). It is undoubtedly a fact that tangential wall stress increases in growing collaterals due to the extreme thinning of the wall at maintained (and rising) intravascular pressures. We had been, however, unable to establish a cause-and-effect-relationship between wall stress and growth. Scheel (15) modelled several physical forces and found that shear stress and tangential wall stress

showed similar time courses with regard to collateral development with only insignificant advantages for tangential stress. I preferred the latter because the thin-walled collaterals had to endure a significant fraction of the arterial pressure and I believed (and still do today) that growth is an important mechanism to reduce wall stress by adding rapidly new layers of smooth muscle.

We deemphasised the tangential-wall-stress-hypothesis when we discovered in our second DNA-labelling study (5) that accompanying veins in the canine ameroid model showed also labelled DNA, much less than arterioles but in significant numbers. Veins may experience similar blood flow velocities but greatly differing tangential stresses.

We interpreted the finding of labelled veins also as proof for the existence of biochemical rather than biophysical transmitters.

Monocyte invasion as a concept of growth

In 1971 and in 1972 (16, 17), we described the invasion of leukocytes into developing collaterals. Originally we thought that they were part of the secondary inflammatory reaction mechanisms that followed the injury phase, but we found in 1973 (18) that monocytes participate in the subintimal cell proliferation that is so characteristic of epicardial collaterals.

A systematic study into the role of monocytes in collateral growth was started in 1974 when we prepared visibly enlarged dog collaterals for study in the scanning electron microscope. We found large numbers of monocytes adhering to endothelium and invading the subintimal space (19) and we felt that they could be the key to the understanding of vascular growth in general and to collateral growth in particular.

Only two years later Polverini et al (20, 21) published their elegant work showing that activated macrophages produced vascular proliferation and were able to induce neovascularization. Activation was found to be important since non-activated monocytes implanted into rat corneas were not angiogenic.

Monocytes were subsequently found to play a very important role also in atherosclerosis where they invade large vessels at places of injury and where they transform into foam cells in hypercholesterolemia (22). The subintimal proliferation in atherosclerosis is believed to have been caused by monocytes. Endothelial cells in regions susceptible to atherosclerosis exhibit increased expression of V-CAM (vascular cellular adhesion molecule (23))

The capability of monocytes to induce angiogenesis lies in their ability to produce growth factors and cofactors. Indeed the list of factors produced by activated monocytes-macrophages is long and still growing: IL-1, CSF, HSF, IFN-β , IL-6, PDGF, TNF-α , FGF, IFN-α, TGF-β, VEGF, Prostaglandins, etc.

The omnipotent monocyte will play a dominant role in the "unifying theory" explained in more detail below: with the detection of monocyte adherence and -invasion at places of high shear stress we have provided a cell-biological foundation for Thoma's law (19).

Other white blood cells have also been implicated in angiogenesis, i.e., next to monocyte induced angiogenesis a lymphocyte-based, a neutrophil-based and a mast cell-based angiogenesis were described (24, 25, 26). In our models all these cell types (including resident tissue macrophages) do play a role, sometimes in a distinct temporal order whereby the lymphocytes appear relatively late in the game.

Arterial "remodelling" as a concept

An early observation from our group (17) was that canine collaterals pass through several well defined stages during development. Extreme thinning of the arteriolar wall is the earliest stage and it is caused by a controlled destruction of certain elements of the vascular wall, i.e., the internal elastic lamina is cut by elastase from invading neutrophil leukocytes and a significant proportion of the smooth muscle cell population is destroyed by invading monocytes. During this phase of remodelling the vessel is able to withstand the intravascular pressure but it becomes leaky and the extravasation of proteins initiates a perivascular inflammation. Remodelling continues for some time in order to generate the space for the new cells but after reaching the final dimensions only new layers of smooth muscle are added until the radius-to-wallthickness ratio has normalized. The expanding vessel demands much more space which is generated by the destruction of the surrounding myocytes which takes place in the wake of the perivascular inflammation. These cardiac myocytes first undergo a loss of myofibrils and some look like a bag filled only with mitochondria. This de-differentiation is accompanied by expression of immunoreactive aFGF. Cardiac myocyte destruction in the perivascular space is usually accompanied by T-lymphocyte invasion.

Arteriolar remodelling can go awry as figure 31 (see chapter by Dr. J. Schaper) shows: when the tissue destruction was inefficient, all the new cells will obstruct the lumen rather than enlarging it. Defective remodelling is probably the most important

difference between the early stages of atherosclerosis and collateral growth which have some features in common especially the intimal injury. Remodelling can also occur in larger arteries: we have observed significant increases of the lumen of the left anterior coronary artery 2 years after chronic occlusion of both the LCX and the right coronary artery. Barger (27) and Heistad (28) showed arterial remodelling even in the presence of obstructive atherosclerosis.

Ischemia as a concept of growth? — A change of paradigm

The strong association between tissue ischemia and the development of a collateral circulation continues to impress all investigators active in the field. Regional tissue ischemia is still the only situation which leads to collateral development (the in situ type vascular growth) in a predictable way; no drug or growth factor was so far reported to do this in the absense of ischemia. The detection of DNA-synthesis also in veins accompanying growing arteriolar collaterals in the dog heart had clearly argued for the presence of a biochemical factor most probably originating from ischemic tissue. The finding of thymidine-labelling not only in renal collaterals but also in the periureteric epithelium following occlusion of the renal artery by Dr. Hollenberg (see chapter) pointed in the same direction. Hollenberg actually tried to isolate a vascular growth factor from ischemic kidney and our group tried to do the same in ischemic myocardium (29). Experiments by Mohri et al from Tomoike's laboratory (30) seemed to have established quite elegantly that myocardial ischemia of a certain minimum duration is required in the repetitive-occlusion model in the dog in order to be effective in producing a functional collateral circulation. However, with each occlusion a strong pressure gradient and increased shear forces across preformed collaterals are also established and they co-variate with the ischemia. The observation that the most rapid development in this model is seen with more advanced degrees of preformed collaterals (and hence with less tissue ischemia) argues for the shear-force hypothesis and for the in situ vasculogenesis (and against an ischemia-related tissue factor) because the most rapid development correlates with the least degree of ischemia.

Several of us (see also the chapter by Dr. Longhurst) have voiced concern about the nature of the relationship beween collaterals and ischemia which may only co-variate with other hidden variables in our experiments. The arguments against a causal and direct relationship are the following:

1. in practically all vascular provinces collateral vessel growth continues, sometimes long after cessation of ischemia, i.e., there is a temporal dissociation between presumed stimulus and response,
2. in practically all vascular provinces collateral vessel growth occurs at a distance from ischemia, i.e., there is a spatial dissociation between presumed stimulus and reponse (with the exception of the porcine heart).

Hollenberg and Schoop (see resp. chapters in this book) give a convincing account on the temporal dissociation in the limb- and kidney circulation. Those who have worked with the canine ameroid model know that the duration of ischemia is short and fleeting and can often only be provoked by exercise at early stages in collateral development (31). In Dr. Tomoike's chapter it is demonstrated that regional function is not visibly disturbed by ameroid closure,a functional disturbance can only be provoked by acutely occluding the stenosed artery.

The extreme sensitivity of the brain toward ischemia makes it rather unlikely that the amazing cases of collateral adaptation in the brain circulation were indeed caused by ischemia. These were most probably caused by long standing pressure gradients during the course of slowly developing stenoses whose flow reducing effects were compensated for by arteriolar vasodilation causing increased blood flow velocity and hence increased shear stress.

The distances between ischemic regions and the predilections for collateral growth can indeed be absurdly large: up to 70 centimeters between a gangrenous big toe and collaterals spanning a popliteal occlusion! In the dog heart usually only the subendocardium becomes ischemic (where no vessels grow) but collaterals develop on the epicardial surface, i.e., the distance between these regions can exceed more than 2 centimeters. The distance beween the center of presumed ischemia and the place of collateral development is also of much concern to Dr. Tomoike (see his chapter).

Recently we studied the spatial relationships between "midzone"-segments of epicardial collaterals and the area of perfusion of the ameroid-occluded artery, and we found that the myocardium in direct contact with the vascular segment did not even belong to the risk region or was situated at the border, i.e., a significant segment of vascular growth clearly occurred outside the region that, for a very short time, may become potentially but perhaps only transiently ischemic.

I find it difficult to believe that any humoral transmitter or tissue factor or growth factor polypeptide can diffuse "upstream", i.e., against the direction of blood flow.

The degree of inconsistency between observed fact and hypothesis has become so large that a paradigm change is necessary. **The new paradigm would postulate that in situ epicardial collateral vessel growth is a strictly local vascular process dominated by shear forces and monocyte invasion.**

A direct temporal and spatial relationship between ischemia and collateral growth is suggested only in the porcine heart: microvessels grow mainly within the ischemic region and the growing season is short. True angiogenesis occurs, i.e., new capillaries sprout, in contrast to the growth-remodeling that occurs in the canine heart. It was this convincing relationship that supported our belief for a long time in the existence of ischemic myocardial growth factors. I will show later that this relationship is less convincing than originally thought because angiogenesis occurs mainly in areas of focal necrosis .

The concept of growth factors produced by ischemic myocytes

The commitment of a cell to enter the G1-phase of the cell cycle is dependent on the presence of a mitogenic peptide that had occupied a cell surface receptor. Our discovery of cell division as the mechanism of collateral development had programmed us to search for a mitogen that is expressed under experimental situations leading to endothelial and smooth muscle mitosis. We have shown (32, 33) that the heart is a surprisingly rich source of growth factors, i.e., the mitogens HBGF-1 (aFGF) and VEGF and the non-mitogenic TGF-β are prominently expressed in myocardium and resident myocardial macrophages and invading monocytes produce TNF-α (see chapter by Dr. Mohri). When we first isolated the HBGF-1 peptide in good yield from the heart several years ago (32) we thought (stimulated by reports from other laboratories (34) that the quest is over and the problem of collateral development finally explained: ischemia releases HBGF-1 (aFGF) which occupies its receptor on endothelial cells and triggers mitosis. We were surprised and became frustrated when we encountered difficulties in gathering evidence for this simple hypothesis: only occasionally were we able to demonstrate increased transcription of the aFGF gene in collateralized pig myocardium although we tried everything to define and find the correct time of tissue sampling: We used the clinical cine coronary angiography equipment not to miss the moment of critical stenosis by modified ameroid (encased in radio-lucent plastic), we used ECG-telemetry for signs of ischemia and we made sure that RNA was extracted from tissue with positive evidence for endothelial mitosis by

in-situ hybridization for histone-H3. We were also misled by a severe case of "beginners luck" in that our first piece of histone-positive tissue from collateralized myocardium showed a positive in-situ hybridization signal with an aFGF-probe over an enlarged collateral vessel (Dr. Kandolf from the Max-Planck-Institute in Munich collaborated). However, Northern hybridization analysis for aFGF mRNA was very inconsistent from one animal to the other but we observed that the strength of the Northern-signal co-varied with the amount of focal necrosis, i.e., aFGF-expression decreased with increasing amounts of necrosis and it decreased to about the same extent as the GAPDH-signal was affected. This was strongly indicative for a myocyte localization of the aFGF mRNA and variations in the strength of the Northern signal reflected not changes in gene expression but rather changes in the size of the FGF-expressing population of myocytes. Our in-situ work with a monoclonal antibody directed against the aFGF-peptide confirmed the localization of the antigen in surviving myocytes surrounding focal necroses in the collateralized porcine heart (35).

The finding of increased expression of aFGF in stressed but surviving myocytes near focal necroses and near places of angiogenesis would have suggested a cause-and-effect relationship: the spatial relationship is ideal but the problem here is: can aFGF leave a live myocyte? Owing to the lack of a leader sequence in the FGF molecule, it cannot make use of established pathways of secretion. Furthermore we could not find aFGF in the extracellular matrix where it was shown to reside by other investigators (36, 37). However, fixation artifacts and cell death (in cell culture), release of FGF and and subsequent binding to extracellular matrix are the more likely explanation for an extracellular localization (38).

This means that the only way aFGF could act as an angiogenic growth factor is by release from dead myocytes. Although myocytes die in our models and although small vessels develop nearby, I find this an unlikely mechanism because although the spatial reationships are right, the temporal relationships are not: aFGF-expression is a relatively late event in pig collateral angiogenesis (see chapters by Dr. J. Schaper and Dr. Mohri).

A look at the dog model shows that aFGF is overexpressed in regions where the growing vessels create mechanical problems for the myocytes in close vicinity: these myocytes usually degenerate under the forces exerted by the expanding vessels in combination with the perivascular inflammation, i.e., again a situation where myocytes come under stress and where some survive.

It is imaginable that aFGF is a survival factor for the heart analogous to the role it plays in the brain: aFGF is present in embryonic and adult brain (neurons) (39), has no relation to the development of brain blood vessels (40, 41) but is a neuronal survival factor and promotes neurite outgrowth (42, 43, 44).

Terminally differentiated cells like neurons are dependent on trophic (or survival) factors (45). It is intuitively understandable that these cells that have lost the capacity to divide need additional protection against the stresses that they are subjected to under extreme physiological situations.[1] **In analogy to the brain, cardiac myocytes are also terminally differentiated and would also require survival factors.**

Although this type of reasoning is speculative and indirect other strong arguments exist that would indicate that some of the so-called growth factors may have other or additional physiological functions: I find it unlikely to assume that at least two very potent in-vitro mitogens should have as the place of their strongest expression an organ with extremely stable cell populations under normal conditions.

A look at the region of expression of aFGF and TGF-β in the normal heart might also furnish a clue for understanding the physiological role of these peptides: both factors are expressed in the Purkinje-system in amounts high enough to be detected by indirect immunofluorescence. The Purkinje-system is known for its superior tolerance toward ischemia compared to the working myocardium. The Purkinje-system is also the least differentiated myocyte subsystem in the heart and we should recall here that aFGF is the tissue hormone regulating differentiation in skeletal muscle: downregulation of aFGF-expression leads to the differentiated state (46, 47, 48) and that may also be the case in the heart. Although adult cardiac myocytes have lost the ability to divide they have not lost the ablility to de-differentiate. A number of stimuli (often pathologic) can induce de-differentiation: i.e., chronic ischemic heart disease in human patients, mechanical stresses in experimental animals, transplant rejection in human patients lead both to morphological signs of de-differentiation (loss of specific organelles) as well as to upregulation of FGF-expression (Schaper and Schaper, unpublished).

An alternative and much simpler hypothesis explaining aFGF-expression in these myocytes near focal necrosis is that they have taken up the factor rather than produced it. These cells although they look alive (but not well) do also take up complement,

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[1] Terminally differentiated cells share also the reluctance to present antigens which is a sensible survival strategy since they will not survive the attack of the cellular immune response.

fibronectin, PAI-1 and uPA without producing these molecules. If true, this hypothesis would reopen the search for the place of production of aFGF.

The conclusion of this elaboration is that **the original idea of growth factor production by the ischemic myocyte is probably untenable.** The arguments in its favor were inspired by the vitalistic "need" that these myocytes have for blood and oxygen and which should have been satisfied by growing collaterals.

The paradigm of this paragraph (i.e., no role of an ischemia-related growth factor originating from myocytes) was chosen for the sake of argument and because indeed no clear evidence had presented itself for the existence of such a factor from our in-vivo work. As said earlier, we originally thought that such a factor should be there and we started a project with isolated myocytes in culture and we could identify a growth factor for endothelial cells but we have at present no clue as to its structure (29) or its possible relationship with the FGF-family. This finding is no argument against our present hypothesis since we have shown hat the presence of a "growth" factor in myocytes does not necessarily imply that it plays such a role in that cell.

Inflammatory angiogenesis as a concept

Already in 1973 we described (6) widespread inflammatory changes originating from micro- and macro-necroses in the pig ameroid model. These changes were documented with radioactive thymidine incorporation and angiogenesis was clearly demonstrated. However, in spite of intense growth the peripheral coronary artery pressure (PCP, measured in the distal stump of the occluded artery) remained much lower than that in the canine model. To address this problem more in depth we performed another set of pig experiments in 1987 (49, 50) where we showed that the relationship between PCP and transmural collateral blood flow (PCP being the true perfusion pressure of the collateral dependent region) rises much more steeply than in the canine model, i.e., each increment in pressure caused a much greater increment in flow in the pig heart. This was direct physiological evidence for a decrease of the minimal vascular resistence of the collateral perfused region caused by microvascular angiogenesis. Similar findings were reported shortly thereafter by Roth and White (51) who chose pump perfusion from the distal stump of the occluded coronary artery in contrast to our approach of controlled changes of aortic pressure with measurement of PCP and collateral flow.

When we started new experiments in the porcine model with the ameroid method in 1985 to search for evidence of growth we excluded all tissue that exhibited evidence for necrosis, micro or macro, in the belief that only living myocytes can produce a growth factor. Morphometric data from this tissue (see Dr. J. Schaper's chapter) showed only very modest increases in indices of vascular growth. Near foci of necrosis the capillary density was greatly and visibly increased — a seminal observation that lead us to conclude that it is mainly the inflammatory reaction to focal necrosis that induced angiogenesis.

We show in the chapters by Dr. Mohri and by Dr. Jutta Schaper that the neo-angiogenesis in the pig ameroid model is caused by an inflammatory reaction in the wake of focal tissue necrosis. **From a conceptual point of view this means that the stimulus (but not the factor) for angiogenesis in the ischemic porcine heart originates from dead myocytes,** thereby refuting the vitalistic "need"-hypothesis which would require live myocytes.

After we observed that angiogenic growth factors (mainly aFGF) are only demonstrable in regions of focal necrosis, we induced focal necrosis by microembolization. This method was used by Chilian and Scheel (52) in the canine model to stimulate collateral flow. We show in the respective chapters that micro-embolization is indeed a powerful model of angiogenesis in the porcine heart that is almost indistinguishable with regard to vascular growth from the ameroid model. Our studies into the mechanisms of angiogenesis in the two porcine models show that many reactions are shared with those of inflammation in other organ systems and only a few observations are specific for the heart, notably the persistence of the new vessels when a pressure gradient continues to exist. Also mast cells do not seem to play a significant role in the inflammatory-type angiogenesis in the porcine heart.

Growth factor receptor regulation and growth factor inhibitors as concepts

"In vivo veritas"

J. Madri

Circulating blood contains growth factors but endothelium in vivo does not readily react to these hormones with a growth response. Even if growth factors are systemically or locally injected, endothelium does not necessarily enter the cell cycle (Braun and Schaper, unpublished; see also Dr. Unger's chapter). These observations lead to the concept that growth is not exclusively controlled by the availability of mitogenic peptides but also by the presence of receptors: endothelial cells in vivo and under normal physiological

conditions apparently do not present their receptors. Only experimental or pathological situations, explantation and in vivo culture or embryonic development induce the expression and presentation of receptors. However, the regulation of collateral vessel development by growth factor receptors need not imply exclusively a change in receptor number: the activity of the effector enzyme (i.e., protein tyrosine kinase) can change through differential splicing (which changes the affinity to the ligand) or through the presence or absence of divalent cations like calcium, manganese and magnesium and by the presence of pyrophosphate (53). Receptors for the FGF family of growth factors can differ substantially in their substrate specificities and their expression patterns (54). Up- and down regulation of growth factor receptors is an important mechanism in embryonic development and differentiation (47). Binding sites for VEGF are present in normal tissues including the myocardium (55) but that does not necessarily mean that the receptor is coupled to the effector system under physiological conditions: the observation by Unger that chronic infusion of substantial amounts of VEGF into the canine coronary system does not lead to endothelial mitoses points to the receptor as a regulatory element.

It appears from these thoughts that the study of receptors should even be as interesting as the study of growth factor production. **A very attractive hypothesis could be that growth factors are always present in basal amounts but that receptors are only presented (or activated) when induced by special situations.** Basal expression (and perhaps more than that) was demonstrated by us for aFGF and VEGF in the normal heart. It has been shown that during development of the chick brain vasculature the expression of growth factors and their receptors is not synchronous (40).

A role similar to that of receptor regulation is played by inhibitors of angiogenesis (56). It is imaginable that the commitment of a cell to enter the cell cycle could also depend on the inhibition of an inhibitor present under physiological conditions in addition to the presence of a mitogen. Known general inhibitors of cell growth are TGF-β, the RB-gene product and the p-53 nuclear oncogene. Specific inhibitors of angiogenesis are steroids (57) in conjunction with heparin, and factors found in avascular organs like cartilage or in areas in the embryo that become avascular again during the development of the limb buds (58).

We searched in normal myocardium for inhibitors of thymidine uptake in endothelial cells and we found a peptide of 11000 Da that, upon partial amino acid

sequencing, exhibited 2 fragments of 10 amino acids each that were novel, i.e., no matching sequences were found in appropriate data banks (59). The significance of this factor for collateral development, its complete sequence, its gene, its function, and its regulation will be the topic of our future research.

A unifying theory?

The development of blood vessels in the embryo is subdivided into angiogenesis and vasculogenesis. Angiogenesis is the sprouting of capillaries from preexisting capillaries and vasculogenesis (type I and type II) is the developmentof capillary vessels from in situ differentiating endothelial cells and subsequently of large vessels (60) (see Dr. Risau's chapter).[2] In analogy to the embryonal situation, the development of collaterals can also be classified into vasculogenesis (arteries become larger by in situ proliferation and differentiation of endothelial and smooth muscle cells) and into angiogenesis (capillaries develop de-novo by sprouting). Both processes (in the embryo) share important molecular mechanisms, i.e., the synthesis of fibronectin in angiogenesis and in vasculogenesis, whereas laminin is an early marker for vascular maturation (60). The migration of angioblasts is believed to be guided by the extracellular matrix proteins. **The central part of our "unifying theory" is that collateral development recapitulates ontogeny and either follows angiogenic or vasculogenic mechanisms or both.** The two experimental models that we describe are either vasculogenesis-dominated (the canine model) or angiogenesis-dominated (the porcine model). Both developmental types, however, do occur, to some extent, together and in both situations: there is also vasculogenesis in the pig heart (existing arterioles and small arteries become larger, see the chapter by Dr. White and Dr. Bloor) as well as there is angiogenesis in the dog model (in the connective tissue around the ameroid

[2]The literature on blood vessel development is inconsistent with respect to the terms vasculogenesis and angiogenesis. In Noden's view (61) the term vasculogenesis encompasses all aspects of embryonic blood vessel development irrespective of how the vessels are being generated. Sherer (62) argues that since the terms vasculogenesis and angiogenesis have the same derived meaning their discriminative use is arbitrary. For Poole and Coffin (63) vasculogenesis type I means the development of the dorsal aorta from angioblasts arising in situ. I follow here Risau's definition for angiogenesis (capillary sprouting) and Poole's definition for vasculogenesis (i.e., development of the larger arteries). For the in situ enlargement of collateral arteries I have formerly used the term "non-sprouting angiogenesis", Burri recently named it "intussuceptible growth" (64). Risau does not agree with my use of the term vasculogenesis because that word is reserved for the development of vessels from stem cells or angioblasts in situ. My view is that the development of small and large collaterals is too different to be given the same name.

constrictor, in regions of micro-infarcts). Vasculogenesis and angiogenesis in coronary collateral growth share with embryonic development the marked increase in the synthesis of fibronectin and laminin. The two developmental types share furthermore the proliferation of endothelial and smooth muscle cells and the proteolysis and subsequent synthesis of extracellular matrix proteins. Collateral vasculogenesis shares with the embryonic development of arteries the vimentin-desmin-switch (vimentin is expressed in embryonal arteries and in early-stage collaterals, desmin in mature arteries and mature collaterals, see chapter by Dr. J. Schaper). Vasculogenesis in the embryo is, as in collateral growth, also strongly influenced by physical factors (65). In our models angiogenesis is of the inflammatory type (leukocyte involvement is less in embryonic development).

Both types of experimental models described by our group in this book are also relevant for the human heart where micro- as well as macro-vessels participate in the development of a collateral circulation, i.e., angiogenesis and vasculogenesis participate in human collateral enlargement.

Another part of the unifying hypothesis is that **in neither animal model and in neither model of vascular occlusion a direct involvement of the "needy" myocyte is detectable in the sense of growth factor production by myocytes in response to ischemia.** In the canine model the ischemic region does not influence its own vascularity at all and in the porcine model the risk-region vascularity increases via granulation-tissue angiogenesis in response to micro-infarction where the growth factor production is dominated by the monocyte-macrophage system (66). This statement is in some contrast to our finding that the growth factor VEGF is transcribed in myocytes (see chapter by Dr. Sharma). However, we could not find a clear and consistent correlation beween the process of collateralization and the expression of the growth factor by cardiac myocytes. Clear and consistent relationships do exist with a multitude of different factors (see chapter by Dr. J. Schaper).

A third leg of the unifying theory is that **the (resident) macrophage-(invading) monocyte system plays a dominant and essential role** but different strategies are used in the experimental models studied.

In the canine model the "remodelling vasculogenesis" approach is employed and in the porcine model the "inflammatory angiogenesis" approach is used. The difference in strategies is dictated by the morphological substrate available. In the canine heart the substrate is interconnecting arterioles in the size class of 50 micrometers. This class of

vessels experiences the highest shear stress (67, 68) already under physiologic conditions. The generation of a marked pressure gradient along these vessels increases the velocity of flow and hence shear stress. The effect of a sustained increase of shear stress is platelet activation, the production of IL-1 by adhering platelets and the upregulation of adhesion molecules in endothelial cells by IL-1 (or by TNF-α, (69)). This mechanism may not be immediately effective since an acute increase in flow increases first the production of EDRF (NO) which inhibits platelet aggregation. It can be envisaged that a chronically increased shear stress exhausts EDRF production which then leads to the sequence of events sketched above.

Remodeling vasculogenesis is finally executed between the invading monocytes on one hand and the resident macrophages and the resident perivascular mast cells (seen in the canine model) present in the adventitial space on the other hand.

An interconnecting network of arterioles is missing in the porcine heart or is very much less developed in comparison to the canine myocardium. Pressure gradients can act only on connections of capillary size where shear stresses are about one order of magnitude less as compared to 50 micrometer arterioles. Consequently we could not detect monocytes invading arteriolar-size vessel walls in the porcine heart. Their invasion was confined to the extravascular space near micronecroses.

The invasion of monocytes in the porcine models is most probably governed by the activation of resident macrophages through the stimulus of ischemic myocyte cell death. It is probably safe to conclude that macrophages with their ability to discriminate between self and non-self detect myocyte cell membranes damaged by ischemia as non-self, initiate the complement cascade and attract first neutrophil leukocytes and thereafter monocytes into the region. The macrophage-monocyte system then controls angiogenesis via the initiation of proteolysis, production of growth factors, and induction of other cell types to produce the extracellualar matrix proteins fibronectin, laminin and the different collagens. Resident macrophages and maturing invading monocytes also enter the cell cycle and mitoses can even be observed with the electron microscope (see chapter by Dr. J. Schaper).

The mitogen for endothelial cells is most probably VEGF secreted by macrophages-monocytes (66) and not aFGF for reasons outlined above and because of the relatively late appearance of immunoreactive aFGF. The adaptation ends by the control of proteolysis via increased expression of PAI-1 and via upregulated expression of TGF-β (70).

Another mechanisms of activation may originate from stressed smooth muscle cells (in the canine model, see chapter by Dr. J. Schaper) that liberate TNF-α which not only activates monocytes but is also a powerful chemoattractant for them (71, 72).

Summarizing the "unifying theory "reads as follows:

1. Coronary collateral development recapitulates the embryonic mechanisms of vasculogenesis and angiogenesis, the latter being more dominant in the porcine and the former more dominant in the canine model.
2. Ischemia is most probably not a stimulant for myocytes to produce angiogenic growth factors in significant amounts.
3. Growth factors are most probably produced by the monocyte-macrophage system.
4. Growth factor receptors on endothelial and smooth muscle cells are important regulatory elements that can be activated under the influence of pathological stresses.
5. Angiogenesis is always seen within the context of inflammation.
6. Vasculogenesis (i.e., the in situ-type growth and maturation of large collaterals) is largely governed by in situ acting local forces (shear stress) leading to monocyte invasion that proceed with controlled tissue destruction (remodelling) and the initiation of cell proliferation.

Drugs, lifestyle and gene transfer as concepts

The promise of the collateral circulation lies in its potential to alter the course of the natural history of coronary artery disease. This it does, but often it fails for a variety of reasons: the speed of coronary occlusion is often too fast to allow for compensatory growth, or: arteries that should serve as "feeders" for collaterals are already stenosed themselves. Therapies should be tried to prevent these failures and to improve collateral flow above the level that is reached spontaneously in the presence of stenoses and occlusions. This level falls markedly short of the physiological range, even under optimal circumstances in experimental animals (73).

There is no therapeutic concept under evaluation today that has as its declared aim the improvement of the collateral circulation. This has certainly to do with the availability of effective and powerful therapies for ischemic heart disease like coronary artery bypass surgery and PTCA and its derivatives. However, these treatments are expensive and are hence not within everybodies reach. A non-surgical therapy directed

at improvement of the collateral circulation could focus on three approaches, i.e., on drugs, on exercise and on the promise of somatic gene therapy.

Although there was a plethora of vasoactive coronary drugs available none had shown any promise nor was there a systematic attempt to influence collaterals. When the interest in this problem was high cine coronary angiography was in its infancy. It must also be questioned whether suitable drugs are (or were) available: from our experiments carried out some time ago it appeared that a powerful and specific coronary vasodilator (chromonar) is not orally active, that an orally active drug (dipyridamole) is only short-acting and not coronary specific, and a third (lidoflazine) was both orally active and long-acting but exhibited too many side effects (arrhythmias) (10). The discussion on shear forces made clear that if the right class of collaterals (50 micrometers) is present the establishment of a pressure gradient should exert a stimulatory influence and specific vasodilators are an assured way to increase pressure gradients.

Drugs that are not vasoactive like heparin, tPA and uPA have been tested in coronary patients but only heparin was aimed at improvement of human and canine collaterals (74, 75). Heparin is a not very precisely defined drug, it has multiple actions (anticoagulant, lipolytic, concentration-dependent potentiator or attenuator of aFGF) and it does not play a well-defined role in angiogenesis except in situations where mast cell degranulation is observed. Heparin does not stimulate collateral growth in the porcine model (76) but is was reported to play a role in a mesenteric window angiogenesis assay in the normal rat following systemic application (77). Heparin was recently tested as an antiproliferative drug to prevent restenosis following coronary angioplasty procedures but was found to increase the incidence of restenosis (78). Heparin, tissue-type plasminogen activator and urokinase-type plasminogen activator are widely used drugs in acute myocardial ischemia in human patients and it is difficult to understand why the actions of these drugs have not been studied more intensely with regard to their actions on collaterals.

The role played by physical exercise in collateral development has received wide attention. For a variety of reasons exercise should be beneficial: in the presence of a stenosis any increase in blood flow increases the pressure gradient over preexisting collaterals; should ischemia play a role: exercise induces it. However, in spite of many efforts the question is not satisfactorily answered (79) (see also the chapters by Dr. Longhurst and Dr. White). The reasons for the lack of a clear answer are probably

manifold: across the published studies in dogs no correlation exists between the level of exertion and the expected effect on the coronary circulation. Often only mild levels of exercise produce an effect in one laboratory (80) but a very much higher workload in another laboratory showed no effect (79). Or, in an animal model (pig) where the size-class of preexisting collaterals would not make an effect of exercise very likely (because on the level of the microvessels flow-dependent shear forces are much less prominent), exercise seems to work much better than in the canine model (81). On the other hand, the effects of exercise on coronary flow are much smaller (about two-fold) than drug-induced maximal vasodilatation (about five-fold) and exercise cannot be repeated as often as a drug application.

Although the transfer of a gene into a coronary artery or into the cardiac myocyte in vivo is now within the realm of the possible (82), it still remains a formidable task to initiate and control angiogenesis in the living human heart under conditions that are safe and reasonably non-invasive. But even if somatic gene transfer has become commonplace in experimental animals it remains to be seen whether the transfer of a single gene (i.e., for a growth factor) would suffice to start the very complex chain of events that angiogenesis is. Dr. Unger's data have taught us already an important lesson: it is useless to infuse growth factors if the target cell does not present receptors or if the receptors are not coupled to their effector system. The expression of a somatically transferred additonal growth factor gene would not seem to alter this situation fundamentally. A difficult practical problem in somatic gene transfer in the heart is at present the occurrence of inflammatory tissue reactions that would render the actions of transferred angiogenic factor genes as difficult to interpret (82).

It would make much more sense to transfer a growth factor receptor gene but even that can be expected to cause problems because the dividing endothelial cells would probably obstruct the lumen rather than making it wider, a situation that occurs spontaneously in a number of cases (see chapter by Dr. J. Schaper)

An interesting idea that can be studied only with gene transfer techniques is to induce increased expression of adhesion molecules for monocytes on the surface of endothelial cells to increase the sensitivity of the endothelium for shear forces. In this way a low grade stenosis and the small pressure gradient it creates would already initiate the processes leading to vascular enlargement.

Unsolved problems

Few, if any, problems are satisfactorily solved in collateral development but a few have not even been properly phrased. In the growth factor field platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) were not mentioned so far in our results. The reasons for these omissions are that we have not so far found any hint that they are involved in the processes studied: neither on the basis of indirect immunofluorescence nor on the basis of mRNA-hybridization (Northern and in-situ) was there a hint that the expression of these factors was changed by the experimental situations described by us. This may be entirely our fault. Sometimes the problem lies in the lack of cross reactivity of antibodies between species: e.g., antibodies directed against the CD-system of human leukocyte surface antigens do not recognize those on porcine cells etc. This markedly impeded progress in understanding the role played by monocytes in the porcine model. PDGF-antibodies raised against the human antigen do not cross-react with the canine and porcine antigens (see chapter by Dr. J. Schaper). Although we could isolate bFGF from the bovine heart by heparin-sepharose chromatography and raised a monoclonal antibody against the human antigen (recombinant) we could not demonstrate immunoreactive structures in the porcine heart under conditions of acute or chronic ischemia. Others were also unable to see a convincing role for bFGF in angiogenesis models like in sterile inflammatory angiogenesis in the mouse cornea or in vascular tumors (83, 84).

Finally, we should address the problem of growth factor receptor expression which adds yet another dimension to the control of angiogenesis.

CONCLUSION

Our studies have led us to conclude that the microvascular response to coronary occlusion in the porcine model is dominated by an angiogenic process that is closely related to wound healing and inflammation in the wake of focal necrosis and tissue repair. This process is not fundamentally different from other types of inflammatory angiogenesis with the exception that in the heart the new vessels do not regress because of the presence of a permanent pressure gradient between the normal and the collateralized region. The necessary growth factors, proteases and cytokines are largely produced by the resident-macrophage-invading-monocyte system and by stimulated endothelium. Myocyte-based growth factors (aFGF) appear too late to be of use as mitogens and they may have other or additional functions in cardiac myocytes.

The macrovascular response in the canine model is dominated by in situ vascular remodelling (collateral vasculogenesis) which is most probably initiated by shear stress with or without endothelial injury and subsequent monocyte invasion (seconded by resident mast cells and macrophages), endothelial mitosis, controlled tissue destruction by proteases and smooth muscle cell proliferation. This process ends when the shear stress had normalized due to marked collateral enlargement and when the control of blood flow is again assumed by the risk region tissue. Tangential wall stress becomes a molding force after remodeling had led to thinning of the vascular wall during the course of radial expansion. The addition of new layers of smooth muscle normalizes the wall tension.

The macrovascular response is characterized by the relatively large distance between epicardial vascular remodelling and the center of ischemia in the subendocardium.

There is little evidence to suggest that ischemic but live myocytes produce angiogenic growth factors in either model. This statement is based on the observation that true angiogenic microvascular responses were only observed in the presence of focal necroses and as a reaction thereon.

Both models exhibit essential aspects of angiogenesis: i.e., migration and proliferation of endothelial cells, lysis and synthesis of extracellular matrix proteins, monocyte attraction and -activation and growth factor involvement.

The micro- and macrovascular responses to progressive coronary occlusion recapitulate angiogenesis and vasculogenesis of ontogeny.

A paradigm change appears to be absolutely necessary because the observed facts had become inconsistent with the reigning theory which was based on growth factor prduction by ischemic myocytes.

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5

COLLATERAL VESSEL DEVELOPMENT IN THE PORCINE AND CANINE HEART

MORPHOLOGY REVISITED

Jutta Schaper and Dorothee Weihrauch
Max Planck Institute, Department of Experimental Cardiology,
6350 Bad Nauheim, F. R. Germany

The principal findings of our earlier studies on collateral development by light and electron microscopy were that after the induction of progressive stenosis of a major coronary artery, preexisting collateral vessels enlarge in three stages (1). The first consists of vessel dilatation and thinning of its wall which sometimes can cause damage to the media and adventitia. In the second stage, mitosis of endothelial and smooth muscle cells lead to growth and remodelling of the vascular wall. In addition, dedifferentiated smooth muscle cells appear. Finally, reorganization (remodelling) of the vascular wall structure is completed. This type of development of collateral vessels occurs on or close to the epicardial surface of the heart.

In the human heart, collateral vessels occur not only as large epicardial arteries, but in many patients they appear as an enlargement of the capillary bed (2). For this situation, the porcine heart is a very suitable model and it was therefore included in our ongoing studies on the mechanism of collateral vessel development.

Progress in the knowledge of the process of angiogenesis (3, 4) and the role of peptide growth factors has especially contributed to the understanding of the process of collateral growth. Morphological studies, however, also attained a completely new aspect by the development of new methods such as immunocytochemistry and in situ hybridization.

Immunocytochemical methods using monoclonal antibodies were introduced to localize proteins involved in vascular development, such as e.g. fibronectin (5), laminin (6, 7, 8), acidic fibroblast growth factor (9), or

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

cytokines such as tumor necrosis factor-α (10).

In situ hybridization represents another modern technique for the investigation of vascular growth. It allows the localization of messenger RNA needed for the synthesis of proteins found to be involved in vascular growth by immunocytochemistry, and of nuclear histones as indicators of mitotic activity.

The aim of this chapter is to present new data on the process of angiogenesis and vascular growth. The work is based on results obtained by ultrastructural, immunocytochemical and in situ hybridization investigations in canine and porcine hearts. We studied in particular the involvement of components of the extracellular matrix, of the cytoskeleton, and of growth factors in the process of collateral development in different animal models.

COLLATERAL DEVELOPMENT IN THE PORCINE HEART

While in the dog collateral growth takes place on the epicardial surface of the heart in preexisting arterioles or small arteries, in the pig heart the collateral vessels develop at the level of the capillary bed. A constrictor as well as a microembolization model were used to study the latter.

The constrictor model for studies of angiogenesis

An ameroid constrictor was placed around the left circumflex artery and the animals were sacrificed 3-24 weeks later. At the time of sacrifice, the hearts were perfusion fixed with buffered glutaraldehyde at a constant pressure of 80 mmHg. After removal from the chest, the hearts were examined for macroscopic evidence of infarction and tissue samples were taken for light and electron microscopy.

In another series of experiments, the hearts were not fixed but tissue samples were preserved in liquid nitrogen for immunocytochemical studies.

Morphology: Light microscopic examinations performed 4, 8 or 24 weeks after applying the ameroid constrictor showed no large infarcts but small areas of patchy fibrosis containing many more microvessels than the surrounding intact myocardium. These were scattered throughout the left anterior and lateral ventricular walls, especially in the subendocardial region.

Number and size of collateral vessels: Morphometric measurements were carried out on tissue containing no "microinfarction" areas and when these were occasionally present, they were excluded from the evaluation. The capillary density in the subendocardium was little affected at 4 weeks but at 8 weeks after placement of the ameroid constrictor a significant decrease below normal had occurred. At the same time the luminal area showed a significant increase. At 24 weeks the capillary density and lumen areas returned to values obtained at 4 weeks, i.e. the number of the vessels had increased while the lumen was smaller than at 8 weeks (Figures 1 and 2). No significant changes were observed at any of the time periods in the subepicardial layer.

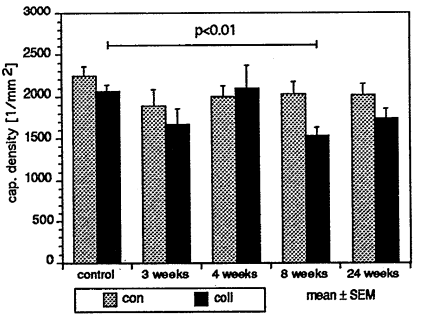


Fig. 1. The capillary density is reduced at 8 weeks in the subendocardial region

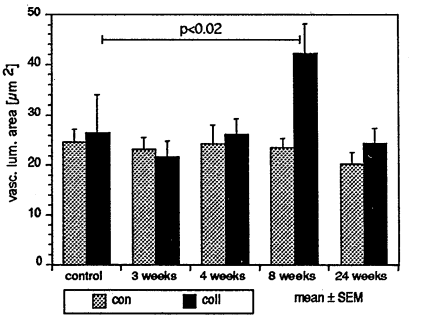


Fig. 2. The vascular lumen is increased at 8 weeks in the subendocardium. For further explanation see text.

These data show that after the onset of ischemia by ameroid constrictor implantation the capillaries become larger and therefore there are fewer vessels present per mm². For the interpretation of these data it is important to remember that vascular density is expressed as number/mm² and therefore only indicates values relative to a reference area (11), but no changes of absolute values. Therefore, the increase of capillary density at 24 weeks and the normal distribution of vessel size may indicate that many new smaller capillaries had developed, i.e. that sprouting of microvessels followed, at later stages after ischemia, the initial increase in capillary diameter. The main adaptation period may therefore happen rather late, between 8 and 24 weeks. Further studies using immunocytochemical

methods are needed to clarify this point.

Electron microscopic studies showed that with the increase in microvascular diameter the thickness of the endothelium also increased, i.e. the ratio between these 2 parameters remained the same at all time intervals indicating endothelial cell growth. In situ hybridization studies in these hearts showed that labelling of endothelial cells for mRNA of the histone H-3 (representative of cells in S phase) was significantly increased, i.e. endothelial cell mitosis was taking place. Addition of smooth muscle cells to these enlarged capillaries, however, was not observed. The occurrence of infarction is prevented in these hearts in a first stage mainly by the dilatation of existing capillaries and growth of microvessels, whereas in a later phase, between 8 and 24 weeks, addition of numerous new small vessels prevails.

Acidic fibroblast growth factor, fibronectin, and laminin. The most marked change observed immunocytochemically in sections from areas at risk consisted of the appearance of acidic fibroblast growth factor (aFGF) staining within myocytes in the vicinity of developing collaterals. (The antibody was developed by S. Bernotat-Danielowski in our institute). This was particularly striking when there was some patchy necrosis-fibrosis nearby (Fig.3).

The extracellular matrix proteins fibronectin and laminin occurred in increased amounts, especially in the areas of fibrosis but also between myocytes and capillaries. This was accompanied by the appearance of numerous new small vessels, well seen with the lectin Dolichus biflorus agglutinin (DBA) (Fig.4). However, the proximity of the myocytes to the microvessels often interfered with proper visualization of the fluorescent immunostains in the surrounding matrix and quantitation of the changes was impossible.

Summary: In the porcine heart, after slow occlusion of the circumflex coronary artery, the occurrence of myocardial infarction is often prevented by the development of a collateral circulation. Vascular growth occurs at the level of the capillaries that first enlarge in diameter and then increase their number by endothelial cell mitosis. Endothelial cell mitosis, however, can lead to enlarged aneurysmal vessels and does not increase their number as seen e.g. in diabetics (Yarom, R and Schaper, J, unpublished

results). For new tubular capillaries to form, the whole cascade is needed to occur, i.e. digestion of basement membranes as well as of extracellular matrix proteins, and this will be elaborated upon in more detail in the next part of this chapter.

The microembolization model for studies of angiogenesis

In the constrictor model it was noted that angiogenesis was most pronounced in areas of patchy necrosis and it was assumed that this could be an important factor for the formation of collateral vessels. Therefore, a model was developed in the pig heart in which micronecroses were produced by microembolization of the left ventricle with microspheres (25nm diameter) injected into the coronary system (following and modifying the method described by Chilian et al in the dog heart (12). Using this model, wound healing by scar formation can easily be observed. Since wound healing is characterized by the early proliferation of microvessels (13, 14), this tissue presents a model suitable to study the process of angiogenesis in porcine myocardium. Furthermore, as shown by Dr. Mohri (see Chapter in this book), collaterals seem to develop when newly formed vessels in the necrotic areas connect with preexisting capillaries nearby.

We studied pigs sacrificed at different time intervals after embolization starting at 12 hrs and continuing with 24 hrs, 3, 7, and 28 days (for more technical details see chapter by Dr. Mohri). Myocardial tissue samples were either fixed in 3% buffered glutaraldehyde for embedding in Epon or frozen in liquid nitrogen and stored at -70°C for immunocytochemistry.

Cryostat sections were prepared for histology (hematoxylin and eosin), for histone in situ hybridization and for immunocytochemistry using antibodies against fibronectin, laminin, collagen I, III, and VI, and vimentin. Dolichus biflorus agglutinin (DBA) was used to identify all vessels, since this lectin stains specifically the basement membranes of pig endothelial cells by binding to the sugar N-acetyl-α-D-galactosamin (15).

Morphology: Light microscopy revealed the presence of contracture bands in myocytes at 12 hrs, cellular infiltrations with polymorphonuclear and mononuclear cells at 24 hrs, and necrosis and more pronounced cellular infiltration were seen at 3 and 7 days. Microspheres were sometimes observed

as small aggregates, but mostly they occurred singly. The number and size of necrotic foci varied between animals, ranging from single cell to small patchy areas of necrosis. At 7 days after microembolization, numerous microvessels were present in the necrotic areas and at 4 weeks scar formation was evident. Like in wound healing elsewhere, the repair process thus consisted of two phases: formation of granulation tissue and scar development (16). In the electron microscope, neutrophils as well as monocytes were seen in the necrotic area as early as 12 and 24 hrs. At 24 hrs the neutrophils became more numerous and monocytes transformed into macrophages, invaded and phagocytosed irreversibly injured myocardial cells (Fig. 5).

At 3 days, the mononuclear infiltrate consisted mainly of monocytes-macrophages and of lymphocytes (Fig. 6).

A few neutrophils could still be observed, especially in the proximity of venules (Fig.7). At 7 days the cellularity was even more pronounced.

Sprouting capillaries were rare at 24 hrs, but edematous swelling of persisting microvessels was evident (Fig. 8).

They became distinct at 3 days as numerous tiny vessels characterized by an almost nonexistent, linear, split-like luminal opening lined by large endothelial cells containing numerous organelles (Figs. 6, 9, 10).

Endothelial cell mitoses were observed most frequently at 3 and 7 days after embolization. They were especially evident in areas devoid of myocytes but filled with monocytes-macrophages (Fig. 9). Mitosis was also observed in pericytes (Fig. 10).

Legends for Figures 3 to 4: ***Fig. 3.*** *Some myocytes surrounding the fibrotic area are positive for aFGF.* ***Fig. 4 a.*** *Staining for laminin using Texas Red as fluorescent dye shows that the fibrotic area contains a rather significant amount of this protein.* ***b.*** *Staining of the same section with DBA reveals that blood vessels including small arterioles as well as capillaries are numerous in the fibrotic area.* ***c.*** *Double exposure of both staining procedures shows that the newly developed vessels are embedded in laminin.*

In all immunocytochemical illustrations the specific fluorescence is green or yellowish-green due to the staining with fluoroisothiocyanate (FITC). Nuclei are stained red with propidium iodide, or they are orange because of overlay of green from FITC with propidium iodide. All magnifications are X 150, except when mentioned otherwise. In printing, however, the illustrations and therefore the magnifications have been reduced to 80% of the original.

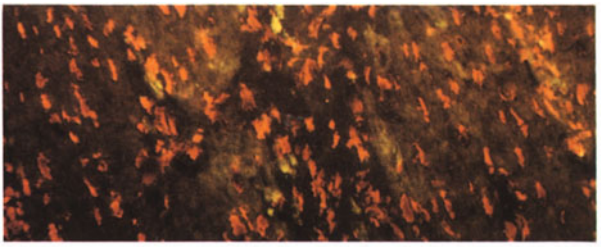


Fig. 3

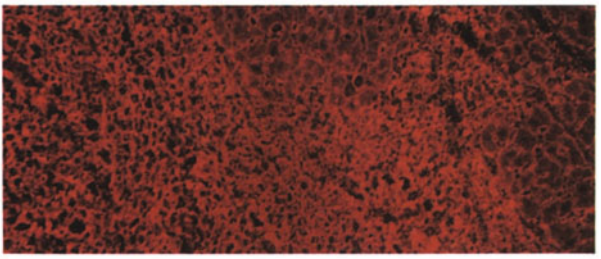


Fig. 4a

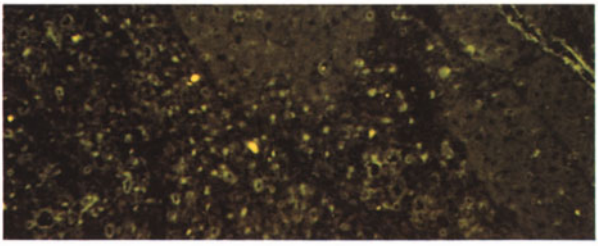


Fig. 4b

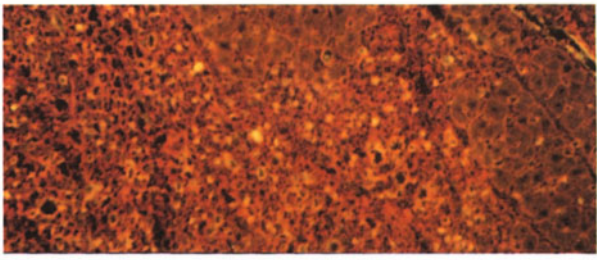


Fig. 4c

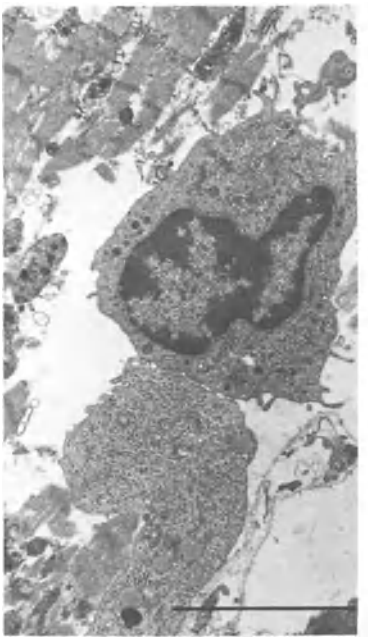


Fig. 5. Macrophages (center) are invading irreversibly injured myocardial cells (left). bar=5μm

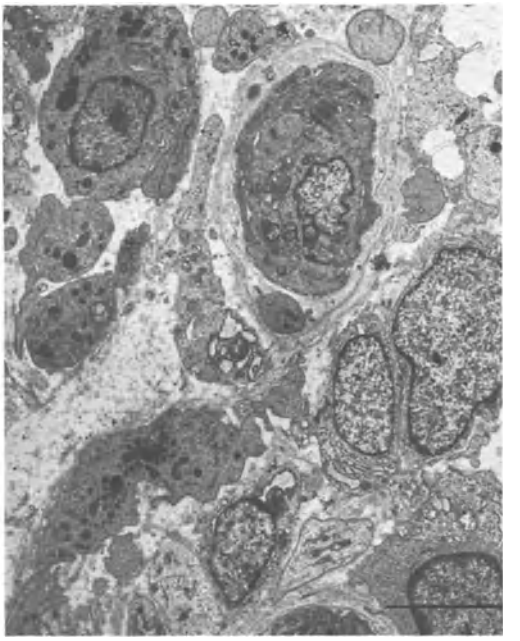


Fig. 6. Macrophages in different stages of maturation are the main constituents of mononuclear infiltrates. Top center: a sprouting capillary (cap) surrounded by multiple basement membranes. bar=5μm

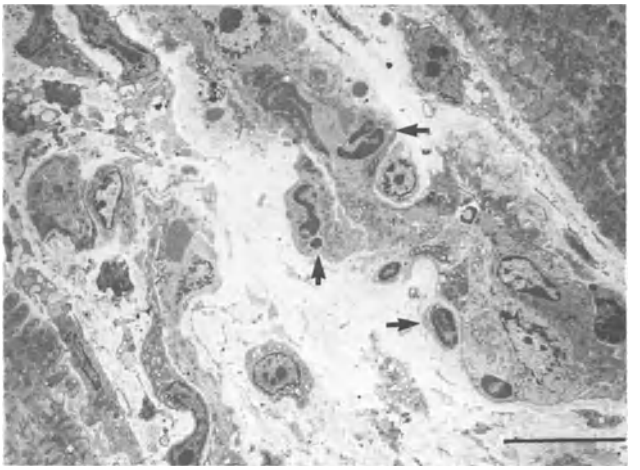


Fig. 7. The interstitial space is filled with neutrophils (arrow) and numerous macrophages. The myocytes are reversibly injured. bar=10μm

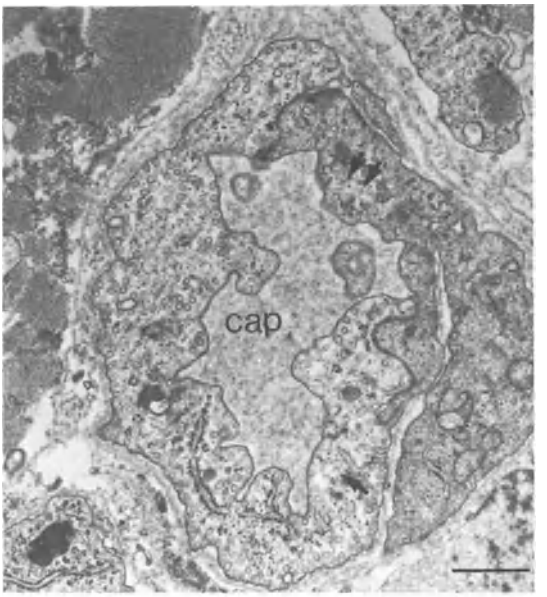


Fig. 8. A capillary (cap) persisting after ischemic injury shows one edematous (arrow) and one normal (double arrow) endothelial cell. Part of an irreversibly injured myocyte is situated at the left side. bar=1 μm

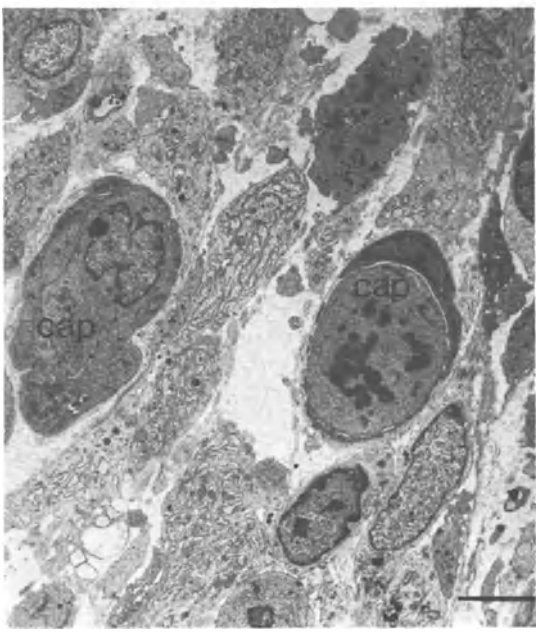


Fig. 9. Necrotic area containing 2 microvessels (cap) and numerous macrophages. The capillary at right shows mitosis of the endothelium. bar=5 μm

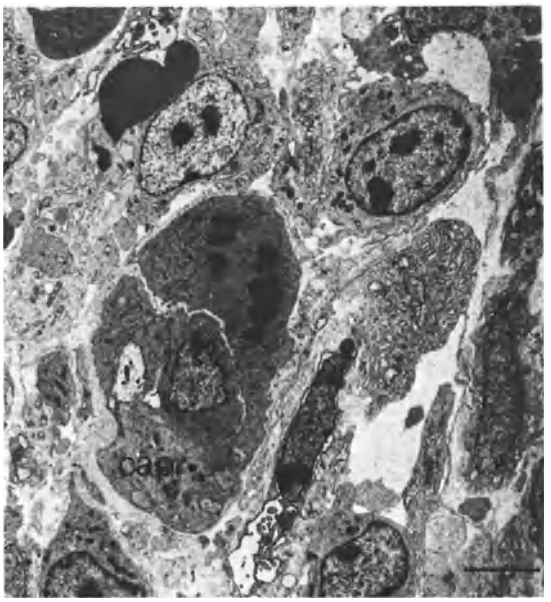


Fig. 10. Necrotic area containing a microvessel (cap) and macrophages. A pericyte is in mitosis (center). bar=5μm

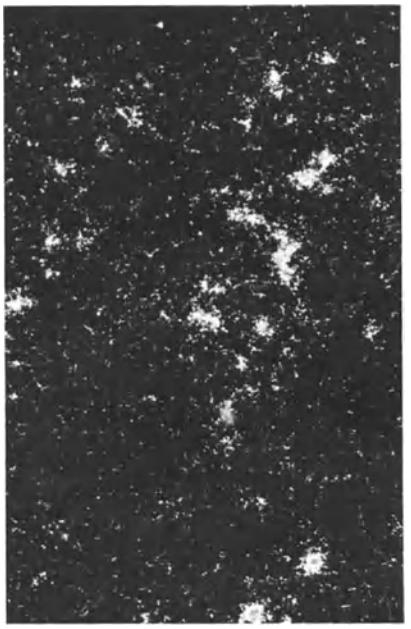


Fig. 11 a. In situ hybridization for histone H-3 Darkfield picture showing labelling of several cells.

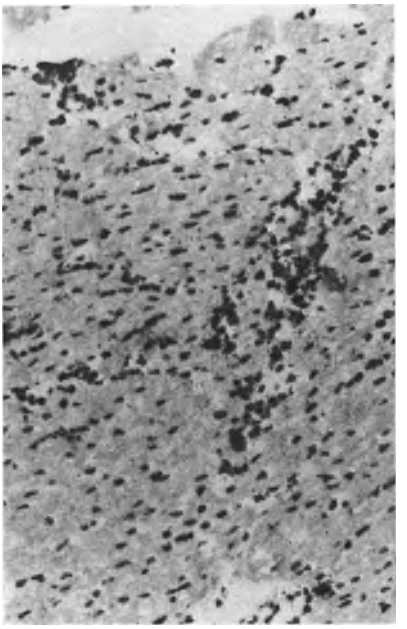


Fig. 11 b. Bright field picture of the same section showing labelling in the infiltrate (top right).

In situ hybridization with the histone H-3 also showed an elevation of mitotic activity by the increased labelling of cells in the necrotic area (Fig. 11).

Myocytes irreversibly injured by ischemia were degraded and removed by neutrophils and macrophages. Viable cells were seen at 7 days intermingled with cellular debris and blood cells (Fig. 12). At 4 weeks, the necrotic myocytes had disappeared.

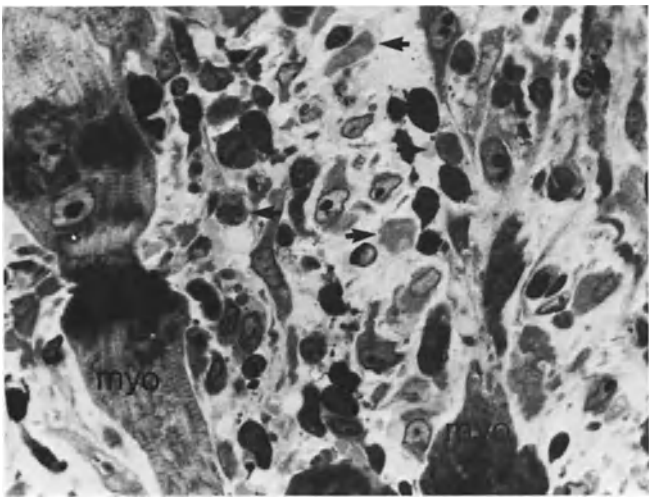


Fig. 12. Light micrograph of a fibrotic area. Surviving myocytes (myo), fibroblasts, a few lymphocytes and macrophages are evident. Remnants of irreversibly injured myocytes are still visible (arrow). bar=20μm

Table 1. Appearance of cells following micronecroses in the myocardium of pigs.

Cell type	Day 1	Day 3	Day 7	4 Weeks
Neutrophils	++	+++		
Endothelium in Mitosis		+++	+	
Monocytes-Macrophages		+++	++	++
Lymphocytes			+	+

+ few ++ numerous +++ many

The time course of these changes is summarized in Table 1.

From these ultrastructural data it was concluded that endothelial cell proliferation occurs as early as 3 days after lethal myocyte injury and that monocytes are especially important in this process.

The extracellular matrix and aFGF in angiogenesis

Results: In the necrotic area, labelled microvessels were present as early as 24 hrs after microembolization. These vessels were more numerous at 3 and 7 days but their number was somewhat decreased after 4 weeks (Figs. 16 and 20). Fibronectin, laminin, vimentin, and the collagens are the main components of the extracellular matrix. Fibronectin is the matrix in which the fibrillar elements collagen I, III and VI and the interstitial cells are embedded. Laminin and collagen IV are found in the basement membrane surrounding endothelial cells and myocytes. Vimentin is an intermediate filament of fibroblasts, fibrocytes and endothelial cells. Figures 13 and 14 show the distribution of laminin and fibronectin in normal porcine myocardium.

Fibronectin was the first of the extracellular matrix proteins to appear in the necrotic area 24 hrs after embolization (Fig. 15). It appeared not only in the extracellular space but also within myocardial cells, most probably by diffusion of plasma fibronectin (17, 18, 19) through leaky membranes of myocytes irreversibly injured by ischemia. At 3 days, fibronectin was abundant, laminin increased (Figs. 16-18) and after 7 days both stained strongly (Figs.19-21). At 4 weeks, fibronectin as well as laminin were still evident (Figs. 22 and 23).

Fig. 13. *Laminin in normal myocardium surrounds all myocytes and blood vessels. Red nuclei in the interstitial space are fibroblasts that do not contain laminin.* ***Fig. 14.*** *Fibronectin in normal myocardium is distributed evenly between myocytes and around blood vessels (middle of right hand side).* ***Fig. 15.*** *Fibronectin at 24 hrs is seen within myocardial cells.* ***Fig. 16.*** *Staining of microvessels with DBA, necrotic area at 3 days* ***Fig. 17.*** *Fibronectin at 3 days is abundant in the necrotic area.* ***Fig. 18.*** *Laminin at 3 days (fluorescent dye is Texas Red) is increased in the necrotic area. The red dot at the left is a microsphere.* ***Fig. 19.*** *Laminin at 7 days is significantly increased.*
Fig. 20. *The number of microvessels of the same tissue section is also increased (DBA).* ***Fig. 21.*** *Double illumination for laminin and DBA shows that the microvessels are situated in the laminin containing area.* ***Fig. 22.*** *Laminin at 4 weeks is still present in the scar tissue between myocardial cells (center).* ***Fig. 23.*** *Fibronectin at 4 weeks is still present in the scar tissue. Top: several surviving myocytes*

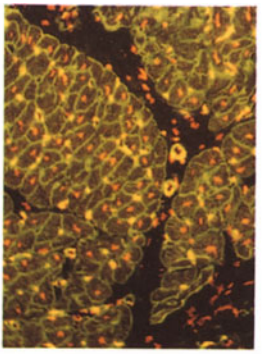


Fig. 13

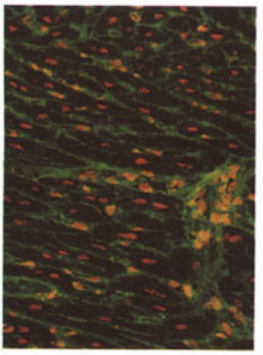


Fig. 14

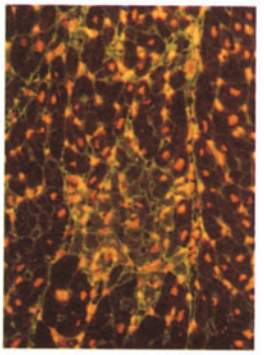


Fig. 15

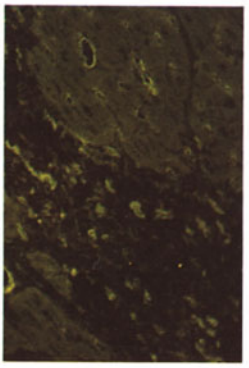


Fig. 16

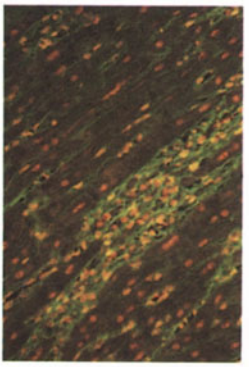


Fig. 17

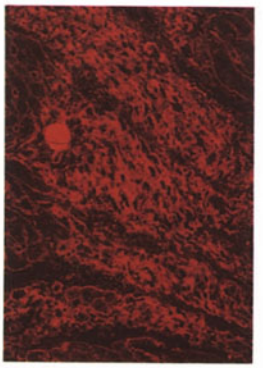


Fig. 18

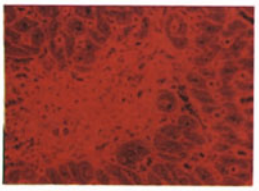


Fig. 19

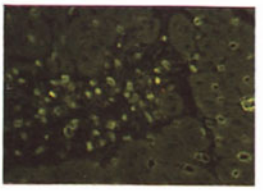


Fig. 20

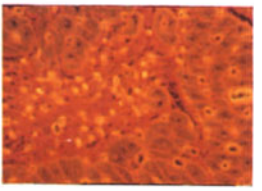


Fig. 21

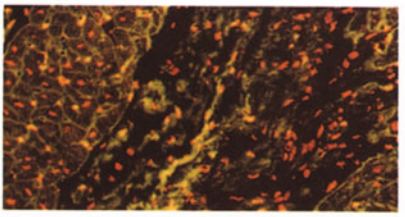


Fig. 22

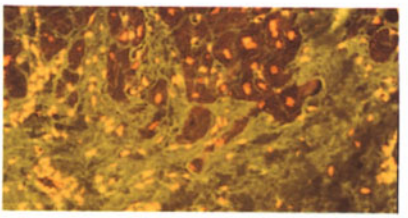


Fig. 23

Vimentin stained weakly at 3 days, but became prominent within numerous fibroblasts and endothelial cells after 1 and 4 weeks (Figs. 24 and 25). The collagens I, III, and VI showed slight labelling at 1 week, and this was increased after 4 weeks (Figs. 26-28). Thus, at 4 weeks, the scar tissue contained numerous fibroblasts and microvascular endothelial cells. Collagens I, III, and VI were more abundant than laminin or fibronectin. To complete the findings, the localization of aFGF at 3 days is shown here (Fig. 29). The labelling of myocytes around the area of injury is similar to that described and discussed in the chapter by Dr. Mohri. ***All data are summarized in Table 2***

Table 2. Appearance of various proteins following micronecroses of pig myocardium.

Protein	6h.	12h.	24h.	3 Days	7 Days	4 weeks
PAI-1	+*	+*	+*	++	++	
uPA	+	++	+++	+++	+++	
Fibronectin			+*	+++	+++	++
aFGF			+	+++	+++	++
Laminin				++	+++	+
DBA				++	+++	+
Vimentin				+	++	+++
Collagen I/III/VI					++	+++

+ small ++ medium +++ large amounts

*Data for uPA, PAI-1 and aFGF were obtained by Dr. Mohri. * proteins probably taken up from plasma*

From these data it becomes evident that the formation of new vessels after myocardial necrosis occurs as early as 3 days after the insult. Endothelial cells are known to be more resistant to ischemic injury than myocytes (20, 21) and therefore it is possible that some of the endothelial cells from persisting capillaries serve as stem cells for new angiogenesis. Monocytes – macrophages may be involved in this process.

Fig. 24. *Vimentin in normal myocardium is visible in all fibroblasts and endothelial cells between the dark myocytes.* ***Fig. 25.*** *Vimentin positive cells are numerous in the scar tissue after 4 weeks of embolization. At top center an unstained myocardial cell.* ***Fig. 26 a.*** *Collagen III in normal myocardium is present in fine septa between myocardial cells.* ***b.*** *Collagen III at 1 week is accumulated in the necrotic-fibrotic area (bottom, right).* ***c.*** *Collagen III at 4 weeks is abundant in scar tissue. Collagen I showed a similar labelling pattern.* ***Fig. 27.*** *Collagen VI in normal myocardium is present in fine septa between myocytes and around blood vessels.* ***Fig. 28.*** *Collagen VI at 1 week is increased in the necrotic-fibrotic area.* ***Fig. 29a .*** *Acidic fibroblast growth factor is present in myocytes around a mononuclear infiltrate. The orange dot at the right hand side is a microsphere. Magn. X 470* ***b.*** *Control with omission of the first antibody. Only the nuclei are stained.*

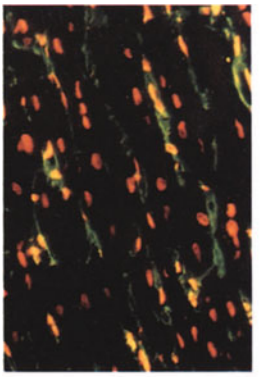


Fig. 24

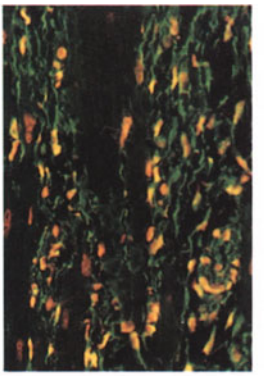


Fig. 25

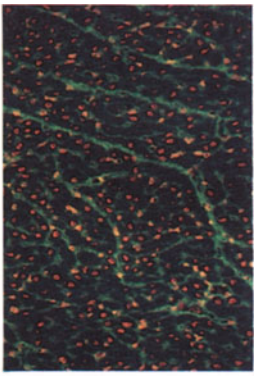


Fig. 26a

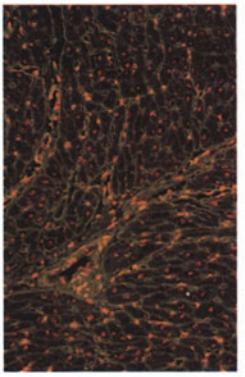


Fig. 27

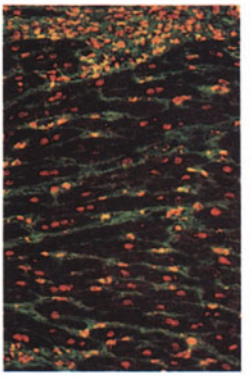


Fig. 28

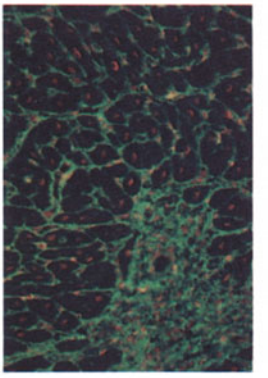


Fig. 26b

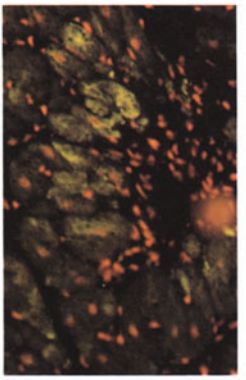


Fig. 29a

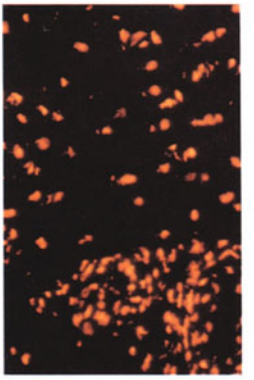


Fig. 29b

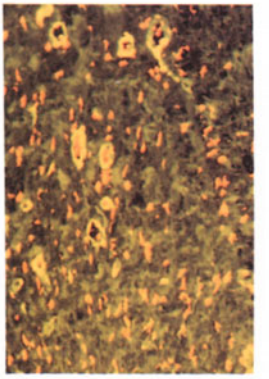


Fig. 26c

General data and Discussion

Fibronectin, a dimeric glycoprotein with a molecular weight of 400 kD occurs in plasma, body fluids and in tissues in a large number of animal species including all mammals (22). The molecule exhibits numerous binding domains, e.g. for collagen, heparin, fibrin and others, and it possesses specific cell attachment sites (22). Specific receptors, the integrins (23, 24), bind fibronectin to cells and via talin-α-actinin-vinculin to the intracellular cytoskeleton.

Fibronectin promotes the adhesion and spreading of cultured cells (5), it plays a role in blood coagulation and wound healing (25, 26, 27) and it has a growth regulatory function in embryonic tissue (28).

Laminin, the most abundant glycoprotein in basement membranes (29, 30), has both structural and biologically active roles (6). It is a cruciform molecule with 3 short and one long arm that exhibits binding sites for entactin-nidogen to bind to collagen IV, for heparin and other proteins, and it has specialized cellular attachment sites (31). The integrins (23) serve as cellular receptors for laminin as well and interact with the cytoskeleton (32). Whereas fibronectin is distributed throughout the entire extracellular space and serves as a matrix for cellular and fibrillar components of the interstitium (22, 33), laminin is confined to the basement membrane of endothelial cells, of myocytes and smooth muscle cells (6, 7, 33). Basement membranes and therefore laminin and collagen IV are essential for the function of endothelial as well as epithelial cells (34), and endothelial cells can rapidly grow and differentiate on a basement-like matrix (35).

In vitro studies have shown that both ***laminin and fibronectin*** are involved in endothelial cell migration, proliferation and multicellular organization as well as in the formation of capillary-like structures (36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46).

The role of fibronectin in angiogenesis in vivo has first been studied in wound healing (26, 25). Clark et al (47) found that endothelial cell proliferation is accompanied by an increase in fibronectin surrounding the ves-

sel. Capillary endothelial cells contain the mRNA for fibronectin (48) and during normal development they produce fibronectin in large amounts (49). The role of fibronectin in the formation of new blood vessels in adults is not yet entirely clarified, and laminin may be the more important growth factor for angiogenesis. It may be that laminin acts more directly as a growth factor on endothelial cells because of its EGF-like repeat (50), but fibronectin influences cell behaviour involving migration and attachment.

From our studies it was deduced that fibronectin, presumably exudating from plasma through damaged blood vessels, and later produced by fibroblasts and macrophages, is deposited in necrotic areas of the myocardium. It probably promotes migration, adhesion and attachment of endothelial cells and it provides the matrix into which sprouting microvessels grow. Laminin may play a similar role in addition to its growth promoting effect. Laminin is a chemoattractant for neutrophils (51) and promotes the oxidative burst (52) whereas fibronectin stimulates neutrophil degranulation (53), i.e., both extracellular matrix proteins are able to influence neutrophil function. Both proteins may also influence monocytes-macrophages by attracting these cells and by promoting their adhesion (54, 55), especially to fibronectin (56). Activated macrophages then produce more fibronectin which acts as chemoattractant for fibroblasts (57). These cells in their turn produce more fibronectin thus establishing a positive feedback mechanism.

Monocytes attracted by fibronectin most probably release various growth factors that stimulate endothelial cell proliferation. Several studies have shown that sprouting of capillaries is accompanied by destruction of basement membrane and matrix components (58, 59, 60), however even fragments of laminin seem to maintain a growth promoting activity (50).

The relative importance of fibronectin versus laminin for vascular growth has been subject to controversy. Recently, Jerdan et al (61) described that sprouting capillaries immediately reestablish their normal extracellular milieu by producing laminin. On the other hand, delayed appearance of laminin as compared to fibronectin has been described in pig embryonic development (62). Wakui et al (63) investigating capillary sprouting in

human granulation tissue reported, in accordance with the results presented here, that fibronectin appeared earlier than laminin, and that only mature capillaries are associated with both, fibronectin and laminin.

Since the mitogenically active domain 1 of laminin is optimally accessible to cells when it exists as a fragment (50), the assembly of laminin in a basement membrane around newly developing vessels may end its growth factor activity and this may be one of the many steps in the regulation of vascular growth.

Collagen: In our study, the time course of appearance of the collagens type I, III, and VI was similar and they will therefore be discussed as a group. All three types of collagen are interstitial fibers. The thick collagen fibers of tendons and scar tissue consist of collagen I, collagen III is a fine network of fibers surrounding vessels and both types of fibers are important for the regulation of tensile strength and distensibility of the various tissues in mammalian organs (64, 65, 66). Collagen VI also occurs in the interstitium of most organs including the heart. It consists of fine fibrils that seem to be connected to the cytoskeleton by specific receptors (67). Collagen VI, like laminin and fibronectin, supports adhesion and spreading of cultured cells (68).

In vitro regulation of angiogenesis by collagen has been demonstrated in several studies (37, 38, 42, 45, 69, 70, 71). Recently, Nicosia (70) suggested that angiogenesis is regulated by collagen production and that "the size of newly formed microvessels is influenced by the degree of collagenization of the extracellular matrix". The regulatory effect of collagen seems to be due mostly to its function as a template for sprouting endothelial cells and to the promotion of cellular adhesion. Collagen, however, probably plays a less decisive role in angiogenesis in our myocardial microembolization model, because it appears much later than fibronectin and laminin but it may influence the size of the vessels.

The interaction of fibronectin and collagen (72, 73) and their role in healing after myocardial injury has been investigated by several groups (17, 18, 74, 75, 76). Knowlton (75) has recently shown that the mRNA for fibronectin appears as rapidly as 72 hrs after coronary occlusion in the

rabbit heart, and Kleinman (77, 78) and McDonald (79, 80) demonstrated the importance of fibronectin for collagen deposition. These data are in accordance with our findings showing that at one week after microembolization the collagens start to appear whereas fibronectin is present at much earlier time points. We therefore believe that fibronectin and laminin are the most significant components to influence angiogenesis, whereas the collagens modify the final structure of the vessels and are important in scar formation.

Vimentin: Staining with an antibody to vimentin, an intermediate filament of mesenchymal cells (81), was used as an indicator of the number of fibroblasts and endothelial cells in the area of interest. It is noteworthy that even at 4 weeks there were still numerous vimentin positive cells present. This is in accordance with the persistence of numerous blood vessels shown by DBA staining at this late stage of repair. Capillary regression may occur at later stages when collagen deposition has reached a more advanced degree and therefore is able to inhibit angiogenesis (42). Apparently, the formation of the final scar with very few connective tissue cells and microvessels occurs later than 4 weeks after the insult.

Summary: The development of collateral vessels in the porcine heart after microembolization also occurs at the capillary level and is especially evident in areas of patchy necroses. Angiogenesis occurs by endothelial cell mitosis. Fibronectin appears at the earliest time point after myocardial injury, laminin appears later, and the collagens show the latest response. Fibronectin and laminin appear to influence endothelial cell behaviour during angiogenesis, and a cellular infiltrate of neutrophils, lymphocytes, and especially monocytes-macrophages, may play an additional role in stimulating angiogenesis by supplying growth factors and cytokines. It seems that the areas of organizing patchy necrosis with their increased vascularization may serve as starting points for the formation of collaterals, thus combining the process of wound healing with that of collateralization.

COLLATERAL DEVELOPMENT IN THE CANINE HEART

The constrictor model for studies of angiogenesis

In contrast to the situation in the pig heart, collateral development in the dog heart takes place in preexisting epicardial arteries (1).

An ameroid constrictor was placed around the circumflex artery in mongrel dogs and the animals were sacrificed 4 or 8 weeks later. Hearts showing the presence of infarction or an open constrictor were excluded from the study. Collateral vessels were found on the epicardial surface of the anterior left ventricular wall. These were removed including a rim of surrounding myocardium, fixed in buffered glutaraldehyde and examined by light and electron microscopy, or they were immediately frozen in liquid nitrogen and investigated using immunocytochemistry and in situ hybridi-

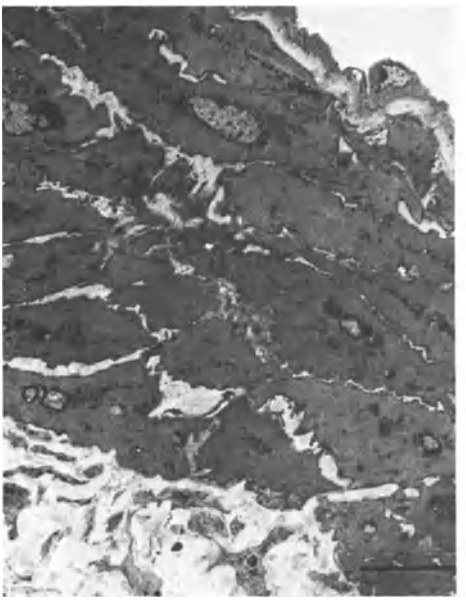


Fig. 30. Arteriole with normal wall structure. The lumen is at the top. The endothelium is normal, the elastic lamina present, and the media contains 5 layers of smooth muscle cells. At the bottom there is a small part of the adventitia. bar=10μm

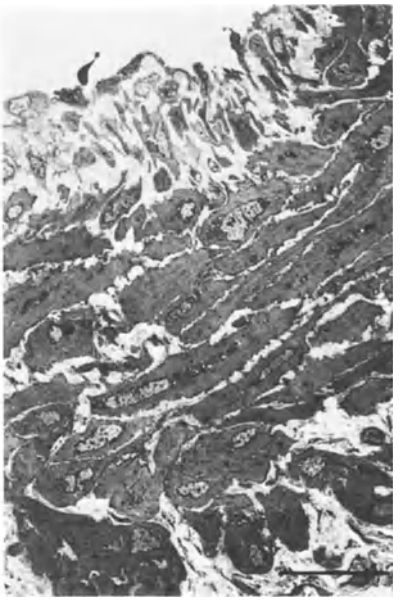


Fig. 31a. Artery with subintimal proliferation zone containing numerous smooth muscle cells. The elastic lamina is absent. bar=20μm.

zation. The following proteins were studied: vinculin, desmin, vimentin, aFGF, tumor necrosis factor (TNF-α), laminin, fibronectin, and the collagens.

Normal coronary arteries were used as control tissue in all staining procedures.

Morphology: Three different types of collaterals could be identified by light and electron microscopy: a. arteries of various sizes with an orderly wall structure and a prominent internal elastic lamina (Fig. 30); b. large and medium sized vessels showing a disorganization of the wall, a lack of the elastic lamina and significant subintimal thickening (Figs. 31a and b); c. smaller disorganized, intramural arteries (Fig. 32) that probably were branches of a larger collateral vessel also involved in the growth process.

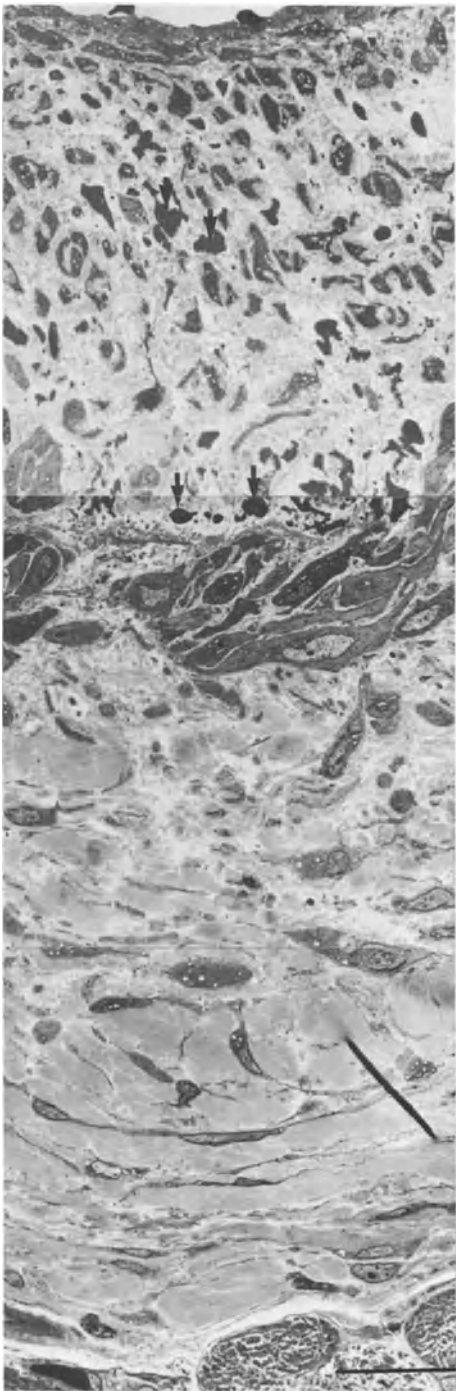


Fig. 31b. Artery with extremely wide subintimal proliferation zone containing numerous smooth muscle cells, necrotic cells (arrows) and a large amount of connective tissue. The elastic lamina is absent. The original media contains only 3-4 layers of smooth muscle cells and is situated in the center of the picture. The adventitia is wide and consists of fibroblasts, collagen fibers and other extracellular matrix proteins. Myocytes are present at the bottom of the picture. bar=20μm

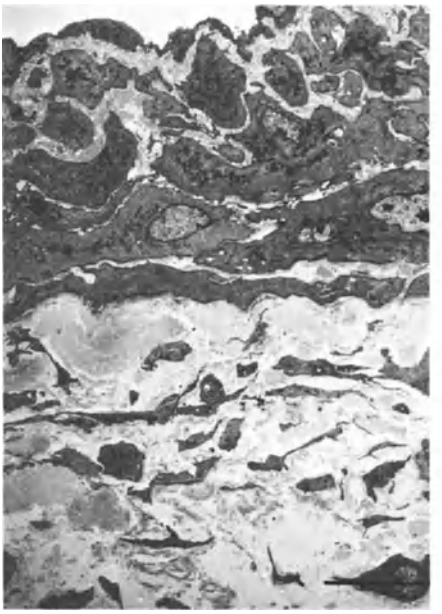


Fig. 32. Small disorganized blood vessel with lack of elastic lamina and "modified" smooth muscle cells underneath the endothelium. bar=10μm

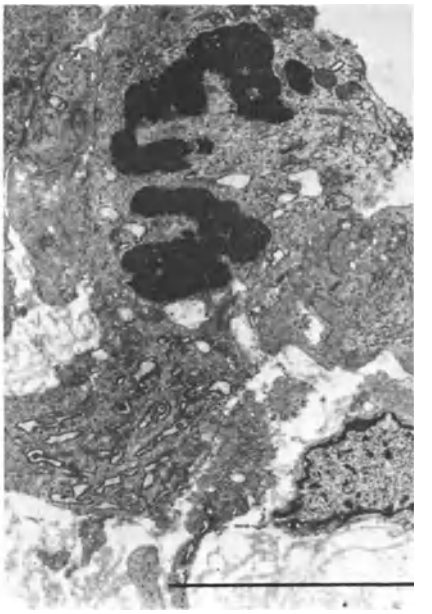


Fig. 33a. Endothelial cell mitosis in a growing collateral arteriole. bar=5 μm

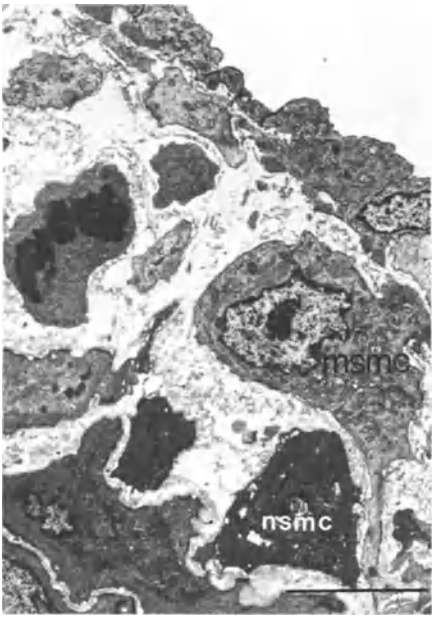


Fig. 33b. Growing collateral vessel exhibiting endothelial cells with numerous cellular organelles, lack of the elastic lamina, modified (msmc) and necrotic (nsmc) smooth muscle cells and one smooth muscle cell in mitosis. bar=25μm

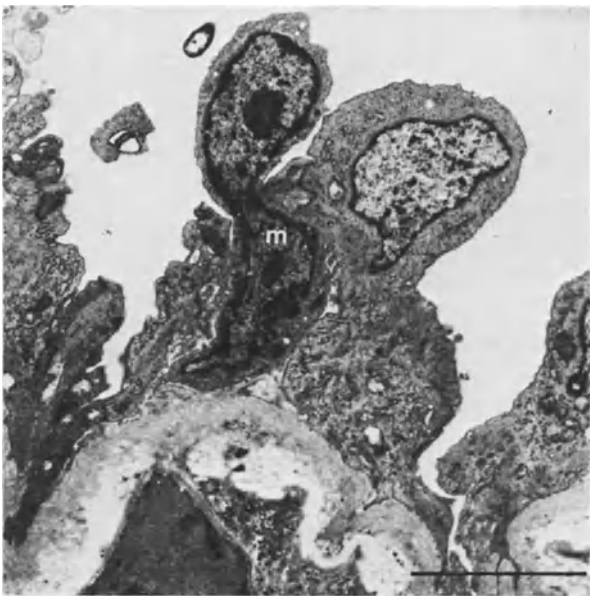


Fig. 34. Monocyte (m) penetrating the endothelial layer. bar=5μm

Endothelial cell and smooth muscle cell mitoses were observed (Fig. 33). Monocytes were seen adhering to endothelial cells or invading the subintima (Fig. 34).

The smooth muscle cells of the subintimal layer frequently were dedifferentiated, i.e., they had lost a certain amount of their contractile filaments but contained numerous cellular organelles such as mitochondria, Golgi apparatus and rough endoplasmic reticulum. Necrotic smooth muscle cells were also observed. It was obvious that the number of mast cells around the growing arteries was greatly increased. The morphology of canine coronary collateral vessels, however, has already been described extensively (82, 83, 84, 85) and will therefore not be discussed at length here.

In this series there were no clear-cut, time-dependent differences in vessel structure which was most probably caused by an early occlusion of the constrictor (8-10 days) so that the stage of early destruction of the vessel had long been passed, and it was also concluded that the rate of collateral growth was a matter of individual variability between animals.

Proteins involved in collateral growth in the dog heart

Vinculin: Vinculin is a 130 kDa protein that was detected in adhesion plaques of cultured cells and purified by Geiger (86), Feramisco (87) and Burridge (88). Vinculin occurs in most cell types (89) including leukocytes and platelets. In cardiac muscle vinculin occurs at the plasma membrane of myocytes localized at the costameres (90, 91), but in smooth muscle it is not connected with the cytoplasmic dense bodies (92). Vinculin is localized at the cellular membranes and it seems to play a role in transmembrane linkage (93). Vinculin has been shown to bind directly to talin (94, 95) and to the actin cross linking protein α-actinin which also binds to talin. The latter interacts with the fibronectin receptor integrin and therefore with the extracellular matrix (89, 94, 96). A tight connection between the intracellular milieu with the extracellular matrix proteins is thereby ensured.

In normal arteries of dog myocardium, vinculin was localized in the smooth muscle cells of the media (Fig. 35). The endothelium stained strongly in veins while in arteries it showed only slight staining. In collateral vessels, vinculin stained similarly except for weaker staining in the subintimal zone of remodelling (Figs. 36 and 37). These cells in the subintima that did not stain for vinculin could be "modified" smooth muscle cells or fibroblast-like cells or monocytes.

Desmin: Desmin belongs to the family of intermediate filaments and it occurs in all types of muscle tissue including vascular smooth muscle (97, 98, 99, 100). The relative proportion of vimentin and desmin varies in different smooth muscle tissues (101, 102, 103), but in coronary arteries, desmin is strongly stained in the cells of the media. The function of desmin like that of vimentin concerns cell stabilization as part of the cytoskeleton. It may also play a role in signal transmission from the cell membrane to the nucleus (104, 105).

In normal blood vessels no desmin is found in the endothelium (Fig. 38), but the smooth muscle cells show intensive staining.

In developing collateral arteries, desmin is well seen in the media but only a few cells are labelled in the subintimal layer (Fig. 39). This staining

pattern resembles that for vinculin which also failed to stain the subintimal cells. It is possible that immature smooth muscle cells proliferating in the subintima express more vimentin than desmin resembling cells during embryonic development (106, 107) and it can be envisaged that this staining pattern will reverse at later stages of vascular growth.

Desmin is clearly seen in all cardiomyocytes labelling the Z-bands and the intercalated discs (108). Therefore, it was easy to detect that the myocytes closest to the vascular adventitia were small and degenerated (Fig. 39). This was confirmed by ultrastructural observation. This myocyte pathology most probably has been caused by the early inflammation followed by repair processes that usually accompany the beginning of vascular growth (82).

Vimentin: Vimentin also belongs to the family of intermediate filaments that possess a thickness of about 10 nm and occur in endothelial cells, fibroblasts and fibrocytes (81, 109). In early embryonic development muscle cells contain both, vimentin and desmin filaments, but in more mature stages vimentin is replaced by desmin (106, 107, 110). The functional importance of vimentin ist not yet clear, but as a component of the cytoskeleton it seems to have an essential role in ensuring cellular stability.

According to Traub (81), intermediate filaments and their derivatives show preferential binding to single-stranded DNA and to histones. This suggests that their final site of action is in the nucleus. It is also possible (105) that intermediate filaments and their associated proteins represent interacting "linkage elements" which connect the nucleus and the cell surface.

In normal blood vessels including the microvasculature, endothelial cells and the fibroblasts of the adventitia contain vimentin (Fig. 40).

In collateral blood vessels, however, vimentin is seen in the media of two different types of arteries: 1. in the disorganized growing vessels where staining is intense throughout the entire vessel wall (Fig. 41) and 2. in the more advanced stages of vessel development where the staining in the media persisted (Fig. 42).

This is different to the staining pattern in normal coronary arteries and may indicate that the cellular components of the media and the subin-

tima are either very immature smooth muscle cells still synthetizing vimentin or that the numerous "modified smooth muscle cells" are related more to fibroblasts than to smooth muscle cells.

An increase in both, desmin and vimentin, has been reported to occur in smooth muscle cells of rat portal veins (102, 111) hypertrophied because of increased pressure overload. This would resemble our results in the reappearance of vimentin in medial smooth muscle cells in abnormal conditions.

Since smooth muscle cells in culture are able to produce vimentin (Fig. 43) and vascular smooth muscle cells coexpress desmin and vimentin in various proportions (110, 112), we believe that the presence of vimentin in the smooth muscle cells of developing collateral vessels is indicative of a regulatory process. Vimentin may be produced in response to the hemodynamic "overload" imposed on collateral vessels (see chapter by Dr. Wolfgang Schaper) during development, and it may be functional in signal transmission from the cell membrane to the nucleus. Further studies need to be done to clarify this hypothesis.

Fig. 35. *Vinculin in a normal artery (cut longitudinally) is present in the media and in the intercalated discs of myocytes.* ***Fig. 36.*** *Growing collateral showing a subintimal proliferation with less intense staining for vinculin than in the media.* ***Fig. 37.*** *Collateral vessel with pronounced subintimal proliferation shows less intense staining for vinculin in this zone. Endothelial cells are negative.* ***Fig. 38.*** *Desmin in a normal artery (cut longitudinally) is present in smooth muscle cells of the media but not in endothelial cells. Myocytes are stained.* ***Fig. 39.*** *Collateral artery with a proliferation zone that is not stained by desmin (top, left hand side). The cardiomyocytes close to the vascular adventitia are smaller than normal.* ***Fig. 40.*** *Vimentin is present in the endothelium but absent from the media of a normal artery. Green autofluorescence of the elastic lamina is evident.* ***Fig. 41.*** *Vimentin is present in all vascular layers of a small growing collateral vessel.* ***Fig. 42.*** *Staining for vimentin persists in collateral vessels after the remodelling phase.* ***Fig. 43.*** *Cultured smooth muscle cell positively stained for vimentin. Magn. X 470*

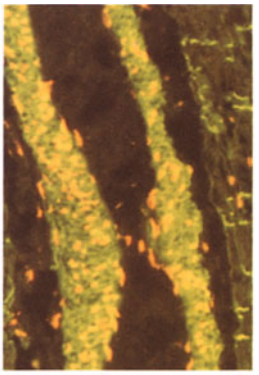


Fig. 35

Fig. 36

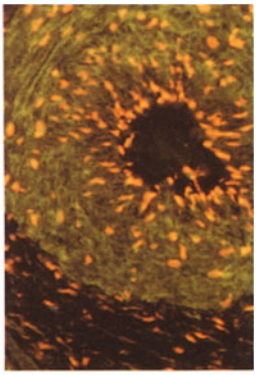
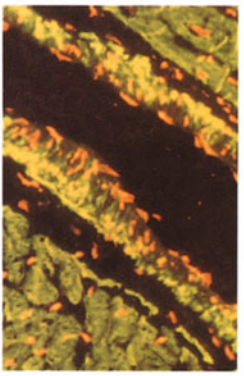


Fig. 37



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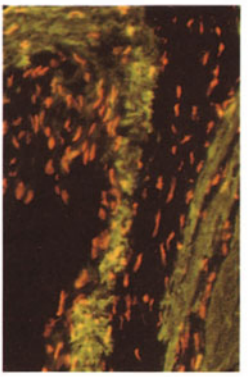

Fig. 39

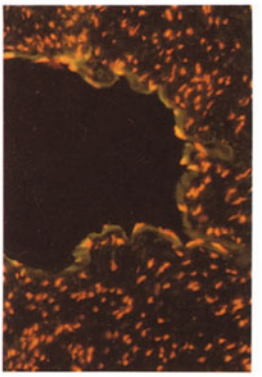

Fig. 40

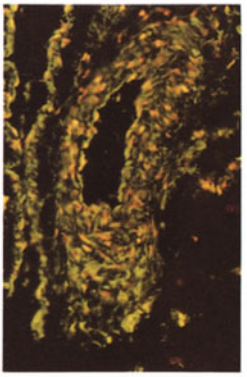

Fig. 41

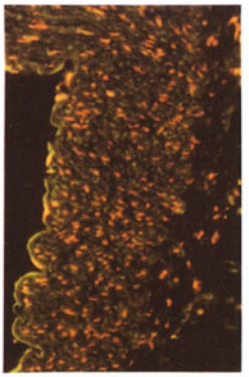

Fig. 42

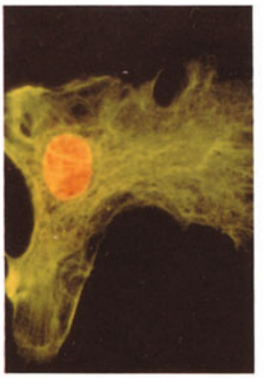

Fig. 43

Acidic fibroblast growth factor (aFGF)

In normal myocardium and coronary vessels, there is no immunocytochemical staining for aFGF. It is, however, seen in myocytes surrounding growing collateral vessels, especially close to disorganized arteries, but also in the vicinity of remodelled collaterals (Fig. 44). Acidic FGF is also occasionally observed in myocytes near small necrotic foci resembling the labelling for aFGF in pig hearts with microembolization (see chapter by Dr. Mohri). It was obvious that aFGF was present only in myocytes showing signs of injury and degeneration. These cells were smaller and thinner than normal, and by electron microscopy revealed a very irregular shape, loss of contractile material, and disorganization of the subcellular organelles. Whether these myocytes produced aFGF or took it up from the extracellular space, is still uncertain. Increased intracellular levels of aFGF may have trophic functions and may be necessary for cell survival.

Several larger, remodelled collateral arteries exhibited aFGF in the media as well as in the subintima, and in very small amounts also in the endothelial cells (Fig. 45).

This was explained as proof that aFGF was one of the growth factors involved in collateral growth, and that it was stored in cells and in the extracellular matrix of the vascular wall after completion of the growth process.

An antibody against platelet derived growth factor (PDGF) (kindly provided by Mochido Pharmaceutical Co., Ltd, Tokyo, Japan) was also tested on porcine and canine blood vessels and myocardium. This antibody was developed against human PDGF and was unfortunately unreactive in animal tissue. An antibody against basic FGF was also negative. The role of these two growth factors, therefore, is still unclear and needs to be clarified in the future.

Tumor necrosis factor-α (TNF-α)

TNF-α is the product of monocytes-macrophages and plays a role in the early inflammatory process, inhibits endothelial cell proliferation, enhances the production of other cytokines and promotes collagen production by fibroblasts (113). TNF-α positive cells, most probably macrophages, were rarely seen in normal canine myocardium or around blood vessels, but they were present in growing collateral arteries (the antibody

was kindly provided by Miles/Bayer AG, USA. Monocytes labelled for TNF-α attached to endothelial cells (Fig. 46) and were present in the media of growing vessels and in adjacent myocardium (Fig. 47) indicating that this cytokine plays a role in vascular growth.

Leibovich et al (114) reported that activated macrophages possess an angiogenic activity that is completely blocked by pretreatment with an antibody against TNF-a and they concluded therefore, that TNF-α promotes vessel growth. This finding would support the idea that TNF-α plays a role in stimulating the development of collaterals.

Staining for TGF-ß was positive for capillary and venous endothelium, but the cardiomyocytes were negative. These data are in contrast to those obtained by Wünsch et al and cited in Dr. Sharma's chapter which may be due to the fact that we used a different antibody than his group did. No changes were observed during collateral development, and therefore the data are not included in this text.

Fibronectin and Laminin: Next to the proteoglycans, these substances are the major components of the extracellular matrix. Their structure and function have been described in the part of this chapter on microembolization in the pig heart and will not be repeated here.

The localization of the different types of collagen between the smooth muscle cells of the media and in the adventitia was similar to that of normal vessels.

Fibronectin occurs in the media and adventitia of normal blood vessels in varying, usually very small amounts (Fig. 48). In growing collateral vessels labelling of the vascular wall is evident (Fig. 49). Disorganized vessels contained an increased amount of fibronectin in all the layers (Fig. 50). Larger vessels already in the stage of remodelling have less fibronectin as determined by the the intensity of the fluorescing light. These vessels became more like normal vessels with regard to their content of fibronectin except for the persistence of a slightly labelled subintima (Fig. 51). Smaller arteries surrounded by myocardial cells often showed strong adventitial labelling. Fibronectin, although mostly extracellular, especially in the adventitia, is also found within endothelial and smooth muscle cells

as well as in injured myocytes (Fig.49).

The increase of fibronectin in growing collateral vessels suggests that it participates actively in angiogenesis. It may do so by influencing cellular adhesion and migration, and by stimulation of chemotaxis for monocytes.

Laminin in normal vessels was present in the media because it is the major component of the basement membrane of smooth muscle cells. In growing vessels, it was seen additionally in the subintima (Fig. 52) where it may influence vascular growth through cell migration and attachment.

However, cause and effect relationships are difficult to establish in vivo and only future in vitro culture work will determine what are the particular functions and temporal role of the extracellular matrix proteins in the angiogenic cascade and in the development of collaterals in the dog heart.

Summary: In the dog heart, collateral formation occurs by growth of preexisting epicardial arteriolar vessels, but smaller intramural branches, most probably of the same small artery, are also involved. Growth is characterized by fragmentation of the elastic lamina, proliferation and mitosis of the endothelial and smooth muscle cells, invasion of monocytes and by the development of a subintimal proliferation zone. The adventitia often shows an inflammatory reaction and the surrounding cardiac myocytes degenerate while expressing aFGF. In later stages of growth, a

Fig. 44. *Acidic fibroblast growth factor in myocytes neighbouring the adventitia of a growing collateral vessel.* ***Fig. 45.*** *Acidic fibroblast growth factor in the endothelium and part of the subintimal thickening zone of a growing collateral artery. The myocytes close to the adventitia are also positive and much smaller in size than normal cardiomyocytes (lower left corner).* ***Fig. 46.*** *Tumor necrosis factor-α stains the smooth muscle cells of a growing collateral artery. Additionally, a positively labelled monocyte adheres to the endothelium (arrow).* ***Fig. 47.*** *Growing collateral vessel with smooth muscle cells stained positively for TNF-α. There are 2 positively stained cells, monocytes or macrophages, in the media and one within the myocardium (arrows).* ***Fig. 48.*** *Fibronectin is absent in normal coronary arteries. The faint green fluorescence at the luminal side is due to autofluorescence of the elastic lamina.* ***Fig. 49.*** *Fibronectin is present in all layers of a growing collateral artery.* ***Fig. 50.*** *Fibronectin is abundantly present in a vessel showing an extreme subintimal thickening (small luminal opening in the center).* ***Fig. 51.*** *Slight staining for fibronectin in a remodelled collateral artery at the end of the transformation process.* ***Fig. 52.*** *Laminin is localized in both, the subintimal layer and the media of a growing collateral artery.*

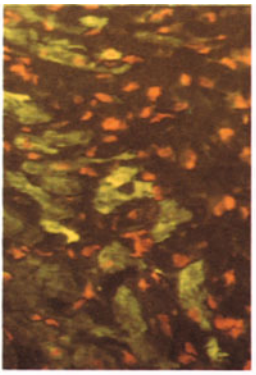

Fig. 44

Fig. 45

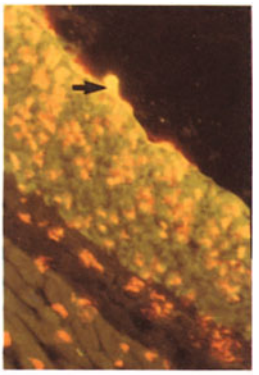

Fig. 46

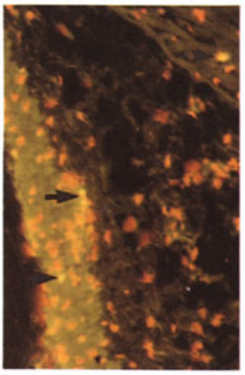

Fig. 47

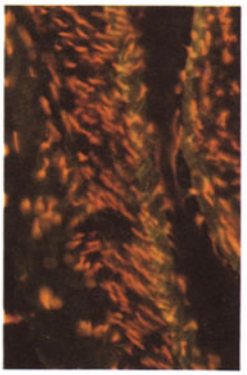

Fig. 48

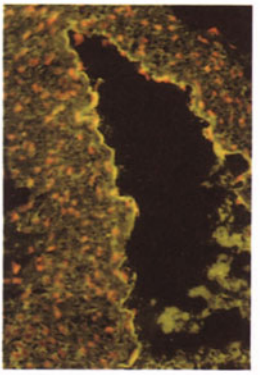

Fig. 49

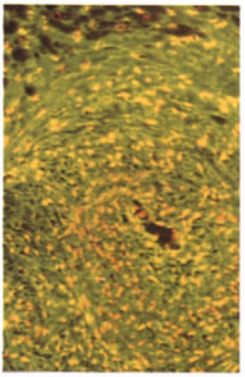

Fig. 50

Fig. 51

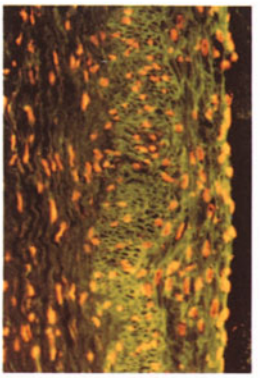

Fig. 52

new elastic lamina is formed and the vascular structure resembles more that of a normal vessels. The smooth muscle cells of the subintima contain no or only slight amounts of vinculin and desmin but are stained intensely for vimentin, indicating the immaturity of the cells. Fibronectin and laminin seem to promote vascular growth, and TNF-α most certainly influences it. Mast cells may play a role.

CONCLUSION

We have studied the expression of specific proteins in growing blood vessels by indirect immunofluorescence in three experimental models known to induce collateral vessel development. In the porcine model, an angiogenic response to vascular occlusion prevailed, i.e., new capillaries sprouted largely within an inflammatory reaction to focal cardiac myocytolysis. Focal necrosis occurred frequently in the ameroid model and was deliberately induced in another experiment by microembolization.This latter model had the advantage that the temporal order of protein expression could be studied in detail. The angiogenic processes in the porcine heart started with the rise of the local concentration of the urokinase-type plasminogen activator (Dr. Mohri) soon to be followed by fibronectin and laminin, by the invasion of leukocytes (neutrophils and monocytes) and by the proliferative activity of resident macrophages. The final result of the vascular adaptation in the porcine ameroid model was large endothelial-lined tubes devoid of smooth muscle.

The in situ growth and differentiation of an arteriole into an artery under the influence of physical and chemical factors, i.e. vasculogenesis, was studied in a canine model of progressive coronary occlusion.

Collateral vessels thus develop in different ways in canine and porcine hearts. In dogs, collateral growth and remodelling processes are rather complex and include proliferation and interaction of endothelial cells, smooth muscle cells, fibroblasts, macrophages and probably mast cells. Judging by the changes in the cytoskeletal proteins desmin, vimentin and vinculin, the dog collateral vessel formation resembles embryonic development of the vasculature. In the pig heart, however, only capillary endothelial cells and macrophages are involved in the growth of collaterals which consists of increased numbers of capillaries. The cytoskeletal pro-

teins do not seem to change in this model.

The extracellular proteins fibronectin and laminin appear to have an active role in collateral growth, similar in both animals. It consists of promoting migration and attachment of endothelial and/or smooth muscle cells and may also involve a growth factor-like activity.

Growth factors do play a prominent role in our models and we can demonstrate their presence on the protein level. In situ hybridization studies for aFGF are now in progress to clarify whether its presence in cardiomyocytes around necrotic areas in pig, or expanding vessels in dog, is due to synthesis or entry from outside.

As demonstrated here, the various factors, cascades and modulatory interactions in the collateral vessel growth process can be studied to advantage by a variety of morphological methods, especially those involving immunocytochemistry and in situ hybridization.

With better understanding of the underlying mechanisms in collateral development a more rational approach to the prevention and therapy of myocardial ischemia can be envisaged.

Acknowledgements: We would like to thank Margarete Arras, D.V.M., Masahiro Mohri, M.D., and Stefan Sack, M.D. who carried out the pig experiments, Zoltan Aranyi, Ph.D. and Sofia Koltai, M.D. (from the National Institute of Cardiology, Budapest, Hungary) who donated some of their canine material and René Zimmermann (Ph.D.), who was our partner for the in situ hybridization studies. We are also grateful to Dehim Mousavipur, M.D., Cornelia Renczes, D.V.M., Markus Schönburg, M.D. and Dimitri Scholz, Ph.D. who carried out the electron microscopy studies, and to our technicians Beate Grohmann, Annemarie Möbs, Renate Möhren and Brigitte Münkel who were involved in all the different techniques in the laboratory. We would also like to thank Rena Yarom, M.D., from the University of Jerusalem, for her constructive criticism of the text.

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6

ANGIOGENESIS IN PORCINE HEARTS WITH CORONARY MICROEMBOLIZATION

Masahiro Mohri and Wolfgang Schaper
Max-Planck-Institute, Department of Experimental Cardiology,
Bad Nauheim, F. R. Germany

INTRODUCTION

Angiogenesis is a complex phenomenon consisting of several distinct processes which include endothelial migration and proliferation, extracellular proteolysis, endothelial differentiation (capillary tube formation), and vascular wall remodeling (1). Although an increasing body of evidence has accumulated regarding to in vitro biological activities of different growth factors and related peptides as well as their roles during in vitro angiogenesis, very little is known about the mechanisms of coronary angiogenesis in the ischemic heart. In 1971, Schaper et al (2) first demonstrated that the enlargement of preexisting coronary anastomoses, mostly of arteriolar size, was accompanied by proliferative activities, i.e. DNA synthesis, of vascular endothelial cells in dogs subjected to chronic left circumflex arterial occlusion. This was later shown to be true also in ischemic porcine hearts, where endothelial cells of various sizes of vessels were found to be proliferating, most prominent at the capillary level (3, 4). These findings have led us to the recognition that coronary collateral development is much more than a simple mechanical dilatation of native anastomotic channels. This chapter will focus mainly on microvascular adaptations that are commonly developed in ischemic porcine and human hearts.

Recently, we have demonstrated that expression of a potent mitogenic peptide growth factor, acidic fibroblast growth factor (FGF), is enhanced both at transcriptional and protein levels in ischemic porcine myocardium after implantation of an ameroid constrictor (5, 6). These findings not only have implicated a possible contribution of peptide growth factor in coronary angiogenesis (for more detailed discussion see chapter by Dr. W. Schaper), but also have raised new questions; what kind of growth factor(s) or related peptides are expressed during angiogenesis?; and when? We have hypothesized that an initial signal for triggering the whole cascade of vascular growth may come from ischemic myocytes, and therefore it is of great importance to define not only spatial but also temporal relationship between an ischemic insult and occurrence of angiogenic

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

responses. Apparently, the ameroid model would not be an ideal means to address these questions, since it is difficult to precisely determine the onset of myocardial ischemia in this model. The hygroscopic ameroid material gradually constricts the vessel and leads to a complete occlusion within 3-4 weeks. Clearly, the onset of myocardial ischemia depends on many factors including the rate of constriction, intraluminal thrombus formation at the stenotic portion, and the level of myocardial oxygen consumption.

THE CORONARY MICROEMBOLIZATION MODEL AS A POSSIBLE ALTERNATIVE FOR STUDYING CORONARY ANGIOGENESIS

Chilian and his colleagues reported a unique model for studying the pathophysiology of coronary collateral growth (7). They embolized coronary microvasculatures of dogs with 25 μm plastic microspheres and found an increase in retrograde flow, an indirect measurement of collateral flow, at 1-3 and 6-8 weeks after microembolization. This was accompanied by the restoration of minimal coronary vascular resistance which suggested the development of new collateral channels. As compared with the ameroid model, this methodology has a great advantage for studies of in vivo angiogenesis in that the onset of ischemia is evident, i.e. at the moment of microsphere injection. Thus, this model seems to be suited for studies on the time course of growth factor expression after an ischemic stimulus. Furthermore, the injection of microspheres could be performed using a standard coronary catheterization procedure and would not require open-chest surgery. These features allow to avoid the effects of non-specific inflammatory reactions related to thoracotomy on the processes under study.

Coronary microembolization in pigs

We aimed to establish a closed-chest porcine microembolization model to investigate growth factor expression in ischemic hearts. In the first example, we used an open-chest preparation to examine the relationship between the number of injected microspheres and the effect on coronary vasodilatory reserve by directly measuring coronary blood flow. After left-sided thoracotomy, the left circumflex (LCx) coronary artery was dissected free, and a coronary blood flow volume probe was placed. Before and after microembolization with non-radioactive polystyrene microspheres of 25 μm diameter into the LCx artery, coronary flow reserve was determined by measuring a reactive hyperemic response to a transient 20-s LCx occlusion. Microspheres were

repeatedly injected until reactive hyperemia was almost abolished with basal LCx flow minimally affected (Fig. 1A). In all pigs with embolization, apparent ischemic electrocardiographic changes were observed. In some pigs, the measurement of coronary flow reserve was repeated at 7 days after embolization. A coronary flow probe was placed again at the same site as in the first measurement, and reactive hyperemic response was

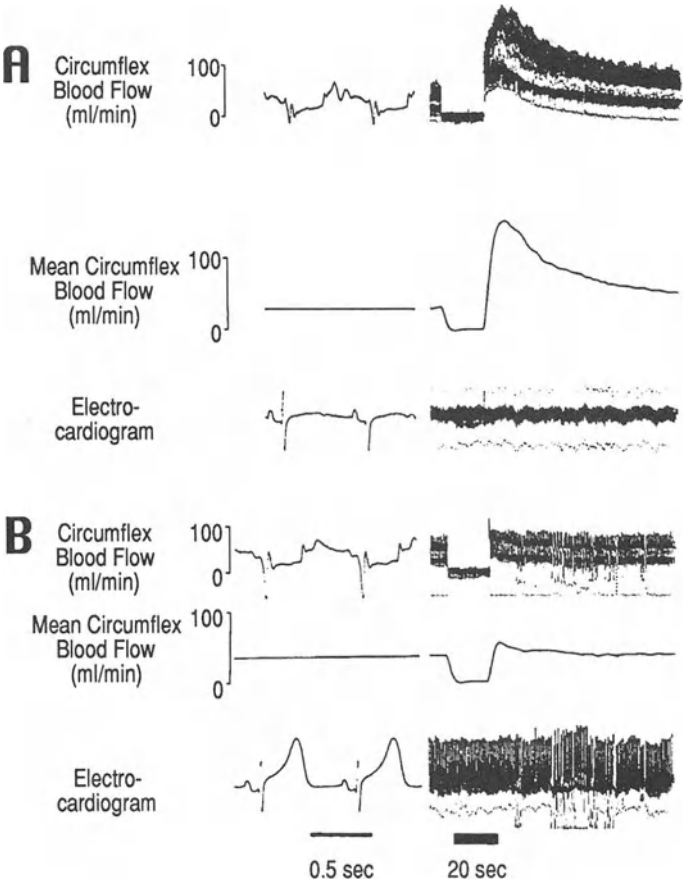

Fig. 1. Coronary flow reserve determined by reactive hyperemic response to a 20-s circumflex (LCx) occlusion at the time of microembolization (A) and at 7 days after microembolization (B) in the same pig. A. Reactive hyperemia prior to microembolization (left panel) and at 10 min after the final injection of 25 μm microspheres. Peak hyperemic flow is reduced by -56% from 34 ml/min to 15 ml/min. B. Reactive hyperemic response is partially restored at 7 days after embolization.

measured in an identical manner (Fig. 1B). In all pigs examined, hyperemic flow response was partially but significantly restored at 7 days after embolization (from 63.7% to 86.7% of pre-embolization value). This restoration of reactive hyperemic response is assumed to be due, in part, to an increase in total coronary vascular cross-sectional areas which resulted from microvascular angiogenesis in the risk region.

Moreover, we found that approximately 8-10 x 10^4 microspheres (as normalized by baseline coronary blood flow volume rate) were required to abolish coronary flow reserve and that the size of the risk area as approximated by baseline coronary flow correlated to body weight of animals. These findings have enabled us to estimate in unthoracotomized pigs the necessary number of microspheres to deplete flow reserve and hence standardize the dose of microspheres.

In the next step, we embolized the microvasculature of the LCx in a closed-chest

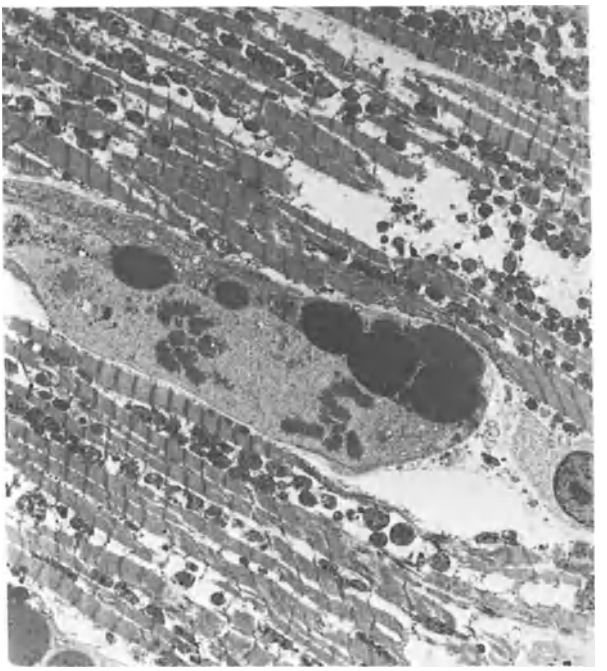

Fig. 2. Mitosis of a capillary endothelial cell in the embolized myocardium (72 h after embolization). Surrounding myocytes appear irreversibly damaged, as characterized by swollen mitochondria with dense amorphous bodies. Erythrocytes are plugged in the lumen.

preparation. A coronary catheter was introduced via the exposed right carotid artery and advanced into the LCx under fluoroscopic guidance. A body weight-adjusted dose of microspheres was then injected through the catheter. Tissue samples were collected from the normal and embolized regions at different time intervals.

CORONARY VASCULAR GROWTH IN THE ISCHEMIC, EMBOLIZED MYOCARDIUM

Although restoration of the reactive hyperemic response following microembolization suggested vascular growth in the LCx region, we sought direct evidence for the occurrence of angiogenesis using different methods based on microscopic analysis.

Upon light microscopic examination of ischemic myocardium, new vessel formation was recognized at 72 h and 7 days following microembolization. These vessels consisted of a single endothelial cell on the transverse section and lacked smooth muscle cell layers; they were found to be located in and around micronecrotic foci. Mitotic figures of capillary endothelial cells, which are very rare in the normal heart, were also demonstrated by electron microscopic analysis (Fig. 2).

Furthermore, we examined cellular proliferative activities (i.e. DNA synthesis) with two different techniques: histone H3 *in situ* hybridization and PCNA/cyclin immunohistochemistry. Histone H3 mRNA expression is known to be tightly coupled with DNA synthesis and can be detected exclusively in dividing cells (8). Similarly, PCNA/cyclin, an auxilliary nuclear protein of DNA polymerase, can be immunohistochemically demonstrated only in cells in proliferation (9). Both examinations showed proliferative activities in endothelial cells of intramyocardial coronary arteries, arterioles, and capillaries (Fig. 3). These results indicate that angiogenesis does occur in response to microvascular occlusions.

Distribution of acidic fibroblast growth factor and its possible roles in angiogenesis

Acidic and basic fibroblast growth factors (FGFs) are members of the heparin-binding growth factor family (10). They are structurally related polypeptides with a 55% sequence homology, and are likely to share the same cell surface receptor (11). Acidic FGF is less potent than basic FGF, but its biological acitivities can be potentiated by heparin. They stimulate proliferation of many cultured cells including

vascular endothelial cells (12), and induce angiogenesis in vivo when exogeneously applied (13, 14, 15). A pivotal role of growth factor(s) in the coronary collateral development has been hypothesized since Kumar et al (16) first reported the presence of angiogenic factors in infarcted human myocardium. Recently, Casscells and co-workers demonstrated mitogenic polypeptides, one of which appeared to be basic FGF, in ischemic foxhound and rabbit hearts (17, 18). Furthermore, acidic FGF was found to be upregulated in collateralized porcine hearts, as examined 2-3 weeks after an ameroid implantation (5). Thus, FGF seems to be one of the most probable candidates that play a role in coronary collateral growth. To obtain an insight into possible roles of FGFs in coronary angiogenesis, temporal changes in acidic FGF distributions were examined in the ischemic myocardium as a funcion of time following microembolization by means of

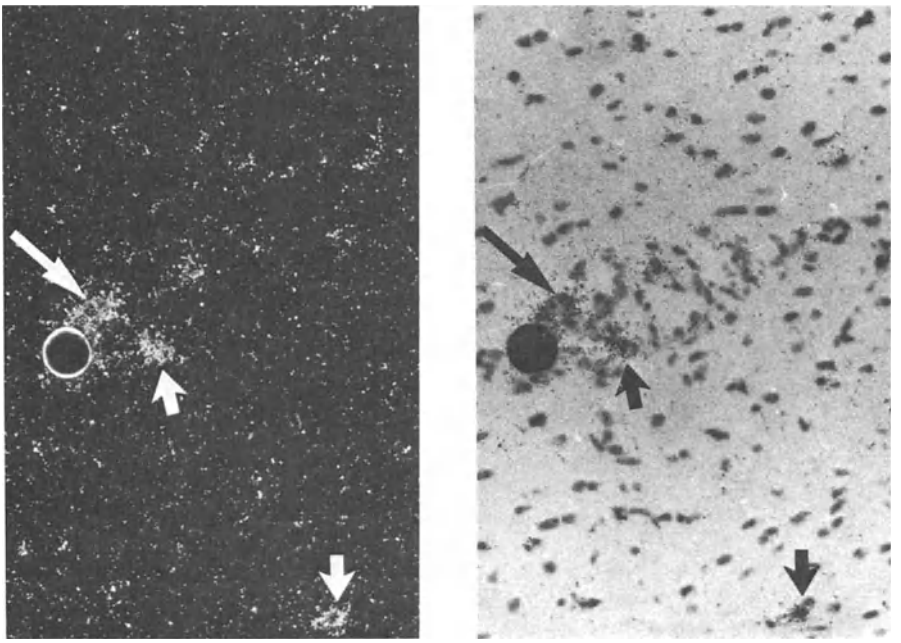

Fig. 3. Proliferative acitivity of vascular endothelial cells determined by in situ *hybridization with radiolabelled anti-sense riboprobe transcribed from a rat histone H3 cDNA (*kindly provided by Dr. David T. W. Wong, Boston*). Positive hybridization signals are recognized in one endothelial cell (large arrow) next to microsphere, and two interstitial cells, possibly fibroblasts (small arrows). Left panel, dark field; right panel, bright field. Section is weakly counterstained with toluidine blue.*

immunohistochemistry using a specific monoclonal antibody that had been prepared in our laboratory.

Immunoreactive acidic FGF was not detected in the normal porcine myocardium (Fig. 4A). This observation does not necessarily contradict previous findings that normal adult hearts contain both acidic and basic FGFs (19, 20, 21, 22). Most likely, the tissue concentration of acidic FGF was below the level of detection with immunohistochemistry. Interestingly, the Purkinje fibers were consistently found to be positively labeled both in the normal and ischemic myocardium.

Immunofluorescence against acidic FGF was not detected in the ischemic region either at 3 h, 6 h, or 12 h following embolization. Acidic FGF was first demonstrated at 24 h after the onset of ischemia. Throughout the LCx region there were multiple focal areas where cardiac myocytes were positively stained (Fig. 4B). At 3 and 7 days, the intensity of acidic FGF immunofluorescence was dramatically enhanced, being most prominent in the spared, viable myocytes in the immediate vicinity of micronecrotic foci with inflammatory cell infiltration (Fig. 4C). Acidic FGF expression was still enhanced at 4 weeks after microembolization, but less remarkable as compared to that at 7 days. Additionally, acidic FGF was also demonstrated in endothelial cells of arterioles and capillaries in the embolized LCx region (Fig. 4B). The extracellular matrix, vascular smooth muscle cells, or endothelial cells of large arteries and veins did not show any detectable acidic FGF at any time point examined. These findings have first demonstrated that acidic FGF is increased in myocytes as well as in microvascular endothelial cells soon after an ischemic stimulus. Its expression peaked at several days after the onset of ischemia and was still enhanced during subsequent weeks.

Possibly, ischemia itself may induce acidic FGF synthesis in myocytes. To confirm this, however, enhanced transcription of acidic FGF mRNA in myocytes should be detected with in situ hybridization method using a riboprobe transcribed from acidic FGF cDNA. Because of technical difficulties, we have not yet succeeded in this. Chiba et al (23) reported that basic FGF expression was upregulated both at transcriptional and protein levels in cultured myocytes exposed to prolonged hypoxia.

Besides mitogenic and chemotactic properties of FGFs, FGF stimulates capillary endothelial cells to produce plasminogen activator and its inhibitor, to invade a three-dimensional collagen matrix, and to form a tubular structure (24, 25). Thus, FGFs seem to have all characteristic features that are necessary for angiogenesis. In contrast to an abundant quantity of in vitro evidence regarding to contributions of FGFs during

angiogenesis, their expression or role in the normal and diseased heart is still largely unknown. The increased expression of acidic FGF in microvascular endothelial cells, which was observed in the present study, suggests that this peptide growth factor is also involved in coronary angiogenesis triggered by ischemia. Although an initial stimulus for upregulation of FGF expression in the endothelial cell is currently undetermined, it could be argued that acidic FGF acts as an angiogenic factor for microvascular endothelial cells in an autocrine and/or paracrine fashion.

It seems, however, that acidic FGF demonstrated in ischemic but still viable myocytes is not necessarily related to its mitogenic activity in these cells. Despite the extensive acidic FGF protein expression in spared myocytes of the risk region, mitotic figure as well as DNA synthetic activities in myocytes were hardly recognized. One could argue that myocardial FGF is a primary messenger for endothelial proliferation after leaving myocytes, but not for myocytes themselves. Foremost among questions to be answered with regard to this hypothesis is that both acidic and basic FGF lack a conventional signal peptide which enables proteins to be secreted through the cell membrane (10, 26). Cell lysis or altered membrane permeability resulting from cell injury has been proposed as a possible pathway for releases of FGFs. Recently, Kandel et al (27) provided evidence that extracellular export of basic FGF out of cells was switched on at a certain stage of tumor growth and was not accompanied with increased amount of cell death and lysis. This observation supports the idea that FGF is released from cells through a yet undefined mechanism(s) (26). A second possibility is that ischemia-induced expression of acidic FGF in myocytes is an adaptive response to ischemic burdens. Acidic and basic FGFs are reported to induce cardiac myocyte hypertrophy (28) and "fetal" β-myosin heavy chain gene expression (29). These might participate in compensatory hypertrophy in regions with depressed contractile function. More interestingly, FGFs possess the ability to promote the survival of injured cells. This has been known as a trophic function and has been best examined in the nervous system (30, 31). Transient forebrain ischemia was also shown to increase basic FGF-like immunoreactivity in the brain (32). Within this context, it is of interest to note that Purkinje cells consistently showed positive immunofluorescence for acidic FGF in the present analysis, and the cardiac conduction system including Purkinje fibers has been shown to have a greater tolerance to ischemic and other types of stresses (33, 34). Although the mechanism of trophic action is unknown, the hypothesis that increased

acidic FGF in response to ischemia exerts "cardiotrophic" effect on myocytes at jeopardy would be worth testing in the future research.

ANGIOGENESIS AND EXTRACELLULAR MATRIX PROTEOLYSIS: ROLES OF PLASMINOGEN ACTIVATORS AND INHIBITOR

The extracellular matrix proteolysis is also considered as an essential step for new vessel growth. Conceivably, focalized destruction of the vascular basement membrane and surrounding extracellular matrix is a prerequisite both for preexisting arteriolar anastomoses to outgrow and for capillary endothelial cells to invade the stroma. The plasminogen activator (PA)-plasmin system has been known to play a central role in the intraluminal and extracellular proteolysis both under normal and pathological conditions including embryogenesis, ovulation, and neoplastic cell invasion (35). PAs are serine proteases and convert plasminogen to its active form, plasmin. Plasmin has a broad spectrum of substrates; it can digest most extracellular proteins and also activates other pro-proteases (e.g. pro-collagenase) to further accelerate matrix degradation. Recently, four distinct molecules that possess the inhibitory activities against PAs have been identified and are generically known as PA inhibitors (PAIs). PAIs modify plasminogen activation and may also participate in extracellular matrix turnover. However, *in vivo* distributions as well as functional significance of endogeneous PAs and PAIs have never been systematically examined either in the normal or diseased heart. We investigated localizations of two different types of PAs (tissue- and urokinase-type PAs) as well as type-1 PAI, a major physiological PAI, in the normal and embolized ischemic myocardium.

Tissue-type plasminogen activator (t-PA)

In bovine heart extracts, Bykowska et al (36) demonstrated PA activity that was inhibited by anti-t-PA antibody. Tissue localization of t-PA in the heart was, however, not determined. On the other hand, t-PA activity was found in human veins of skin (37) and limbs (38). In the normal porcine myocardium, we found t-PA localized in the vascular endothelial cells of veins (Fig. 5A) and, less frequently, of arterioles. In contrast, capillary endothelial cells did not show any detectable labeling for t-PA. Our immunohistochemical examination using a specific antibody against porcine t-PA has provided evidence that venous endothelial cells are also main sources for cardiac t-PA.

The distribution of t-PA in the embolized myocardium was not significantly changed. Positive labeling was infrequently recognized in the endothelium of small vessels located in micronecrotic foci (Fig. 5B). These vessels were mostly larger than capillaries and lacked smooth muscle layers, thus appearing to belong to the venous system. However, no detectable immunofluorescence for t-PA was found in the extracellular matrix, capillaries, vascular smooth muscle cells, or cardiac myocytes. These findings are in accordance with the notion that t-PA serves as an intraluminal protease to maintain the tubular structures including blood vessels (35, 38). In summary, t-PA seems to play no central role in the extracellular matrix degradation nor perivascular inflammation in coronary angiogenesis. Rather, it may remain bound to endothelial cells or be released into the vascular lumen, possibly functioning against venous thrombosis.

Urokinase-type plasminogen activator (u-PA)

Immunohistochemistry using anti-porcine u-PA antibody (kindly provided by Dr. Y. Nagamine, Basel) showed that the endothelium of capillaries and small veins were weakly labeled for u-PA in the normal porcine myocardium (Fig. 6A). Distribution of u-PA was, however, strikingly altered in the ischemic myocardium. At 6 h following embolization, fluorescence for u-PA was found to be increased in the

Fig. 4. Immunohistochemical demonstration of acidic FGF in the normal and ischemic myocardium. In all photomicrographs, nuclei are counterstained orange-red with propidium. A. Normal myocardium. B. Ischemic myocardium at 24 h following microembolization. Positive immunofluorescence (bright green) for acidic FGF is obvious in myocytes as well as in endothelial cells in a small vessel (center). C. 7 days following microembolization. Acidic FGF expression is markedly enhanced in myocytes surrounding a focal necrosis (center). Original magnification 50x (A, C), 100x (B).

Fig. 5. Immunohistochemical demonstration of t-PA in the normal (A) and ischemic (B, 72 h) myocardium. Nuclei are counterstained orange-red. A. Positive immunofluorescence for t-PA (bright green) is bound to venous endothelial cells. B. t-PA is also found at venous endothelial cells. In the center, a micronecrotic focus is present with cellular infiltration. Original magnification 50x

Fig. 6. Immunohistochemical demonstration of u-PA. In photomicrographs except panel C, nuclei are counterstained orange-red. A. Normal myocardium. Endothelial cells of capillaries and veins are positively labeled. B. Ischemic myocardium at 6 h after microembolization. An increase in the intensity of immunofluorescence for u-PA is evident in the extracellular space (center). C. 72 h after embolization. Immunoreactive u-PA (bright green) is localized in the extracellular matrix around a microinfarct. Many tiny vessels in and around a microinfarct are also positively labeled at their walls. To facilitate the detection of u-PA labelings, nuclei are not stained. D. 7 days after embolization. Immunofluorescence for u-PA (bright green) is clearly demonstrated in the extracellular space around a microinfarct (center). Original magnification 50x

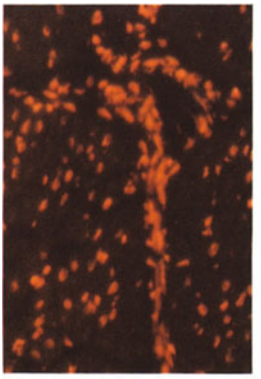

Fig. 4a

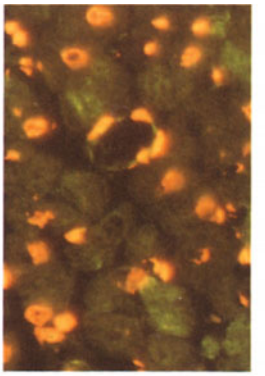

Fig. 4b

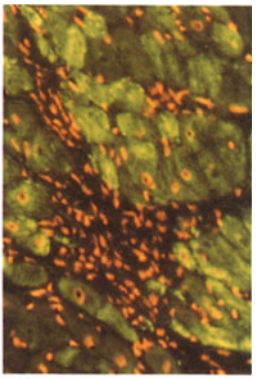

Fig. 4c

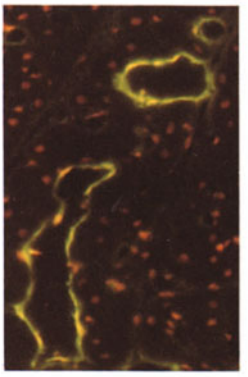

Fig. 5a

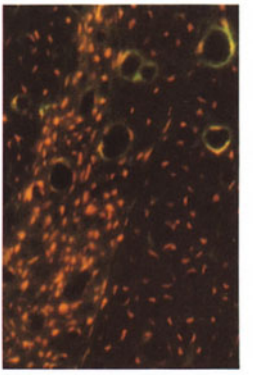

Fig. 5b

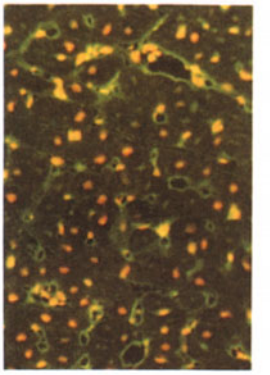

Fig. 6a

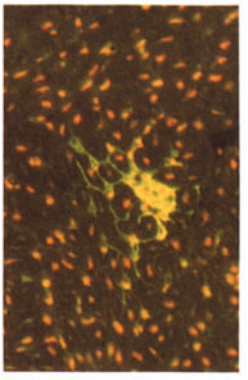

Fig. 6b

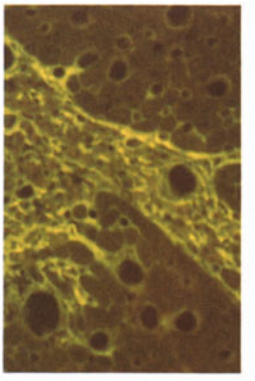

Fig. 6c

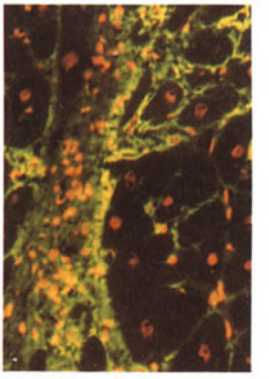

Fig. 6d

extracellular space (Fig. 6B), and positive regions were scattered in the embolized area. The u-PA-positive extracellular matrix further expanded at 24 h. At 72 h and 7 days, microinfarcts were histologically evident with the loss of myocytes and inflammatory cell infiltration. u-PA staining was markedly increased in the extracellular matrix around viable myocytes that surrounded micronecrotic foci (Figs. 6B, 6C). Furthermore, microvessels found in and around microinfarcts occasionally showed positive labeling for u-PA. These observations suggest that u-PA, rather than t-PA, is a key enzyme for the extracellular matrix breakdown in the ischemic myocardium. Most probably, u-PA-mediated proteolysis is involved in the migration of inflammatory cells (perivascular inflammation) and microvascular endothelial cells (angiogenesis).

It is of great interest to note that acidic FGF and u-PA distributions were localized close to each other. Figure 7 shows immunohistochemical localizations of acidic FGF and u-PA on the same section determined by a double-staining method with both antibodies sequentially applied. It is noteworthy that ischemic myocytes exhibiting enhanced acidic FGF expression are surrounded by the extracellular matrix with increased u-PA expression. It is possible that u-PA-mediated proteolysis releases FGF stored in the extracellular matrix (39, 40, 41), and that an active form of FGF then is bound to cell surface receptors of myocytes to increase FGF synthesis (42). Released FGFs may also act on vascular endothelial cells. Moreover, basic FGF is known to stimulate endothelial cells to produce u-PA (43, 44). Taken together, these observations strongly suggest a close interaction between u-PA and FGF(s) in the ischemic myocardium.

The PA-plasmin system may also be of great importance in remodeling processes of damaged myocardium. Components from dead myocytes should be lysed to allow new matrix proteins to be deposited. Particularly, fibronectin and laminin are shown to play substantial roles in endothelial cell migration and differentiation during microvascular angiogenesis (45, 46, 47). We have found that fibronectin began to accumulate by 12-24 h after embolization and that laminin and collagens followed. Such early a deposition of fibronectin, as demonstrated in the ischemic heart, may be most probably related to angiogenesis rather than infarct healing (for further discussion on extracellular matrix proteins see chapter by Dr. J. Schaper).

Chronic myocardial ischemia is known to upregulate transforming growth factor-β (TGF-β) in porcine hearts (48), and plasmin has been implicated to activate TGF-β (49).

However, no direct evidence is currently available to indicate a significant role of u-PA-mediated activation of TGF-β during angiogenesis.

In summary, u-PA seems to play a central role in the extracellular matrix proteolysis to facilitate angiogenic processes in the ischemic myocardium.

Type-1 plasminogen activator inhibitor (PAI-1)

PAI-1 is a single-chain glycoprotein with an approximate molecular weight of 50,000, and appears to be the major inhibitor of plasminogen activation among known PAIs (50). PAI-1 was originally isolated in endothelial cells and is now found in various types of cultured cells. PAI-1 is present in plasma and platelets, but very little information is available as for its in vivo localization in organs. A binding protein for PAI-1 is also identified, and known as vitronectin. Vitronectin is present in plasma as well as in the extracellular connective tissue and is believed to modify stability and function of PAI-1 (51). A monoclonal antibody against PAI-1 (a kind gift from Dr. K. T. Preissner, Bad Nauheim) was applied to determine tissue distribution of the protein in the normal and ischemic heart.

PAI-1 was widely distributed in the adventitia of the arterial wall and in the extracellular connective tissue (Fig. 8A). Additionally, PAI-1 was also demonstrated to be bound to the capillary and venous endothelial cells (Fig. 8B). Levin and Santell (52) found that PAI-1 rapidly deposited in the underlying compartment of cultured human endothelial cells and suspected a protective role of this inhibitor against proteolytic degradation of the basement membrane of the vessel. Our results have added first in vivo evidence to support this idea. We furthermore determined in vivo vitronectin localization in the heart with a monoclonal antibody (kindly provided by Dr. K. T. Preissner) and found that vitronectin was also distributed in the arterial wall and in the extracellular matrix. Thus, PAI-1, possibly bound to vitronectin, is associated with the extracellular connective tissue, the vascular wall of large vessels, and the endothelium of capillaries and veins in the normal heart. Most likely, this inhibitor protects the vascular wall and basement membrane from an excessive PA-mediated proteolysis.

At 6-12 h following the onset of ischemia, PAI-1 was found to accumulate in the cytoplasm of myocytes in the embolized region (Fig. 8C). Labeled and unlabeled myocytes were clearly demarcated. The staining was rather homogeneous within the cells and did not seem to correspond with any specific structure. At 24 h, PAI-1 was bound to apparently dead myocytes which lacked stainable nuclei within the cytoplasm.

At 3 and 7 days following embolization, PAI-1 was localized in the non-cellular extracellular matrix components in microinfarcts (Fig. 8D). Early appearance of PAI-1 in myocytes is likely to represent an accumulation of plasma- and/or platelet-derived PAI-1 by passive diffusion through the cell membrane with increased permeability. This kind of diffusional increase in plasma-derived proteins has been well documented in ischemically injured myocardial cells (53, 54). In addition to early redistribution of plasma-derived PAI-1 into myocytes, a marked increase in PAI-1 mRNA transcripts was demonstrated by the Northern hybridization in the ischemic myocardium at 12 h following microembolization (Sharma et al, unpublished observations). This indicates that de novo synthesis of PAI-1 also takes place in the ischemic heart. Recently, we have shown that acidic FGF induces PAI-1 mRNA in *cultured* endothelial cells of adult rat hearts (25).

Figure 9 shows a simultaneous detection of u-PA and PAI-1 localizations in the ischemic myocardium. Clearly, u-PA is bound to microvessel walls in and around microinfarcts, while PAI-1 is located in the extracellular space between microvessels. Interestingly, myocardial ischemia by microvascular occlusion simultaneously enhances expressions of u-PA and PAI-1; however, a net proteolysis-antiproteolysis balance may be determined in part by spatial differences in u-PA and PAI-1 localizations, as observed in Figure 9. PAI-1 in the extracellular space is likely to counterbalance an

Fig. 7. Simultaneous demonstrations of acidic FGF and u-PA in the ischemic myocardium at 3 days following microembolization. Acidic FGF is labeled with rhodamin (red), and u-PA with fluoresceine isothiocyanate (bright green). One microsphere is present in the micronecrosis (center). Original magnification 50x

Fig. 8. Immunohistochemical detection of PAI-1. A and B. Normal myocardium. PAI-1 is recognized in the extracellular connective tissue (A) and in the capillary and venous endothelial cells (B) of normal myocardium. C. Ischemic myocardium at 6 h following embolization. PAI-1 accumulates within myocytes. Labeled and unlabeled myocytes are clearly distinguished. D. 7 days following microembolization. PAI-1 is localized in the extracellular matrix of a microinfarct. Nuclei are counterstained red in all photomicrographs. Original magnification 50x

Fig. 9. Simultaneous demonstrations of u-PA and PAI-1 in the ischemic myocardium at 3 days following microembolization. u-PA is labeled with fluoresceine isothiocyanate (bright green) and PAI-1 with rhodamine (orange). Microvessel walls are positively stained for u-PA, whereas the extracellular space between them are labeled for PAI-1.

Fig. 10. Immunohistochemical detection of a macrophage labeled with fluorescence (bright green) for TNF-α in the ischemic myocardium at 7 days after embolization. Surrounding myocytes appear to be intact. Nuclei are counterstained red. Original magnification 100x

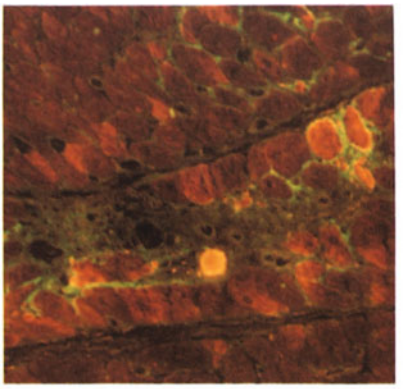

Fig. 7

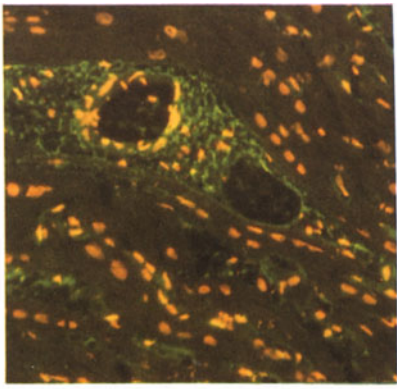

Fig. 8a

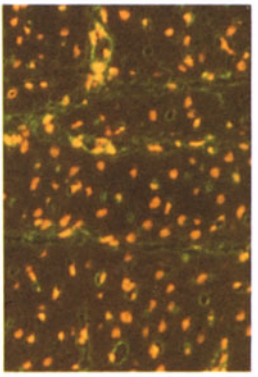

Fig. 8b

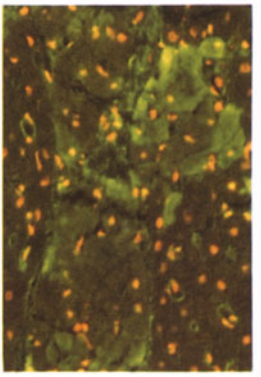

Fig. 8c

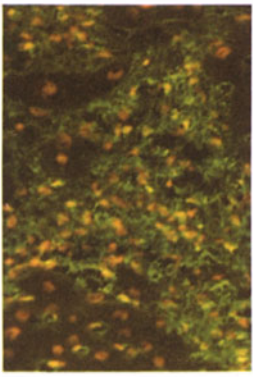

Fig. 8d

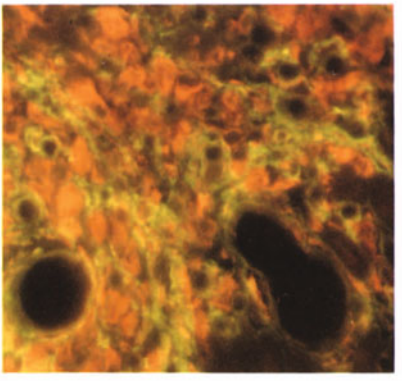

Fig. 9

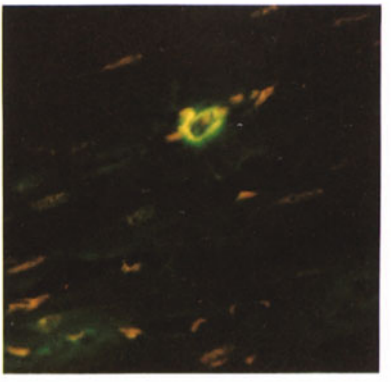

Fig. 10

excessive u-PA-mediated degradation and may protect the deposition of new extracellular proteins such as fibronectin, laminin, and collagens. These proteins may serve in part for endothelial migration and vascular basement membrane formation during microvascular angiogenesis.

TUMOR NECROSIS FACTOR-ALPHA (TNF-α) IS PRODUCED BY ACTIVATED MACROPHAGES IN THE ISCHEMIC MYOCARDIUM

TNF-α is one of the secretory products of macrophages, and is known to be a particularly important mediator of inflammatory responses (55). Additionally, TNF-α was shown to be angiogenic in vivo (56, 57). However, its in vitro effects on cultured cells seem to be complicated, depending on many factors including the concentration of TNF-α, the culture conditions, and the presence or absence of other growth factors and cytokines. Generally, TNF-α inhibits endothelial proliferation, induces capillary tube formation, promotes growth of fibroblasts and mesenchymal cells, enhances biosynthesis of other growth factors and cytokines, and accelerates collagen production from fibroblasts and synovial cells (58). Thus, these features appear to be related to endothelial differentiation and tissue reorganization processes, rather than vascular proliferation itself. We determined, using a monoclonal antibody against TNF-α, tissue distribution of this cytokine in the ischemic myocardium.

As expected, the normal myocardium did not contain TNF-α-positive macrophages. The infiltration of macrophages laden with immunoreactive TNF-α was significantly increased at 24 h following microembolization (>60 cells/cm^2 of tissue section) and remained in the embolized area for up to 4 weeks (Fig. 10). Most positive macrophages were found near microinfarcts. Apparently, the size of infarction in the risk area did not significantly influence the number of TNF-α-positive macrophages at an early stage of ischemia (24-72 h), but did so at a later stage (1-4 weeks). Thus, it may be speculated that TNF-α has distinct functions at early and late stages. Although important roles of monocytes-macrophages during collateral development in dogs have been well documented (59, 60, 61), the functional significance of macrophages and TNF-α for coronary microvascular growth in pig hearts still remains to be elucidated.

SUMMARY

Coronary microvascular angiogenesis develops in the porcine heart subjected to myocardial ischemia following microembolization. The present analysis has revealed

possible contributions of different growth factors, proteases and their inhibitor, as well as extracellular matrix proteins at certain stages of vascular growth. Undoubtedly, the biosynthesis and biological activity of these molecules may be mutually influenced, and also regulated by other factors that have not yet been determined. Availabilities of monoclonal antibodies against these and other peptides and the recent development in molecular biological techniques will certainly make way for a better understanding of this complex phenomenon.

Acknowledgments: We thank Dr. Jutta Schaper for continuous encouragement and valuable discussion throughout the study. Dr. M. Mohri was supported in part by Overseas Research Fellowship for Cardiovascular Investigation from Japan Heart Foundation, Tokyo, Japan.

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7

THE ROLE OF GROWTH FACTORS DURING DEVELOPMENT OF A COLLATERAL CIRCULATION IN THE PORCINE HEART

Hari S. Sharma and Wolfgang Schaper
Max-Planck-Institute, Department of Experimental Cardiology,
Bad Nauheim, F. R. Germany

INTRODUCTION

Angiogenesis, the formation of new capillary blood vessels is an important component of various normal and pathological conditions such as wound healing, fracture repair, folliculo-genesis, ovulation and pregnancy (1). These periods of angiogenesis are of relatively short duration and they are tightly regulated. However, if not properly controlled, angiogenesis can also represent a significant pathogenic component of tumor growth and metastasis, rheumatic arthritis and retinopathies (2). Angiogenesis, being a complex biological process, involves extracellular matrix degradation and proliferation, migration and maturation of endothelial cells (3). Efforts have been made to identify and characterize the factors controlling motility and proliferation of endothelial cells. Several polypeptide growth factors have been shown to contribute to the molecular events involved in the regulation of new blood vessel growth (1, 2, 3, 4, 5, 6). Being modulators of cell proliferation and differentiation, polypeptide growth factors bind to the specific cell surface receptors to exhibit their functions for subsequent alterations in the gene expression in the responsive cell. The biological role(s) of various growth factors have been studied mainly in cell culture systems, and relatively little is known about their physiological significance in vivo.

Under normal physiological conditions, angiogenic events associated with the development of coronary collaterals are infrequently observed in the normal adult heart, however, progressive slow coronary artery occlusion can induce the process of myocardial angiogenesis (7, 8, 9). This compensatory vascular growth in response to ischemia is an important event in the prevention or attenuation of ischemic heart disease and myocardial infarction. It is often observed that patients suffering from chronic stable angina pectoris usually have occluded major coronary arteries but well developed collaterals that can meet the basal demand of the heart for blood flow and nutrients. Thus, a detailed study on the molecular responses to ischemia leading to

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

collateralization would be an interesting endeavor to unravel the complexity of mechanisms involved in the coronary heart disease.

In an attempt to understand the molecular basis of ischemia-induced myocardial angiogenesis, we examined the expression of polypeptide growth factors in the porcine heart after a progredient chronic coronary artery occlusion by an ameroid constrictor. Using reverse transcriptase-polymerase chain reaction (RT-PCR), Northern and in situ hybridization techniques, we studied the expression pattern of angiogenic growth factors, like acidic and basic fibroblast growth factors (FGF), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF) and transforming growth factor-β1 (TGF-β1).

Angiogenic growth factors

Within the past few years, several polypeptide angiogenic growth factors have been purified, and well characterized for their amino acid and gene sequences (for reviews see 4, 5, 6). These polypeptides include vascular endothelial growth factor (10, 11) family of fibroblast growth factors (12), angiogenin (13), transforming growth factor-α (14) and transforming growth factor-β1 (15), platelet derived endothelial cell growth factor (PD-ECGF) (16) and tumor necrosis factor-α (17). Additionally, several low molecular weight angiogenic factors such as prostaglandins PGE1 and PGE2 (18, 19), nicotinamide (20), adenosine (21) and certain degradation product of hyaluronic acid (22) have been described in the literature. Analysis of these factors strongly suggests that there must be several different mechanisms for stimulating angiogenesis.

As the endothelial cell is considered to be the primary cell involved in all forms of angiogenesis, it is of utmost importance to identify molecules governing endothelial cell motility and proliferation. Endothelial cells under normal conditions form remarkably stable populations. However, in a number of pathological conditions endothelial cells rapidly proliferate and form new blood vessels and whole capillary network (2). Specific angiogenic molecules including polypeptide growth factors can initiate this process and specific inhibitory molecules can stop it (5). On the basis of their action on either motility or proliferation of endothelial cells, polypeptide growth factors can be grouped as shown in table 1. Some angiogenic peptides stimulate motility or proliferation or both, whereas some have no effect or inhibit endothelial cell proliferation suggesting their direct or indirect role in angiogenesis. Folkman and Klagsbrun (4) have proposed that angiogenic factors that stimulate locomotion and

mitosis of vascular endothelial cells in vitro have the vascular endothelial cells as their immediate target in vivo. VEGF, FGF, and TGF-α are examples of such direct angiogenic factors. On the other hand, molecules such as TGF-β1 and TNF-α that have no mitogenic effect on vascular endothelial cells in vitro, but act by mobilizing host cells (for example, macrophages) to release endothelial growth factors can be categorized as indirect angiogenic factors.

Table 1. Effects of Angiogenic Peptides on Endothelial Cells

Angiogenic Growth Factor	M_r (kD)	Endothelial cell Proliferation	Migration	Reference
VEGF/VPF	45 (dimer)	Yes	Yes	(10,11)
FGF	18 (monomer)	Yes	Yes	(6,12)
PD-ECGF	45 (monomer)	Yes	Yes	(16)
TGF-α	5.5 (monomer)	Yes	Yes	(14)
TGF-β	25 (dimer)	Inhibition	Inhibition	(15)
TNF-α	55 (trimer)	Inhibition	Yes	(17)
Angiogenin	14 (monomer)	No	No	(13)

VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; FGF, fibroblast growth factor, PD-ECGF, platelet-derived endothelial cell growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor

Recently, several groups have reported the purification and characterization of heparin-binding growth promoting polypeptides from hearts of various species including pigs (23, 24, 25). In 1971, Schaper et al (26) discovered that cell proliferation and DNA synthesis were the basis of myocardial collateralization. These findings encouraged further studies exploring the stimulus for vascular growth.

Angiogenic growth factors in the heart

Recent investigations demonstrated that the adult heart produces a number of growth promoting polypeptides which are well characterized to be angiogenic in vitro and in vivo. Quinkler et al (23) from our group have isolated and characterized heparin binding peptide mitogens from normal adult bovine, porcine and canine hearts. Amino acid analysis of these polypeptide mitogens showed a very high degree of homology to the previously known acidic and basic fibroblast growth factors isolated from brain (27, 28) and kidney (29). Following our report on these cardiac growth factors, Casscells et al (24) and Sasaki et al (25) have also purified and characterized on the basis of Western blotting, heparin binding polypeptide growth factors identical to acidic and basic FGF.

Recently, we and others have demonstrated the expression pattern of TGF-β1 in the myocardium of various mammals (30, 31, 32). Using molecular biological methods, we have also found that the angiogenic polypeptides, VEGF (33), and TNF-α (Sharma and Schaper, unpublished) are also expressed in the adult porcine myocardium. Results depicted in figures 1 and 2 demonstrate that mRNAs for VEGF, TGF-β1, aFGF and TNF-α are transcribed in the normal adult porcine heart and can easily be detected by RT-PCR. Using RT-PCR on cDNA templates derived from normal (C) and collateralized ischemic myocardium (E), we specifically amplified respective DNA fragments of 407bp (VEGF), 472bp (aFGF), 119bp (TGF-β1) and 383bp (TNF-α). The existence of potent angiogenic growth factors in the adult heart, where practically no angiogenesis occurs under normal physiological conditions, raises questions. One explanation could be that the heart may require these factors to maintain its normal functioning including vascular permeability, repairs and/or they are vital for the normal

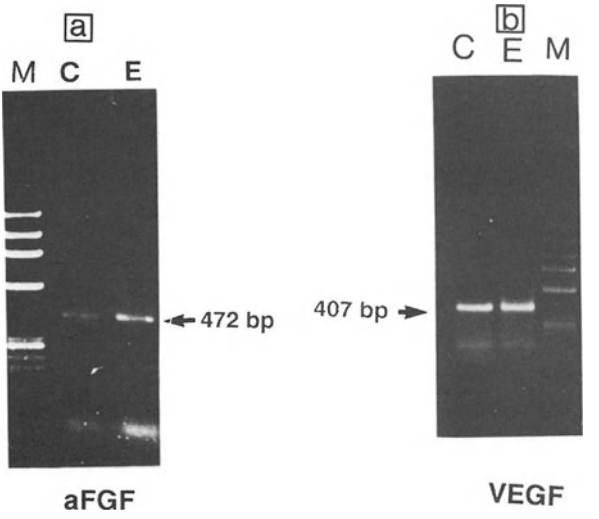


Fig.1. Detection of mRNAs encoding aFGF and VEGF in the porcine myocardium by RT-PCR.
(a): cDNA templates derived from the total RNA isolated from normal (C) and collateralized (E) myocardial tissue were amplified using oligo-nucleotide primers designed from human and bovine aFGF cDNA sequences. An aliquot of the RT-PCR mixture was analysed on a 1.2% agarose gel by ethidium bromide staining. A dominant band of expected size (472bp) encoding aFGF was amplified. Size marker (M) is Hae III-digested Phi-X 174RF DNA.
(b): RT-PCR product analysed by ethidium bromide staining of a 1% agarose gel showed a major band of 407bp spanning two VEGF specific oligonucleotide primers.M: Phi-X 174RF/Hae III DNA size markers.

survival of myocytes and can be regarded as trophic factors.

Heart is the major source of aFGF and VEGF

The presence of aFGF and bFGF immunoreactive material in normal hearts of various mammals is well established (23, 24, 25), but the true concentration of such peptide mitogens as well as their physiological function in the heart remain unclear. We describe here that the expression of aFGF-mRNA is higher in the heart than in a wide range of other organs tested. Acidic and basic FGF are potent mitogens in vitro for a wide variety of cells originating from vascular, nervous, connective and muscle tissue and are angiogenic in vivo (1, 6, 12). The recently described vascular endothelial growth factor (VEGF) is a heparin binding, homodimeric peptide mitogen with target specificity to vascular endothelial cells derived from small and large vessels and it has been shown to induce angiogenesis in vivo (10, 36). The presence of these angiogenic peptides in the adult heart where practically no angiogenesis takes place and where any other cell proliferation is extremely low (37) was unexpected and remains unexplained.

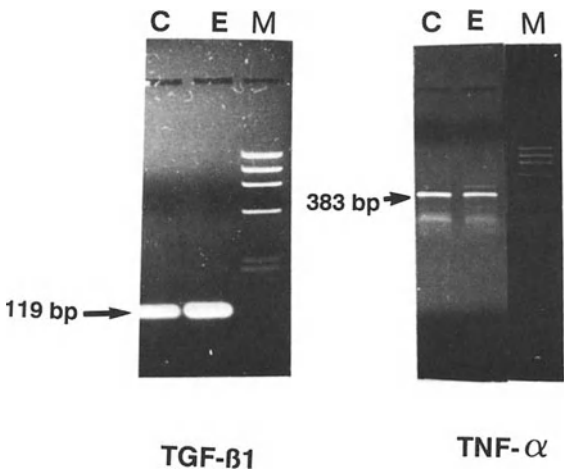


Fig.2. Detection of TGF-β1 and TNF-α mRNAs in the porcine heart by RT-PCR.
(a) Agarose gel analysis of TGF-β1 RT-PCR products derived from normal (C) and collateralized (E) tissue. An expected size DNA fragment (119bp) was amplified from both myocardial tissues. M: size markers as mentioned in Fig. 1.
(b) A 383bp DNA fragment (analysed by agarose gel electrophoresis) spanning two oligo-nucleotide primers for TNF-α was amplified by RT-PCR from cDNA templates derived from normal (C) and collateralized (E) myocardium. M = as mentioned in Fig. 1.

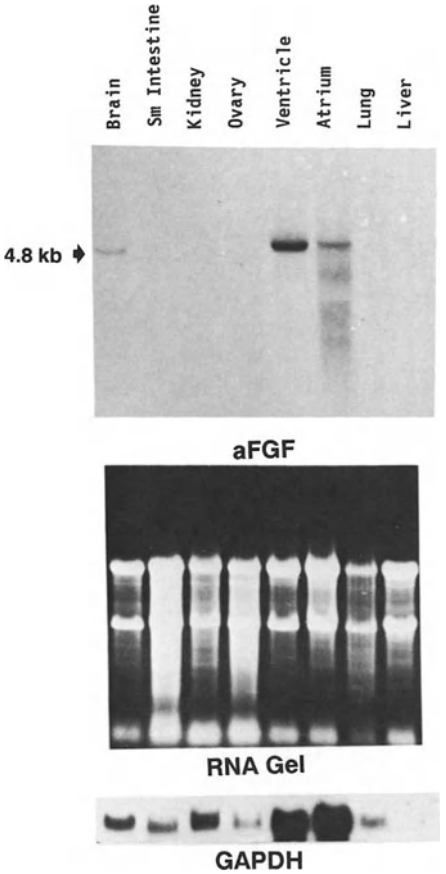


Fig. 3. Expression of aFGF mRNA in different porcine tissues.
15-20 µg of total cellular RNA from various porcine tissues was electrophoresed on an agarose gel containing formaldehyde, transferred to nylon membrane and hybridized with a radioactive aFGF cDNA probe as described in the text. A major transcript of 4.8 kb encoding aFGF is predominantly expressed in the ventricle, followed by atrium, brain and faint signal in ovary. Ethidium bromide staining of the gel revealed intact 28S and 18S ribosomal RNAs (middle panel). Lower panel shows the GAPDH hybridization signals (1.4 kb) which vary from tissue to tissue and hence can not be used as a reference for the loading and quality of RNAs.

The mRNA expression pattern for aFGF showed a 4.8 kb transcript in the normal adult ventricle, atrium, brain and ovary but no mRNA band could be seen in small intestine, kidney, lung and liver (Fig. 3). The size of mRNA detected in the adult porcine heart was identical to the aFGF mRNA reported in human brain as well as in different cell types (38). The mRNA expression for aFGF was maximal in the ventricle followed by atrium, brain and ovary suggesting that heart, particularly ventricle, is the major source of this peptide growth factor. Densitometric analysis of aFGF expression revealed that the mRNA levels in adult ventricle were 3-fold higher than atrium and 6 fold higher than brain and more than 40-fold higher as compared to ovary. This is (as far as we know) the first report on mRNA detection encoding aFGF in the adult

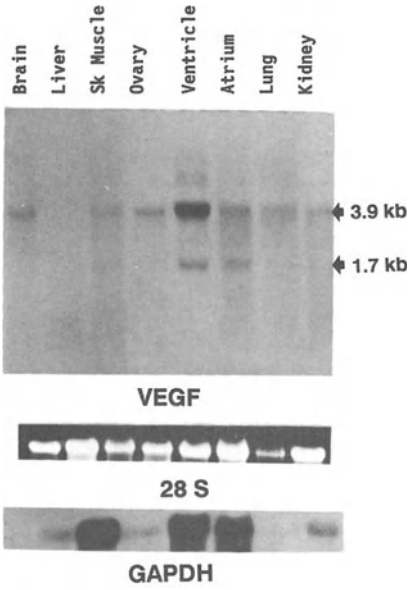


Fig. 4. Distribution of mRNAs encoding VEGF in different porcine tissues.
A ^{32}P-labelled human cDNA insert encoding VEGF was used for RNA blot analysis as described in the text. Ventricle showed the maximal expression of two mRNA species of 3.9 kb and 1.7 kb as compared to all other organs. Ethidium bromide stained 28S rRNA band depicts the quality of RNA (middle panel) and as a reference GAPDH hybridization signal revealed varied expression in different tissues.

myocardium by Northern hybridization. However, we recently have reported the cDNA sequence of porcine aFGF after amplifying the reverse-transcribed mRNAs derived from the normal and collateralized myocardial tissue (39). There are several reports showing the presence of aFGF protein in the normal myocardium (23, 24, 25) and mRNA expression either in neonatal rat cardiac myocytes (40) or immunoreactive aFGF in primary cultures of isolated adult rat myocytes (41).

Similar to the expression of aFGF, VEGF showed the highest expression of one major (3.9 kb) and one minor (1.7 kb) mRNAs in the adult ventricle and basal expression in other organs except liver (Fig. 4). It seems that the 1.7 kb mRNA band is heart specific as it was not seen in brain, skeletal muscle, ovary, lung and kidney. The tissue concentration of VEGF mRNA was uniformly higher than aFGF mRNA concentration. The densitometric analysis of VEGF blots revealed that the mRNAs levels in adult ventricle were 3-6-fold higher than those of atrial and almost every other organ studied. We were the first to provide evidence for the presence of VEGF at least at the level of mRNA in normal adult heart (33, 42, 43). However, Berse et al (44) very recently published their results on the expression of VEGF in various adult tissues including heart which support our previous findings.

From our results it can be inferred that the heart is a major source of aFGF and VEGF. However, since cell proliferation in the normal adult heart is extremely rare, these peptide mitogens may play a role other than or in addition to angiogenesis. VEGF has been reported to enhance the permeability of blood vessels (45) therefore it could probably be involved in regulating a baseline microvascular permeability needed for influx of nutrients and waste product removal in the adult heart. In brain derived endothelial cells, VEGF increases free cytosolic calcium (46). An influence of VEGF on the Ca^{++}-homeostasis in the heart (47) may be possible but needs further clarification. VEGF as a regulator of endothelial cell growth and differentiaton has been shown to play an important role in the embryonic brain and renal angiogenesis (48). We have demonstrated that brain derived microvascular endothelial cells express VEGF probably for its autocrine function in such cells (49). The large amount of mRNA for aFGF and VEGF in the heart raises questions of the function of these growth factors. The wide distribution and the broad spectrum of biological functions of aFGF make itself a possible candidate for being a repair-mediating protein.

Ameroid constrictor model of collateralization in pigs

In a series of pigs (minipigs and German landrace pigs), a hygroscopic ameroid constrictor was implanted around the left circumflex coronary artery (LCx) as shown in the schematic diagram (Fig. 5). Stenosis of a coronary artery and collateralization were verified by in vivo angiography after 2-4 weeks of ameroid implantation. At more than 90% of LCx stenosis, pigs were sacrificed, the hearts were removed quickly and rinsed in ice-cold saline. Ventricles were sliced into 3-4 rings as shown in figure 5. Small tissue pieces from the LCx region (macroscopically examined to exclude any infarcted tissue) and normally perfused interventricular septum were excised and snap frozen in liquid nitrogen and stored at -80°C until analysed. Myocardial tissues from macro-infarcted and normally perfused areas from a minipig as well as from a German Landrace pig were also collected for analysis. Using molecular biological techniques, we examined the expression of various angiogenic growth factors: acidic FGF and basic FGF, VEGF, TGF-β1 and TNF-α in the tissues mentioned above.

Induced expression of angiogenic growth factors during collateralization

In dogs and pigs, our group has reported that progressive stenosis of a coronary artery leads to the mitosis of endothelial and smooth muscle cells (7, 37). The entire vasculature in the region at risk of infarction in the pig heart responded to ischemia

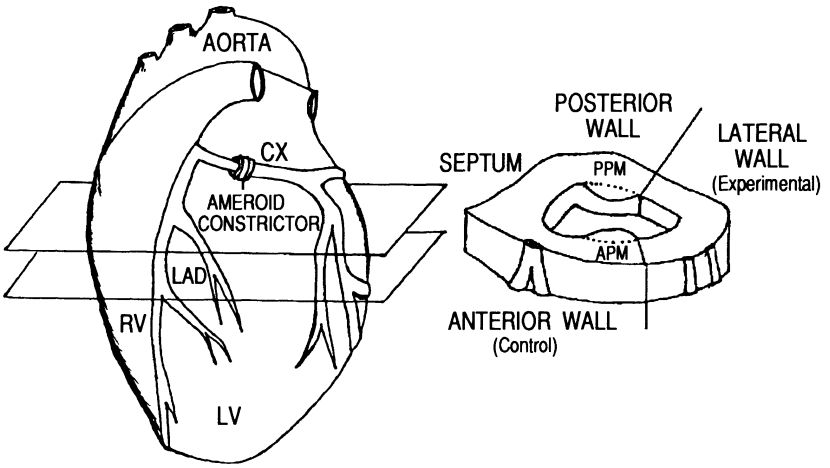


Fig. 5. Schematic diagram showing the experimental model of myocardial angiogenesis and tissue sampling.
After lateral thoracotomy in healthy pigs, a hygroscopic ameroid constrictor was implanted around the LCx and the degree of stenosis as well as collateral vessel growth was verified by in vivo angiography. After sacrifice, the ventricle was cut into rings and tissue was collected from the LCx (experimental) and from the interventricular septum (control) areas as shown in the right panel.

with the growth and enlargement of capillaries as evaluated by in vivo coronary angiography (9). R. Zimmermann and J. Schaper from our group have developed a technique based on in situ hybridization with a radioactive histone-H3 cRNA probe in order to identify cells under division. A significantly higher labelling of vascular cells was found in the ischemic region where the existence of collaterals had angiographically been verified as compared to the normally perfused myocardial region (Fig. 6). Previously, using ^{3}H-thymidine incorporation into DNA, Pasyk et al have shown that progressive stenosis drastically increased DNA synthesis (37). After verifying the myocardial tissue for vascular cell mitosis, we studied the expression of the following angiogenic polypeptides in the ischemic collateralized tissue and compared it with that of the normally perfused myocardium.

Acidic fibroblast growth factor

Endothelial cell growth factor-α (α-ECGF), a precursor of β-ECGF and aFGF is a heparin binding anionic polypeptide mitogen of about 18 kD with nearly 55% amino acid homology to basic fibroblast growth factor (6). It is well documented that the

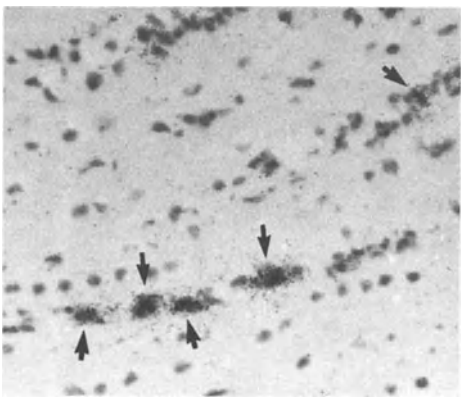


Fig. 6. In situ hybridization for histone-H3 mRNAs in the collateralized myocardium. 5 μm tissue sections were hybridized with histone-H3 antisense cRNA probe and washed under stringent conditions. Bright field micrograph of the ischemic collateralized region. A high density of grains is focally distributed over the nuclei of endothelial cells (arrow head).

acidic FGF is mitogenic in vitro for cells of vascular, connective and muscle tissue origin (6, 50) and induces complete angiogenic response in vivo (4). aFGF promotes neurite outgrowth and under specific conditions it may act as differentiating and maintenance factor for nerves (51, 52). Unlike other angiogenic factors an intriguing feature of aFGF is that it lacks a signal sequence to direct secretion from the cell and it appears to be cell associated (6, 53).

We searched for the mRNAs encoding aFGF in the porcine myocardium employing reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern hybridization techniques. Frozen tissue was pulverized in the presence of liquid nitrogen and total cellular RNA was isolated and tested according to published methods (55, 56). In order to perform RT-PCR, cellular RNA from collateralized and normal myocardium was reverse-transcribed into cDNA and an aliquot of its cDNA was used for PCR amplification (57). aFGF specific oligonucleotide primers were synthesized from the published bovine and human cDNA sequences (53, 58). Highly conserved regions were chosen to design sense and anti-sense primers spanning the whole coding region of the mRNA. cDNA products were amplified using sense and anti-sense primers in an automatic thermal cycler for 30 cycles. As an internal control, cDNA products were also used to amplify a part of 28S ribosomal RNA (59). The PCR products were analysed by agarose gel electrophoresis. To characterize the PCR products encoding aFGF, Southern hybridization was performed with a radiolabeled cDNA insert encoding human aFGF (kindly provided by Dr. T. Maciag).

By RT-PCR we could amplify a DNA fragment of 472bp encoding aFGF in the collateralized and normal myocardium (39, 43). Southern hybridization with a radiolabeled human aFGF cDNA probe further verified the specific amplification of the aFGF in the pig myocardium. Recently, we have also established the cDNA sequence of aFGF from porcine heart generated by asymmetric amplification of cDNAs derived from normal and collateralized myocardium (39). The nucleotide sequence analysis of porcine aFGF revealed a homology of 94% to the human and 92% to the bovine cDNA sequences respectively. Hence, we have shown that the mRNA encoding aFGF is transcribed in the normal and collateralized myocardium, but RT-PCR in its conventional form is unsuited for the study of quantitative differences.

We performed Northern blot analysis to study the differences in the aFGF expression in the collateralized and normal myocardium. Total RNA from each tissue was size fractionated on a formaldehyde containing agarose gel in the presence of ethidium bromide. RNA was transferred to a nylon membrane by vacuum blotting, UV crosslinked and hybridized with aFGF specific cDNA and/or PCR product under stringent conditions. We detected a mRNA species of 4.8 kb (Fig. 7) after an overnight exposure of the autoradiograph in the lane of collateralized myocardium while a faint signal corresponding in size (4.8 kb) was seen in the normal myocardium (60). In pigs

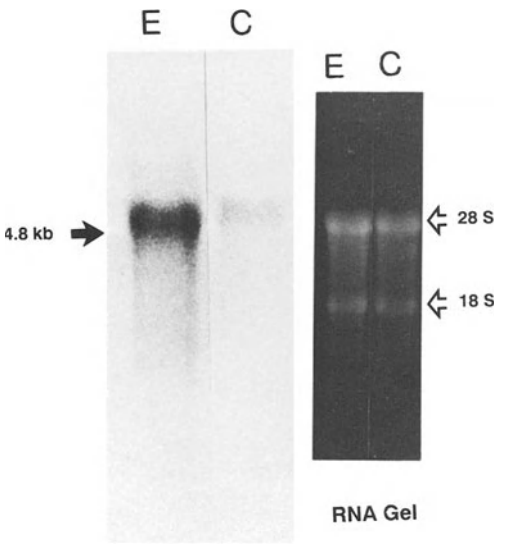


Fig. 7. Expression of aFGF mRNA in the collateralized myocardium.
Similar amounts of total cellular RNA isolated from the collateralized (E) and normally perfused (C) myocardium was subjected to Northern blot analysis as described in the text. A single dominant band of 4.8 kb encoding for aFGF was expressed in the E region whereas a faint signal was seen in C. Ethidium bromide stained gel (right panel) shows the intact 28S and 18S rRNAs verifying the quality of RNA.

with less than 90% of coronary stenosis and no collateralization, aFGF expression was not altered in the ischemic and normally perfused myocardial regions (data not shown). However, in a pig with ameroid constrictors and visible infarction, aFGF expression was drastically decreased in the experimental area (Fig. 8, left panel). In another pig with tight stenosis and no visible collaterals, aFGF expression was slightly increased in the experimental area as compared to the control (Fig. 8, right panel). Enhanced expression of aFGF during collateralization points to its participation in ischemia induced myocardial angiogenesis.

It is clear that aFGF is present in the porcine myocardium but the cell type(s) producing this factor under normal and experimental conditions are not well known. To localize the aFGF transcripts, we performed in situ hybridization. Porcine normal and collateralized myocardial tissue sections were hybridized with the [^{35}S] labelled DNA probe encoding human aFGF. In one of the several experimental pigs studied, aFGF transcripts were mainly seen in the vascular wall cells presumably in the endothelial and smooth muscle cells (61). Previously it was shown by immunohistochemical methods that aFGF is localized in viable cardiac myocytes in the zone surrounding focal necrosis

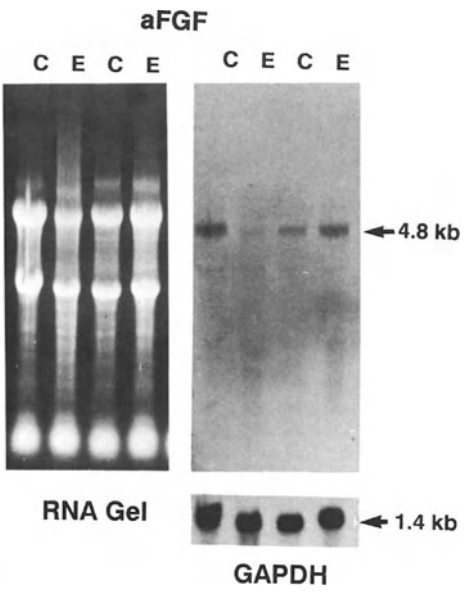


Fig. 8. aFGF expression in the ischemic myocardium.
Northern hybridization was performed for the total RNA isolated from C and E (left panel, E = infarcted tissue and right panel, E = ischemic without visible collaterals) using a 2.1 kb human specific cDNA probe encoding aFGF as mentioned in the text. Expression of a 4.8 kb mRNA was significantly reduced in the infarcted tissue and slightly induced in the ischemic tissue without infarction. Left panel depicts the ethidium bromide stained RNA gel and lower right panel shows the 1.4 kb GAPDH mRNA used as reference.

(62, see also Dr. Mohri's chapter). No immunoreactive aFGF was seen in the normally perfused myocardial tissue sections with the exception of the Purkinje-fibres of the cardiac conduction system. Weiner and Swain have also shown that cultured neonatal myocytes produce aFGF as they could detect the mRNA and protein in extracts of myocytes (40).

Transforming growth factor-β1

Within the transforming growth factor-β (TGF-β) family, TGF-β1 is the most common form which modulates the growth of normal and neoplastic cells (63). TGF-β1 is a 25 kDa (homodimeric polypeptide) differentiation factor largely found in platelets and most organs, including heart (31, 64). In the cardiovascular system, TGF-β1 is found in almost every cell type including cardiac myocytes, arterial and venous smooth muscle cells, endothelial cells and fibroblasts as well as macrophages, neutrophils and platelets (30, 65). Although, TGF-β1 is an inhibitor of endothelial cell proliferation, it has been found to elicit a potent angiogenic reaction when injected subcutaneously into new born mice (15) or when applied locally in wound healing experiments (66). The angiogenic response to TGF-β1 application is an indirect one, the primary response is the chemo-attraction for monocytes which then in turn stimulate angiogenesis. An interesting property of TGF-β1 is that it is secreted in a biologically inactive latent form which can be activated by heat, acidification and proteases (67). Recently Lefer et al (68) have evaluated the cardioprotective role of TGF-β1 in response to ischemic injury and found significantly decreased circulating TNF-α which is involved in aggravating the tissue damage by ischemia. The physiological roles of TGF-β1 in the heart remain to be well elucidated, however, its properties in vitro suggest that it might be an important molecule in various myocardial situations such as cardiac embryogenesis, hypertrophy, atherogenesis, healing of myocardial infarction and development of coronary collaterals (65, 69).

In our experimental model of angiogenesis caused by progressive coronary occlusion, we investigated whether TGF-β1 is expressed in the myocardium in response to chronic ischemia and how its expression is altered during collateralization. We used RT-PCR for the detection of TGF-β1 in various myocardial RNA preparations. TGF-β1 specific oligonucleotide primers were designed from the published porcine cDNA sequence (70). We amplified a DNA product of 423 bp in cDNA preparations derived from normal and collateralized myocardium. The PCR product was verified for TGF-β1

specificity by Southern hybridization with a human cDNA insert encoding TGF-β1 (kindly provided by Dr. R. Derynck) as well as by reamplification of a smaller fragment using internal primers (71). In a semi-quantitative approach, higher amplification of a TGF-β1 specific DNA fragment of 257 bp was observed after 15 cycles of RT-PCR in collateralized as compared to the normal myocardium (71). In all experiments, a blank was included (cDNA substituted by water) to check the contamination of reagents used in RT-PCR and no amplification in the blank tube was observed. By RT-PCR, we learned that TGF-β1 transcripts are present in the swine myocardium and that this growth factor is increasingly expressed during collateral growth. This difference became more apparent when TGF-β1 expression was evaluated by Northern hybridization. Using this technique, we found a significantly enhanced expression of a 2.4 kb (major) TGF-β1 mRNA in collateralized myocardium as compared to control in pigs with critical stenosis but without major infarction (Fig. 9). In another set of experiment, we

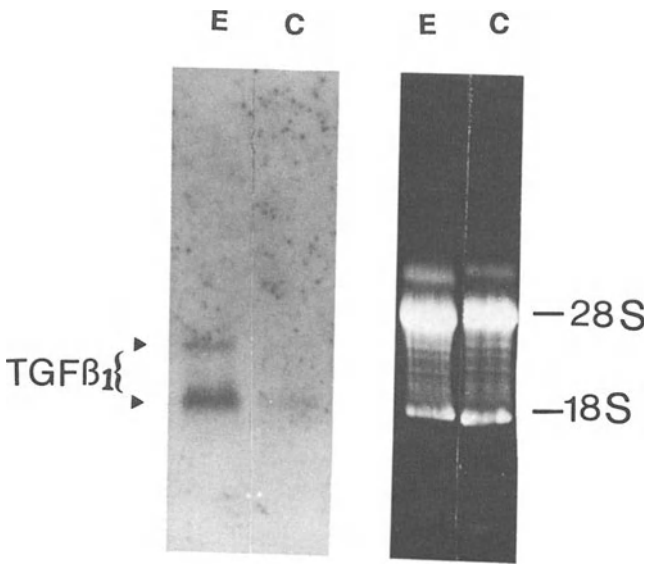


Fig. 9. Expression of TGF-β1 in the collateralized and normally perfused myocardial tissue
20 μg of extracted total RNA from collateralized (E) and normally perfused (C) tissue was separated on a 1% agarose gel, transferred to a nylon membrane and subjected to Northern hybridization with a human cDNA insert encoding TGF-β1. The autoradiograph depicts two mRNA bands 2.4 kb (major) and 3.5 kb (minor) after three days of exposure which were drastically icreased in E as compared to C.

used a porcine specific 257bp DNA fragment amplified by RT-PCR in Northern hybridization and found a significantly increased mRNA (1.9 kb) expression in the collateralized tissue as compared to the normally perfused tissue (Fig. 10). The presence of TGF-β1 mRNA in the normal adult pig heart suggests that there is an ongoing transcription, translation and utilization of this peptide.

Immunoblot analysis with a polyclonal anti-TGF-β1 antibody showed a specific band of 25 kD in myocardial protein extracts from normal and collateralized tissue (30). No differences in the signal intensity between normal and collateralized myocardium was observed. This could be explained by assuming a higher turnover of TGF-β1 in the collateralized tissue. Employing in situ hybridization, we found TGF-β1 specific mRNA transcripts predominantly in cardiac myocytes near fibrotic tissue and not in the area of inflammatory infiltrate (30). The results of indirect immunofluorescence studies on tissue sections are difficult to interpret and were conservatively described already in our paper (30). The problem is that different commercially (and other) available antibodies produce a different staining pattern. Of the 5 different antibodies that we

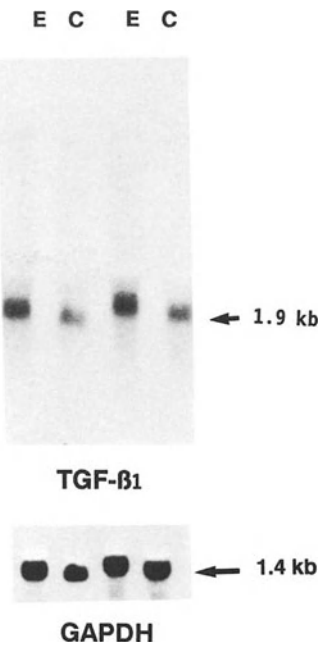


Fig. 10. Northern blot analysis of the TGF-β1 mRNA in the collateralized tissue.
Total RNA extracted from the collateralized (E) and control (C) myocardial tissue was used for the Northern hybridization with a radioactive 257 bp porcine specific DNA fragment prepared by RT-PCR. A 1.9 kb mRNA band for TGF-β1 was significantly increased in E as compared to C. Lower panel shows the GAPDH mRNA which was used as reference.

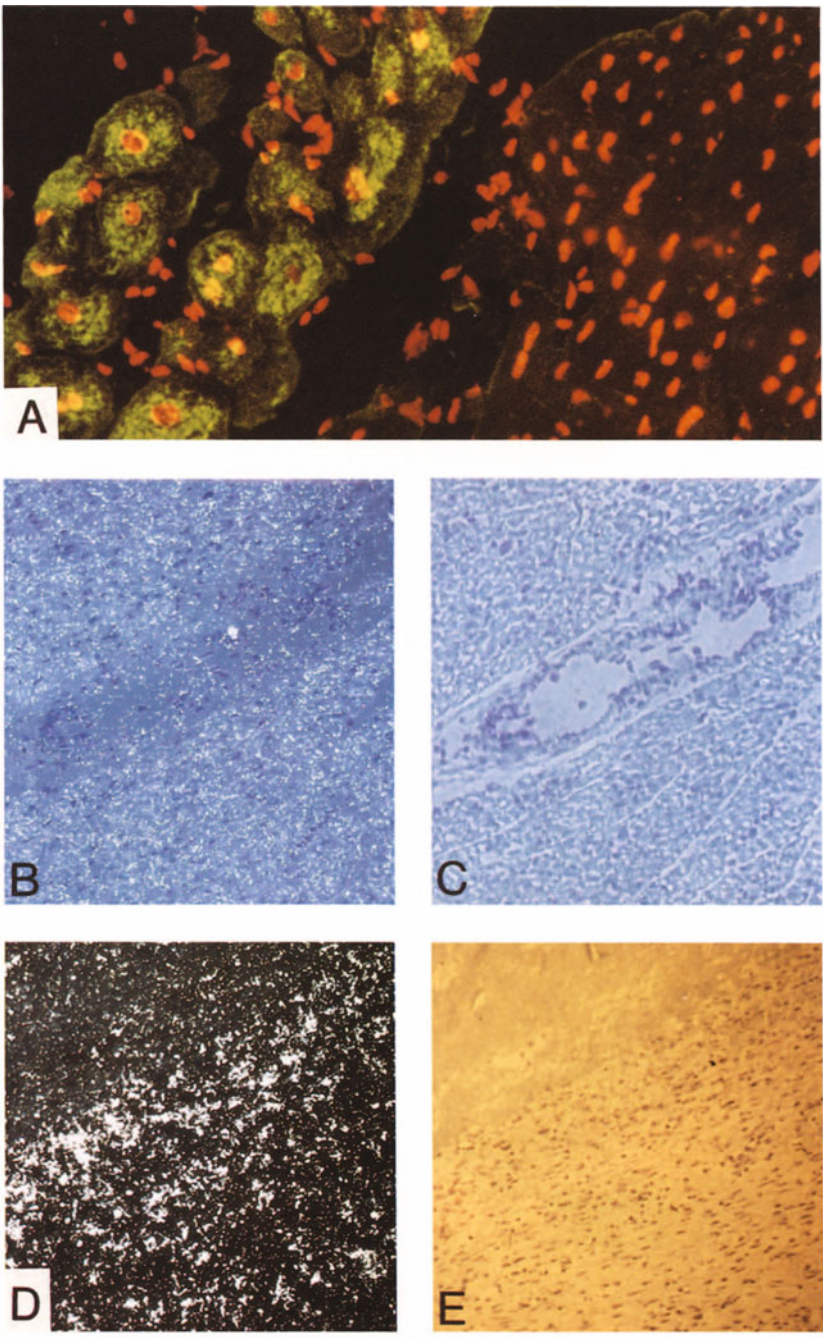
have tried, one is no longer available (the one rabbit that produced it for a British company had died) and the only other one which produces consistent results (chicken-anti-human) shows a localization only around capillaries and in capillary endothelial cells (Sack et al, unpublished). There was no change in localization between normal and collateralized myocardium. Purkinje cells of the conduction system were consistently stained with TGF-β1 specific antibodies (Fig. 11A). Possibly, TGF-β1 and other growth factors influence the degree of differentiation in these cells.

The cellular source of TGF-β1 in the heart is controversial. Thompson et al (31) found TGF-β1 mRNA and protein in the cardiac myocytes, whereas Eghbali (32) could demonstrate mRNA expression of TGF-β1 only in the non-myocyte fraction of cardiac tissue. She observed that the immunofluorescence staining for TGF-β1 was intense around the blood vessels and radially diffuse in the surrounding myocardium (32). In contrast to the findings of Thompson et al (31), we could not find a significantly enhanced expression of TGF-β1 in the infarcted tissue (30). By in situ hybridization technique, we found that cardiac myocytes predominantly express TGF-β1 mRNA and not the cells in damaged tissue or capillaries where only few silver grains were seen (Fig. 11B, C).

Vascular endothelial growth factor

Vascular endothelial gowth factor (VEGF) is a heparin binding, highly glycosylated cationic homodimeric protein of 46 kD (34), very similar to vascular permeability factor which contains a 24 amino acid insertion (35). VEGF cDNA has been cloned and analysis of its nucleotide sequence suggests that it is a member of the platelet derived growth factor (PDGF) family with 21-24% amino acid

Fig. 11. In situ localization of TGF-β1 and VEGF in the porcine myocardium.
A) Immunoflourescence micrograph depicting localization of TGF-β1 in the porcine myocardial tissue. Strand of Purkinje cells are brightly stained with anti-TGF-β1 antibody.
B) In situ hybridization of TGF-β1 mRNA in the collateralized swine myocardial tissue. Darkfield micrograph showing toluidine blue stained section of a collateralized tissue with dense silver granulae over the intact myocardium indicating TGF-β1 mRNA expression in the myocytes and not in vascular cells.
C) Bright field microphotograph of the above myocardial tissue section stained with toluidine blue. Histologically intact myocardium with a longitudinally cut blood vessel are visible.
D) In situ localization of VEGF mRNAs shows a dense accumulation of silver granulae in the granulation tissue of an infarcted area.
E) Destroyed myocytes are visible in the left corner, below there is granulation tissue. Bright field view of the same section as in D.


A
B
C
D
E

homology (34, 72). VEGF may exist by alternative splicing of mRNA as four different molecular forms with 121, 165, 189 and 206 amino acids polypeptides. The two shorter forms are secreted proteins, whereas the longer ones seem to be cell associated (36). VEGF has been isolated from a number of normal and tumor cells and its expression has been shown in the corpus luteum and developing brain and kidney (48,73). It is a highly specific peptide mitogen for endothelial cells derived from small and large vessels and is a potent angiogenic factor in vivo (34, 36).

As described for aFGF and TGF-β1, using similar molecular biological methods, we examined the expression of VEGF in the normal and collateralized myocardium. As a diagnostic search, we performed RT-PCR on cDNA templates prepared from normal and collateralized tissue. We amplified a DNA product of 319 bp by PCR derived from the normal as well as from collateralized myocardium. This PCR product was subjected to Southern hybridization using a cDNA insert of 930 bp encoding human VEGF (kindly provided by Dr. N. Ferrara). A clear dominant band of 319 bp hybridized to the

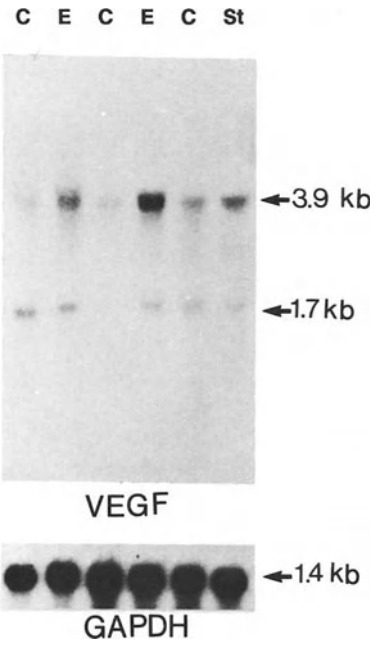


Fig. 12. Expression of VEGF in the normal (C) and collateralized (E) swine myocardium.
Northern blot analysis with a porcine specific cDNA probe was performed as described in the text. One major (3.9 kb) and one minor (1.7 kb) mRNA are expressed in both regions. In a stunned myocardial tissue VEGF expression is slightly induced (right lane) while in the collateralized tissue expression is induced by several folds. GAPDH mRNA (1.4 kb) was used as a reference.

human VEGF insert confirming the specific amplification of the VEGF (74). Furthermore, the PCR product was cloned into a plasmid vector and recombinant double stranded plasmid DNA was sequenced. The nucleotide sequence analysis of the PCR fragment cloned in the plasmid revealed a homology of 94% with the published human cDNA sequence. Using the porcine specific cDNA insert of 319 bp encoding VEGF, we detected two mRNAs of 3.9 (major) and 1.7 kb (minor) in the normal and collateralized myocardium (Fig. 12). The expression pattern of VEGF in various ischemic collateralized hearts revealed an overall enhanced expression of VEGF in the collateralized tissue as compared to the normally perfused tissue (Fig. 13). In the pig, where we observed infarction, VEGF expression was significantly reduced indicating the myocardial cells as a major contributor of VEGF (Fig. 13, right panel).

By in situ hybridization, VEGF mRNA could be demonstrated predominantly in cardiac myocytes and somewhat less over interstitial cells of normally perfused myocardium (Fig. 11D). Enhanced silver grains showing VEGF mRNAs were seen in the granulation tissue and in the viable myocytes somewhat remote from fibrotic regions of ischemic myocardium (Fig. 11E). These results clearly indicate a potential role of VEGF in the collateral vessel development in response to progredient chronic ischemia. Enhanced expression of VEGF in the collateralized myocardial tissue could also contribute to the enhanced permeability of endothelial cells. It is interesting to mention here that in a porcine model of myocardial stunning with two cycles of 10 min ischemia and 30 min reperfusion we observed significantly enhanced VEGF expression in the stunned tissue as compared with control (75). This suggests a rapid response of the VEGF-gene to ischemia and its contribution to the angiogenic processes (as for instance in the model of repetitive coronary artery occlusions). The rapid induction of VEGF in the ischemic myocardium would not rule out its properties as an intermediate molecule which may be needed to induce proteases involved in the extracellular matrix degradation. Pepper et al (77) have very recently shown that VEGF induces tissue and urokinase type plasminogen activators and their potential inhibitor PAI-1 in isolated microvascular endothelial cells in culture. The contribution of VEGF in inducing other angiogenic molecules in vitro as well as in vivo, particularly in the ischemic myocardium, is not yet clear.

Angiogenic response from the ischemic myocardium

Since myocytes are highly energy consuming cells in the heart, even a restricted blood supply would immediately affect the phenotypic mRNA expression of these cells. As we know that the vascular growth results in the reduction and prevention of myocardial ischemia, we assume that the growth adaptation is regulated in relation to the myocardial needs, i.e., a signaling pathway must exist from the ischemic myocytes to the arterial and capillary microvessels (61). Possible candidates involved in such a

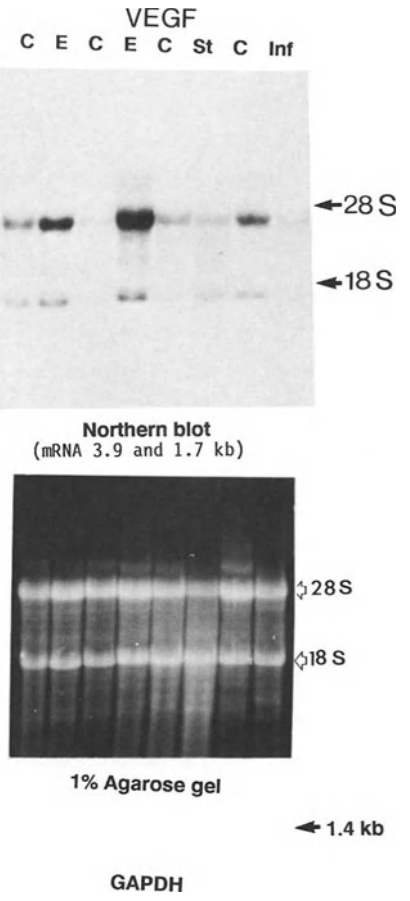


Fig. 13. Northern blot analysis of VEGF mRNAs in the various collateralized, stunned and infarcted myocardial tissues.
Using a porcine specific radioactive cDNA probe, an enhanced expression of two mRNAs encoding VEGF was observed in the collateralized myocardium. In the infarcted tissue VEGF expression was significantly reduced (right lane) while in the stunned tissue VEGF expression was unchanged. The ethidium bromide stained gel shows the integrity and quality of the RNA (middle panel). Lower panel depicts the expression of GAPDH, which was drastically decreased in the infarcted tissue (right lane).

signaling pathway could be TGF-β1, and the potent angiogenic secreted peptide, VEGF itself. We have shown that the myocytes in the ischemic region of the heart express significantly higher amounts of TGF-β1 and VEGF hence, they probably induce the process of myocardial angiogenesis in a paracrine manner to act on neighbouring endothelial cells, the building blocks of a new blood vessel. In addition, ischemic myocytes may also make and secrete a yet unknown ischemia-dependent mitogen activator which binds to the cell surface receptors on endothelial cells and thereby inducing a cascade of events leading to the myocardial angiogenesis. (The fallacy of such deductions based on vitalistic "needs" of the myocyte are elaborated in the chapters by Dr. Hollenberg and Dr. W. Schaper). Furthermore, mitogenic peptides may also be carried by the circulating blood into the ischemic myocardium for the vascular growth. Circulating monocytes and platelets have been reported to adhere to the endothelium of the growing epicardial collaterals (61, 78). Adhered platelets and invading monocytes are known to produce a number of polypeptides such as PDGF, TGF-β1.

CONCLUSION

The heart is an organ with only limited capabilities to protect itself from acute ischemic episodes. However, the heart can partially compensate for its inability to produce new myocytes by growing new blood vessels "wherever and whenever needed". In this study, we have shown that the progressively slow chronic coronary artery occlusion leads to the induction of angiogenic processes, which probably require a cascade of molecular and cellular events in the myocardium. We have provided evidence that this ischemia induced myocardial angiogenesis involves a variety of polypeptide growth factors. Using molecular biological techniques, we have shown that myocardial collateralization requires aFGF, VEGF and TGF-β1 in a very regulated manner for their functions. The existence of a number of angiogenic polypeptides in the *normal* adult heart and continuous expression of their genes indicate yet unknown mechanism(s) for their functions. However, it can be stated that these molecules are probably vital for the cardiac myocytes and their vascular network.

Acknowledgements: We would like to extend our sincere thanks to Drs. R.J. Schott and S. Sack for the surgical work during ameroid constrictor experiments. We are grateful to Drs. R. Zimmermann, R. Kandolf and M. Wünsch who carried out or helped during in situ hybridization studies. Technical assistance of Mrs. C. Ullmann,

B. Münkel and E. Neubauer is highly acknowledged. We also thank Mrs. A. Möbs for photographic work and Mr. G. Stämmler for the computer help.

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8

FUNCTIONAL ASPECTS OF COLLATERAL DEVELOPMENT IN ANIMAL MODELS

Hitonobu Tomoike
The First Department of Internal Medicine, Yamagata University, School of Medicine, Yamagata, Japan

INTRODUCTION

Coronary collateral circulation is a defense mechanism, in cases of gradual coronary narrowing or obstruction (1, 2). However, collateralization is not unique to the coronary circulation. Derangement in perfusion, in general, accompanies angiogenesis and/or further dilation of pre-existing anastomotic channels toward the perturbed area in the brain, lung, skeletal muscle, kidney and so on (3). Although unused pathways between major coronary arteries can be present in the intact heart, even in the newborn (4), the presence of an anastomosis cannot be equated with collateral channels in hearts with ischemic heart disease (5). Not only angiogenesis or de novo vascular formation but also expansion of pre-existing collaterals involve active cell proliferation (6). Recent advances in molecular biology are revealing interesting and clinically important phenomena related to collateral development (7). Nevertheless, factors related to collateral growth in vivo are complex, and are a matter for conjecture.

Experimentally induced or clinically observed collateral channels have been evaluated in detail in postmortem specimens (8). To elucidate mechanisms of collateral growth, cause-effect relationships with regard to vascular formation and/or remodeling need to be examined under chronic conditions and repeatedly during the maturation of collateral channels. Quantification of collateral growth includes morphological, functional, chemical, and signal transductional assessments in newly formed vessels.

METHODS OF MONITORING COLLATERAL FUNCTION

Anastomotic connections have been examined experimentally and clinically, using coronary angiography, regional myocardial blood flow, retrograde flow, reactive hyperemia, peripheral coronary pressure, or viability of the myocardium. The extent of infarction or ischemia, reactive hyperemia and regional wall motion abnormality reflect the nutritional level of collateral blood flow (9). Parameters not related to the evolution

Wolfgang Schaper and Jutta Schaper, (eds.), Collateral Circulation.

of infarction are favourable for repeated measurements during the process of vascular growth.

Since the middle 1970's, it has been possible to record regional wall motion at the micrometer level with use of miniaturized ultrasonic crystals (10). This technique provides accurate and instantaneous measurements of the distance between crystals, even in a running animal (11). Several studies have shown that myocardial shortening is related to the amount of coronary blood flow (12, 13). We repeatedly confirmed an inverse linear relation between the reduction of reactive hyperemia and recovery from contraction abnormalities during temporary coronary occlusion after implantation of an ameroid constrictor (14, 15, 16) (Figs. 1 and 2). The appearance of regional hypokinesia in the presence of ischemia is clearer than are changes in systemic hemodynamics and at the ECG ST level (17). In addition, the degree of hypokinesis at the center of ischemic area in the ameroid model correlates with the amount of regional myocardial blood flow, as assessed with radioactive tracer microspheres (16). Accordingly, it is now well accepted that regional myocardial shortening during abrupt coronary occlusion provides

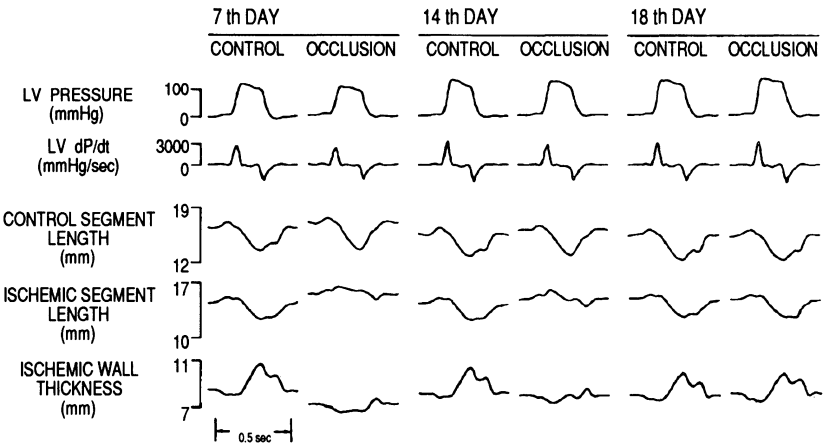

Fig. 1. Responses to abrupt coronary occlusion during progressive coronary stenosis induced by an ameroid coronary constrictor. Left ventricular (LV) pressure, LV dP/dt, and regional segment lengths in control and ischemic areas shown. Basal hemodynamics for 7th, 14th, and 18th days after ameroid implantation shown, in left-hand columns, and conditions 1 min after coronary occlusion in those on the right. In a representative dog, the ischemic response on the 7th day with increased end-diastolic length and holosystolic lengthening during ejection; response less marked on 14th day and disappeared on 18th day. Note segmental dysfunction accompanied by reduction of LV systolic pressure and LV dP/dt. Darkened lines produced for illustrative purposes by adding a 300-Hz sine wave to the original tracing (data from Tomoike et al (15)).

direct and quantitative information concerning the functional states of collateral channels. Two pairs of ultrasonic crystals were chronically implanted to measure regional wall motion at the ischemic and non-ischemic areas along with ECG and left ventricular pressure. These parameters were continuously measured in conscious animals (16, 18).

Effects of gradual coronary stenosis on collateral development

Characteristics of an ameroid constrictor: Gradual coronary occlusion over a period of weeks especially in a canine model, leads to the development of collateral vessels (19). The ameroid constrictor has been one of the most popular devices for use in

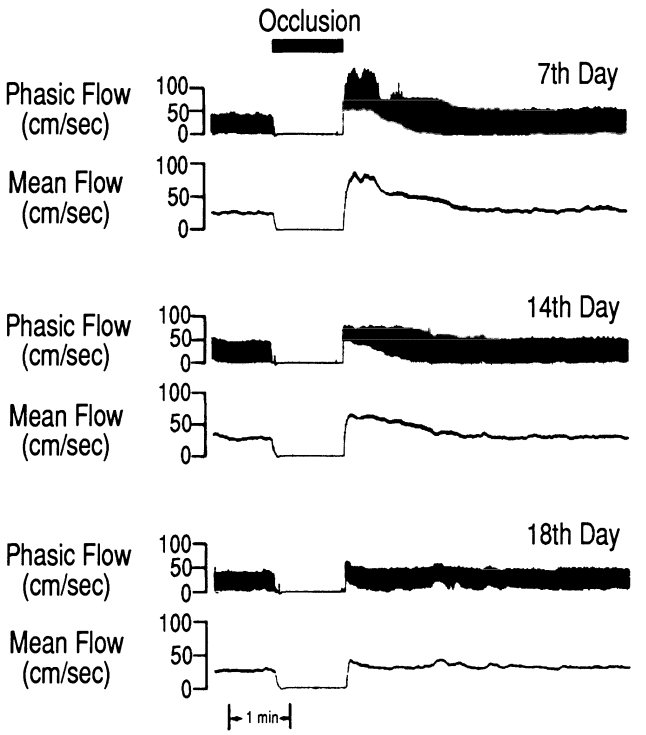

Fig. 2. Responses of coronary blood flow velocities to short coronary artery occlusions during the course of ameroid-induced coronary artery obstruction in the representative experiment shown in figure 1. Phasic and mean flow velocities shown on each day. In this example, resting blood flow velocity did not change significantly, but serial tracings illustrate a progressive decline in reactive hyperemia (data from Tomoike et al (15)).

experimental models of collateral development. The appearance of collateral channels was evaluated not only anatomically or angiographically, but also by measuring peripheral coronary pressure, retrograde flow, reactive hyperemia and regional wall motion abnormalities that appear during temporal coronary occlusion. The relationships among collateral flow, regional wall motion and the grade of coronary stenosis during ameroid-induced chronic coronary constriction were investigated (16).

To define the extent of coronary stenosis, the radiolucent ameroid constrictor devised involved placing a plastic ring around the ameroid instead of a stainless steel case (20). Gradual inward expansion of the ameroid was quite different, depending on conditions such as between saline and in situ. As shown in figure 3, the extent of coronary stenosis in situ progressed strikingly to 57 and 88% on the 10th and 30th day of implantation. There was a big dog-to-dog variation in a speed of luminal narrowing, compared with events seen with saline. Histological examination revealed an abundance of inflammatory responses at the outer layer of the media and the adventitia along with intimal thickening, fibrinoid degeneration and edema (20). Accordingly, arterial tissue reactions also play an important role in the progression of coronary narrowing at the time of complete occlusion. As the nature of the device is uncontrollable, experiments should need their own control before the occurrence of any significant coronary stenosis.

An animal model: Under sterile surgical techniques and a positive pressure respiration, a radiolucent ameroid, a Doppler flow probe and a cuff occluder are placed on the left circumflex coronary artery, and two pairs of piezoelectric crystals are

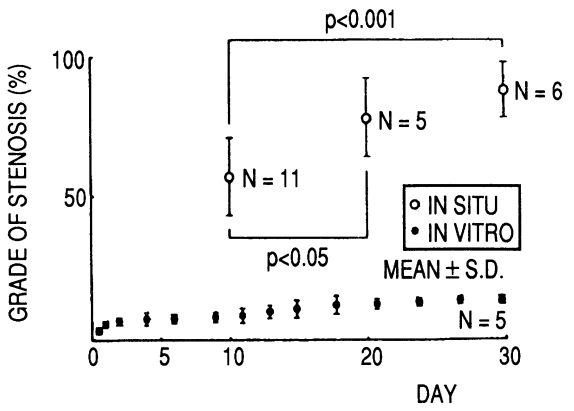

Fig. 3. Chronological changes in luminal diameter of an ameroid constrictor in saline (in vivo) and in situ conditions. Coronary stenosis in situ was assessed angiographically (data from Inou et al (20)).

implanted subendocardially at the LAD and LCX areas. In 22 conscious dogs, regional wall motion and regional myocardial blood flow were assessed simultaneously, using a multi-channel sonomicrometer and a tracer microsphere technique, respectively, during temporary occlusions of the LCX by a cuff occluder.

Time course of collateral function: Representative serial changes in regional myocardial shortening before and after abrupt coronary occlusion are shown in figure 4 (16). Percent shortening of the LCX area decreased to around 2% of control and there was no obvious amelioration for 25 days along with 5-8% hypershortening at the LAD area. There were progressive recoveries of regional hypokinesia associated with reduction in EDL and finally the regional shortening was unchanged before and after coronary occlusion. Regional myocardial shortening before coronary occlusion was maintained fairly constant during the entire period, hence, gradual coronary occlusion did not lead to myocardial infarction. As also shown in figure 4, amelioration of regional shortening during occlusion was rapid. The average duration from the

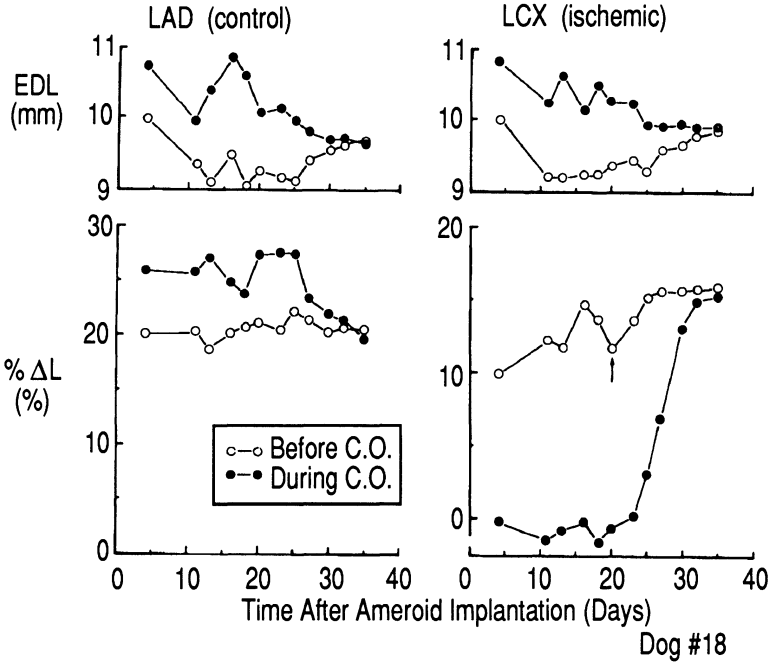

Fig. 4. Serial changes in end-diastolic length (EDL) and % systolic shortening before and after temporary coronary occlusion in a representative case (data from Tomoike et al (6)).

attenuation of regional myocardial hypokinesis to full recovery during temporary of coronary occlusion ranged from 5 to 29 days (14±3 days). A 50% recovery to full recovery ranged from 4 to 16 days (7±2 days). Disappearance of flow signal occurred from 11 to 53 days (28±6 days) in 6 dogs. Accordingly, the time course of collateral growth correlated with the progression of luminal narrowing.

Correlation between regional blood flow and wall motion in gradually-induced coronary stenosis: When the functional state of collateral vessels was minimum, early after instrumentation, regional myocardial blood flow at the LCX endocardial region decreased strikingly, from 1.21±0.28 ml/min/g (Ac in Fig. 5) before occlusion to 0.28±0.07 ml/min/g during occlusion (Ao in Fig. 5). After a functionally full growth of collateral vessels, in which flow measurements were performed on about the 23rd day

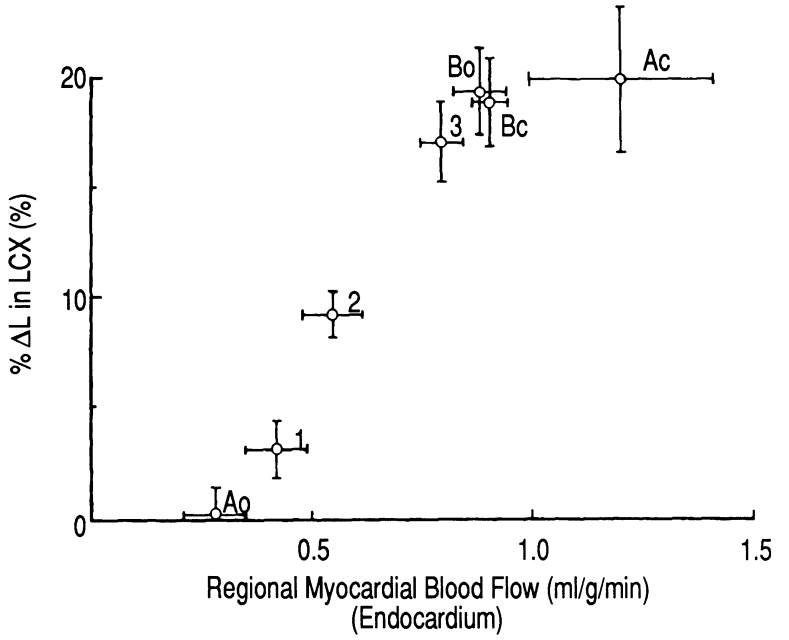

Fig. 5. Relationship between regional myocardial flow measured by tracer microspheres and % shortening (% L) measured sonomicrometrically at the endocardial site of the LCX area in the ameroid model. Ac and Ao = before and during temporary coronary occlusion early after the ameroid implantation. Bc and Bo = before and during temporary coronary occlusion after collateral development. The numericals 1, 2, 3 indicate the beginning, 50% and 100% development of collaterals, respectively, from the functional aspects (data from Tomoike et al (16)).

of instrumentation, regional myocardial blood flow in the LAD and LCX areas was unchanged before and during coronary occlusion (Bc vs Bo in Fig. 5).

Including data obtained during the time of collateral growth, a fairly linear relation was noted between regional myocardial shortening at the endocardial site and regional myocardial blood flow at the site of crystal implantation during coronary occlusion (y=31x-9, r=0.99). Thus, the dependency of regional myocardial performance on regional flow was confirmed in the case of chronic increases in collateral flow as it was repeatedly documented in acute reductions of antegrade coronary flow (12, 13).

Critical coronary stenosis for collateral development: There was a time delay (for example, 25 days in Fig. 4) in the day-to-day recovery of regional systolic wall motion during abrupt coronary occlusion by a cuff occluder. Such a delay may be related to the progression of gradual coronary stenosis to the critical level for myocardial ischemia (21). Figure 6 shows the time course of changes in regional wall motion and coronary flow before and after temporary occlusion. Changes in reactive hyperemia also show that progression of coronary stenosis to the critical level occurred over several days (Fig. 6, lower panel).

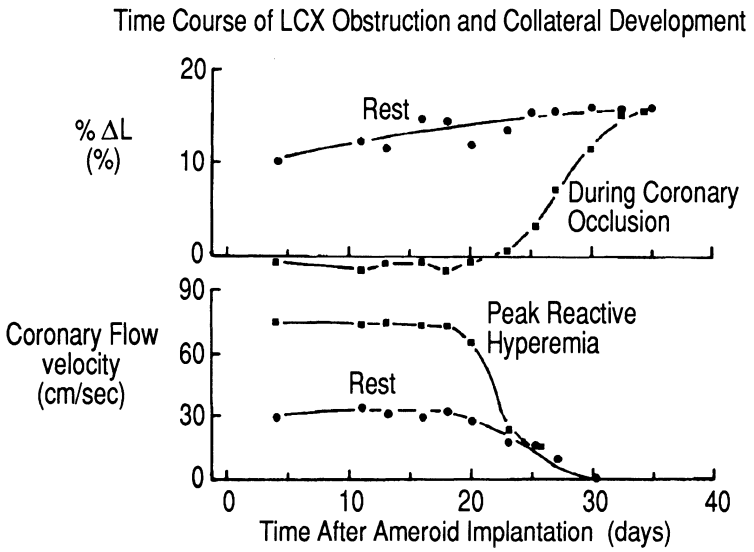

Fig. 6. Chronological changes in regional wall motion (upper panel) and coronary blood flow at rest and during reactive hyperemia (lower panel), in a representative case.

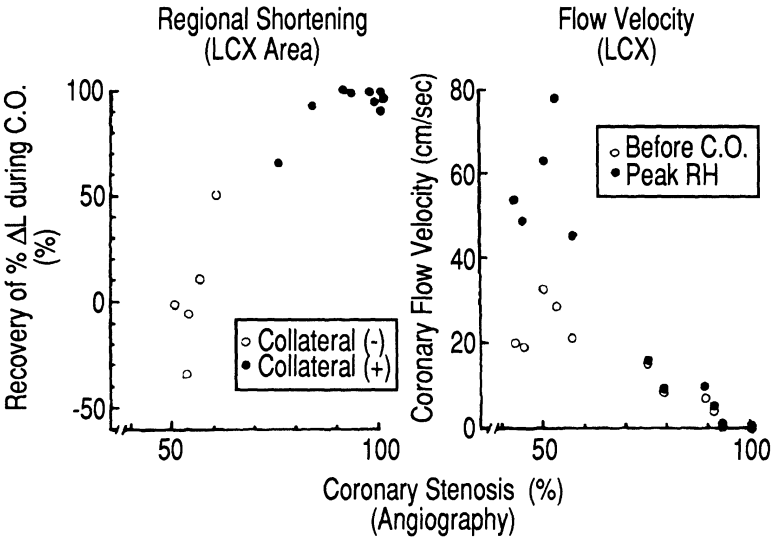

Fig. 7. Relationship between coronary stenosis (angiographically determined) and regional shortening (left) and coronary flow velocity (right) (data from Tomoike et al (16)).

Coronary angiography under anesthesia was done using a Kifa catheter before and during temporary coronary occlusion. With the amelioration of regional hypokinesia, the LCX distal to the occlusion was opacified from the intact LAD. As shown in figure 7 left, collateral channels were evident angiographically when the LCX stenosis exceeded 75%. This critical level of coronary stenosis coincided with the time when the resting and hyperemic flow became equal.

Thus, inherent collaterals may functionally be stimulated at the level of 75% coronary constriction, an event which resulted in the presence of a pressure gradient or of myocardial ischemia. Since the myocardium at the LCX area at autopsy had no significant fibrosis, transient appearance of the derangement in oxygen supply-demand balance in daily life stimulated collateral formation in the presence of critical coronary stenosis.

Repetitive brief coronary occlusions and collateral development

Angiographic studies in patients with vasospastic angina revealed a transient staining of collateral channels during vasospastic attacks (22, 23). This phenomenon was confirmed in a canine model (18, 24), as shown in figure 8. These lines of evidence support the postulation that intermittent myocardial ischemia and/or pressure gradients

between the proximal and the distal part of the occluded epicardial coronary artery can stimulate the evelopment of coronary collateral vessels, as observed in the classical animal model with a gradual coronary stenosis.

In the repetitive coronary occlusion model, the potential stimulus is controllable, such as changes in duration, repetition rate or mode of coronary occlusion. Quantitative analyses of cause-effect relationships in collateral development are thus feasible. The level of collateral development was assessed functionally, based on changes in regional wall motion during coronary occlusion or by reactive hyperemia responses following reperfusion.

The mechanisms and transmitters related to collateral development in repetitive coronary occlusions remained unclarified, in the experiment as well as in the setting. A

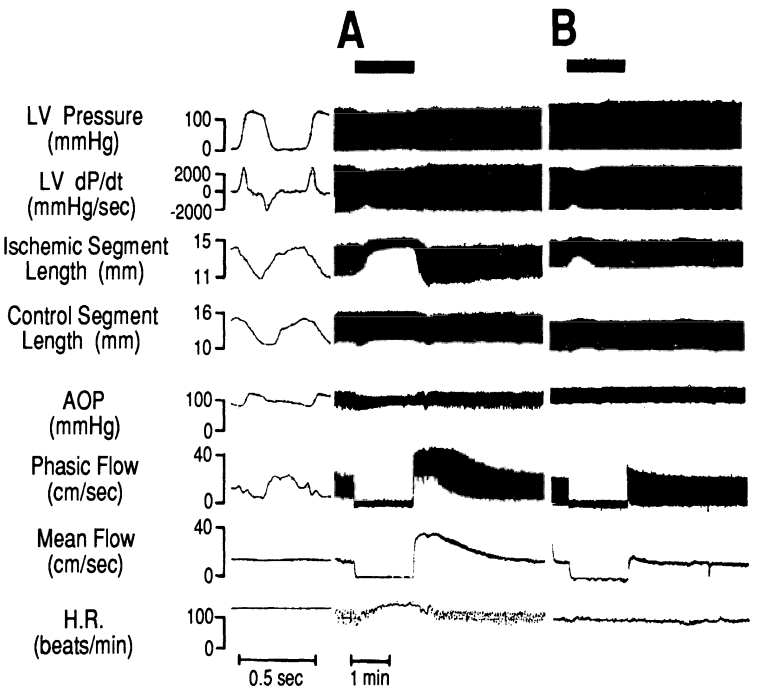

Fig. 8. Effects of a 90 second coronary occlusion before (panel A) and after (panel B) collateral development in the same conscious dog, on left ventricular pressure (LV pressure) left ventricular dP/dt (LVdP/dt), segment length at the LCX and LAD areas, aortic pressure (AoP), phasic flow and its mean, and heart rate (HR). Note that, after collateral development, regional hypokinesia in an ischemic area was transient and recovered before the release of LCX occlusion. Resultant reactive hyperemia was markedly attenuated (data from Yamamoto et al (8)).

2-minute coronary occlusion was empirically chosen for enhancing functional capacity of coronary collateral vessels (18), because regional dysfunction completely recovers by reperfusion within 45 seconds, and the occurrence of reperfusion-induced fatal arrhythmia seems tolerable (17). Little is known about the duration of ischemia as a stimulus for collateral development. Effects of repeated 15 second coronary occlusions at an interval of 4 minutes were studied in a different group of dogs (25). The total cumulative time of occlusion per day was 90 minutes, the same as for the 2 minute occlusion group. Fifteen seconds of occlusion was chosen because the peak level of reactive hyperemia following reperfusion was similar to that seen with the 2-minute coronary occlusion (26). The difference between the 2-minute and 15-second occlusion was the level of regional hypokinesia, not the level of myocardial blood flow.

An animal model: Adult mongrel dogs of either sex, weighing 20-32 kg, were anesthetized with sodium pentobarbital 25 mg/kg i.v. and were ventilated with room air with a positive pressure respirator. Using sterile surgical techniques, a left thoracotomy was performed at the 4th intercostal space and the pericardium was incised. An 8F polyvinyl chloride catheter was inserted into the left internal thoracic artery to monitor arterial pressure. A micromanometer (P-22, Konigsberg Instrument, Pasadena, California) was placed inside the left ventricular cavity through a stab wound at the apex along with an 8F polyvinyl catheter for calibration. A pneumatic cuff occluder and a 20 MHz pulsed Doppler flow probe or an electromagnetic flow probe (Nihon Koden, Tokyo, Japan) were positioned around the proximal site of the left circumflex coronary artery. Two pairs of 5 MHz piezo electric crystals were implanted subendocardially into the left ventricular free wall, as shown in figure 9. Epicardial electrodes were sutured to the right atrial appendage, the right ventricular wall and the center of LCX area for electrocardiograms (Fig. 9).

The pericardium was left open, and all wires and tubings were passed subcutaneously to the base of the neck and secured between the scapulae. The chest was closed, and antimicrobials were given intramuscularly for 7 days (25).

Protocol: All studies were carried out 10-14 days after the initial surgery. All dogs were active and fully recovered from the surgical procedure. The experimental room was dimly illuminated and kept free from noise or other activities.

After control recordings of electrocardiograms, left ventricular pressure (LVP), wall motions at the LCX and LAD areas, coronary flow of the LCX and arterial pressure,

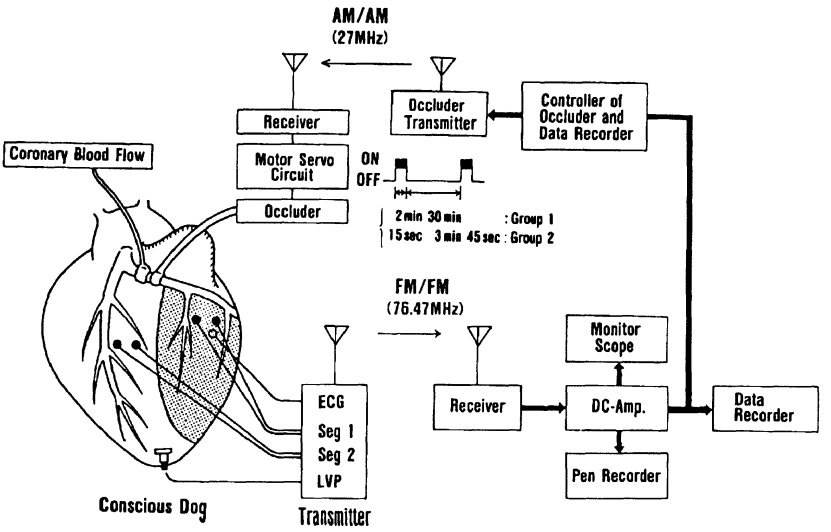

Fig. 9. Diagram of experimental system. ECG, electrocardiogram; Seg, segment length; LVP, left ventricular pressure; DC-amp, direct current amplifier (modified from data Mohri et al (25)).

an abrupt coronary artery occlusion was maintained for 2 minutes by a cuff occluder to record the level of regional and systemic derangements of the first coronary occlusion.

To continuously monitor a high fidelity LVP, two sets of segment lengths and an electrocardiogram from an untethered animal were obtained without interruption day and night, and an FM/FM telemetry system was designed to transmit these signals to a remote station (Fig. 9). Amplifiers for these parameters and an FM transmitter were incorporated into a small back pack (140 mm x 90 mm x 20 mm, approximately 150 g in weight), which were easily carried by the dogs. The system functions with six R 14 batteries which are renewed once a day. An occluder and a radiocontrolled miniature servo motor were also carried by the dog in the same back pack and were controlled by an AM/AM telemetry system. The dog was kept in a cage in an air conditioned room for the experiments. The dogs were randomly allotted to two groups (2 min and 15 sec occlusions) in this series of studies. Signals from LVP, two sets of regional segment length, and epicardial ECG via an FM/FM telemetry system were continuously monitored and were recorded on a pen-recorder at a paper speed of 5 mm/min and on magnetic tape throughout the experiment.

Group 1 consisted of 16 dogs in which a 2 minute coronary occlusion was repeated every 32 minutes. Group 2 consisted of 18 dogs in which a 15-second coronary

occlusion was repeated every 4 minutes, in the same manner as group 1. In group 2, this protocol was repeated at least for 10 days (3,600 or more occlusions) and a 2-collateral development. Total cumulative time of LCX occlusion was 90 min/day, in both groups.

When regional shortening during a 2-minute coronary occlusion recovered to the preocclusive state, repetition of the protocol was stopped. The dogs in both groups were then re-anesthetized with an intravenous administration of sodium pentobarbital and ventilated with a positive pressure respirator. Selective coronary arteriography was performed to determine the level of coronary stenosis around the cuff occluder and the degree of collateral vessel development during the coronary occlusion. Figure 10 shows a representative case after collateral development. To assess hemodynamically the level of collateral development, a rigid polyvinyl catheter was also inserted into a Kifa catheter and peripheral coronary artery pressure was monitored during an abrupt occlusion of the implanted cuff.

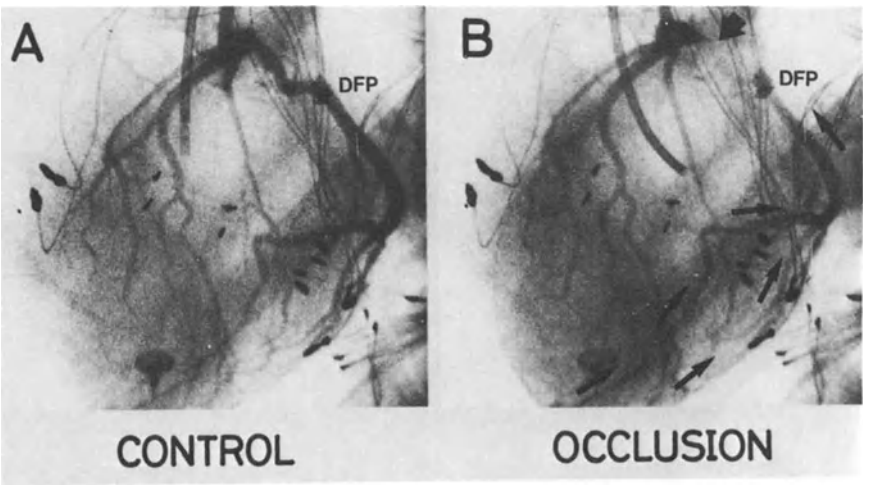

Fig. 10. Coronary angiograms before (left side) and after coronary occlusion (right side), in a representative case with well developed collaterals. The large arrow indicates the site of placement of the cuff occluder. DFP, Doppler flow probe.

At the end of the experiment, the dog was given a lethal dose of potassium chloride intravenously, the heart was excised, and a barium-gelatin mixture was perfused into the coronary arteries at a pressure of 120 mmHg. The heart was unrolled according to the Schlesinger's technique by separating the right ventricular free wall and the interventricular septum from the left ventricle at the border (27). The unrolled heart was fixed in 20% buffered formalin and stereo-angiograms were taken.

Background on data analysis: Five of 16 dogs in group 1 and 5 of 18 dogs in group 2 died of ventricular fibrillation during repeated coronary occlusions. In another 5 animals in group 2, significant coronary artery stenosis above 90% luminal reduction was noted at the site of the occluder. In these five dogs, collaterals had developed from the LAD to the periphery of the stenosed LCX, and these dogs were excluded from the

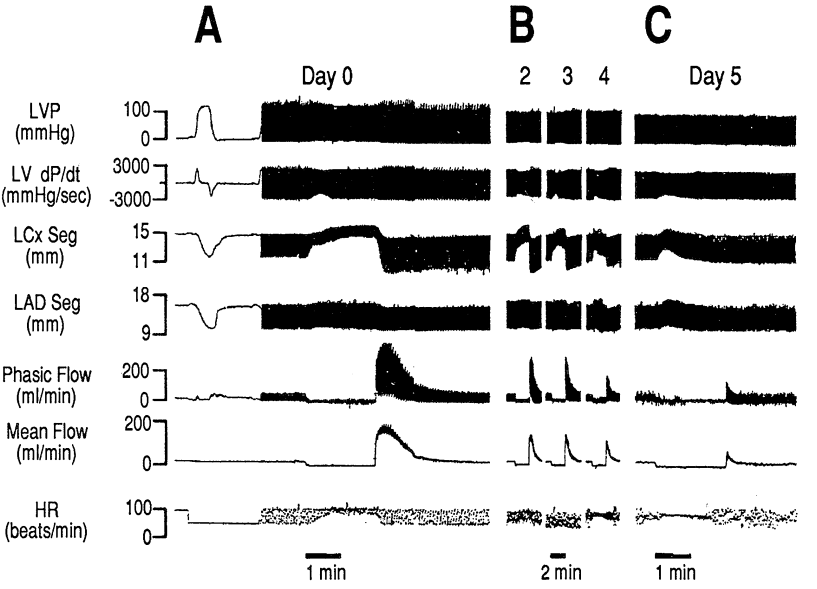

Fig. 11. Original tracings of left ventricular pressure (LVP), left ventricular dP/dt, segment lengths (Seg) in the left circumflex artery (LCX) and the left anterior descending artery (LAD) areas, phasic and mean LCX flow, and heart rate (HR) before (A) during (B; 2,3,4 = 2,3, and 4 days after the beginning of repetitive occlusions), and after (C) repeated 2-minute coronary occlusions in group 1. Regional hypokinesia in the LCX area and hemodynamic derangement during 2-minute coronary occlusion gradually improved as a function of time, along with a progressive diminution in reactive hyperemic response. On days 5, hemodynamic variables and segment shortening at 2 minutes of LCX occlusion returned to the preocclusive levels (data from Mohri et al (25)).

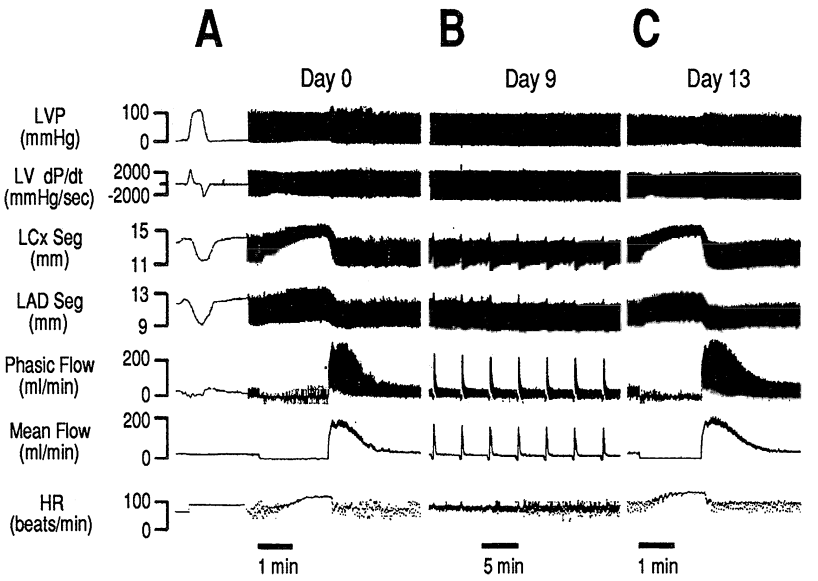

Fig. 12. Original tracings from a dog in group 2. The degree of regional hypokinesia (during 2 minutes) of the left circumflex coronary artery (LCX) occlusions changed little before (A) and after (C) the procedure of repeated 15-second coronary occlusions. Panel B shows typical tracings on day 9. A 15 second coronary occlusion was repeated at 4-minute intervals, continuously day and night. LVP, left ventricular pressure; LAD left anterior descending coronary artery; HR, heart rate (data from Mohri et al (25)).

following analysis. Therefore, we examined the effect of repetitive brief coronary occlusions on the collateral development in 11 dogs in group 1 and in 7 dogs in group 2.

Collateral function after repetitive brief coronary occlusions: Regional wall motion abnormality during 2 minutes of coronary occlusion in group 1 was attenuated progressively with continuation of repetitive procedures of coronary occlusion, as shown in figure 11. Changes in LV peak pressure, LV dP/dt and heart rate during the occlusion and the reactive hyperemic response following reperfusion were gradually attenuated to the levels of the preocclusion state at the end of repeated occlusions. Namely, resting regional systolic derangement disappeared after 125-478 (265±106) coronary occlusions. The time required for functional development of collateral circulation following occlusion stimuli was 3-11 days.

As shown in figure 12, 3,500-5,450 coronary occlusions (4,400±630) were repeated every 4 minutes in 7 animals of group 2. Despite 11-16 days of repetitive ischemic stimuli, changes in EDL and regional % shortening during 2-minute coronary occlusion were unaltered compared to findings at the first 2 minute of coronary occlusion. Figure 13 shows changes in % systolic shortening during the 2-minute coronary occlusion before and after repetitive ischemic stimuli in groups 1 and 2.

Measurement of peripheral coronary pressure (PCP) was feasible in 5 of 11 dogs in group 1 and in 4 of 7 dogs in group 2. After complete occlusion of the LCX by inflating the implanted cuff occluder, PCP fell in both groups and was significantly higher in group 1 (69±12 mmHg) than in group 2 (23±4 mmHg) ($p<0.001$, group 1 vs 2).

Accordingly, functional recovery in the presence of coronary occlusion in group 1 (Fig. 13) accords well with the hemodynamic improvement in PCP and angiographic documentation of collateral channels. Repetitive 2 minute ischemic stimuli induced dilation of anastomotic channels and/or angiogenesis, as was observed

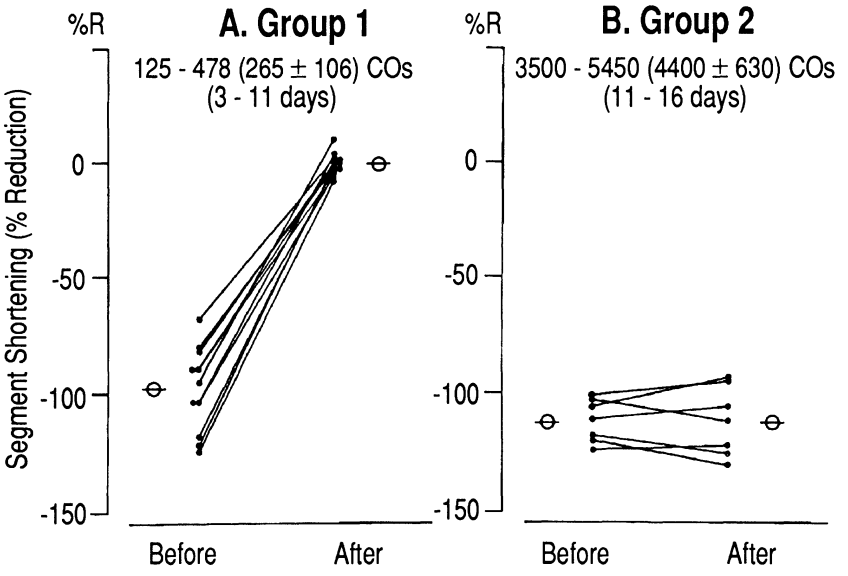

Fig. 13. Changes in the degree of regional dysfunction induced by a 2-minute occlusion of the left circumflex coronary artery before and after repeated coronary occlusion (COs) in 11 dogs of group 1 (panel A) and in seven dogs of group 2 (panel B). Segment shortening during 2 minutes of occlusion is presented as a percent reduction (%R) from a preocclusive level (data from Mohri et al (25)).

in the gradual occlusion model.

Duration of ischemia as a determinant of collateral development: We defined the rate of collateralization as integrated numbers of abrupt coronary occlusion. The functional capacity of coronary collateral vessels can be enhanced with repetition of 2-minute coronary occlusions every 30 or 60 minutes, as confirmed by our observations and those of Franklin and colleagues (24). In contrast, a 15-second coronary occlusion failed to stimulate collateral development functionally as well as hemodynamically, despite a longer period of occlusion stimuli. In our experimental design, the ischemic stimulus for collateral development was applied only during the brief period of coronary occlusion. There was no myocardial ischemia or pressure gradient between the major coronary arteries, during the reperfusion periods.

Quality of ischemic stimulus needed for collateral development: Olsson and Gregg found that 5-7 seconds of coronary occlusion elicit maximal dilation of the resistance vessels in the conscious dog (26). Yamamoto et al noted in the same conscious animal model as used in the present study that the debt repayment ratio was almost at the peak level in occlusions of 5 to 20 seconds (18). Olsson also showed that a 15-second coronary occlusion increased the level of intramyocardial adenosine by a factor 5 (28). Thus, the amount of a vasodilating substance produced during the ischemic period may not be sufficient in case of a 15-second occlusion, or such a vasodilator substance may not be of primary importance in initiating collateral development, in the present animal model.

Cumulative periods of coronary artery occlusions did not play an important role in collateral development. Accordingly, we assume that there is a qualitatively critical difference between the present two types of brief coronary occlusion with regard to the capacity to stimulate collateral formation. These lines of evidence suggest the presence of a threshold in ischemic stimulus for developing coronary collateralization. Recent advances in knowledge of cell adhesion and angiogenesis are expected to elucidate these events.

Functional development follows a growth curve

Chronological changes in systolic wall motion during transient coronary occlusion were plotted (Fig. 14). The profile of the time course of collateral development mimicked that of a biological growth curve. In general, a biological growth curve

includes sequentially a lag, an acceleration and a plateau phases. Whether the biological significance noted in bacterial growth equates collateral development awaits further investigation.

Time course of collateral development in the repetitive occlusion model was similar to that seen with the ameroid model shown in figure 4. The physiological process during the lag phase was the time course of progression of coronary stenosis in the ameroid model and was the accumulation of ischemic burden in the repetitive occlusion model. Because angiograms revealed the presence of epicardial collaterals in these canine models, there may be a unifying process along the epicardial anastomotic channels located far from the center of ischemic derangement.

To accurately compare the time interval for acceleration, a rigid comparison of two models in one particular animal should be studied or genetically controlled animals should be grouped for comparison. However, collateral development is unidirectional and irreversible which hinders the strict comparison of two models in a particular dog or groups of animals.. Expansion and/or proliferation of anastomotic epicardial channels necessitates at least a flow signal across these channels.

In our studies, the profile of the acceleration phase during the maturation of

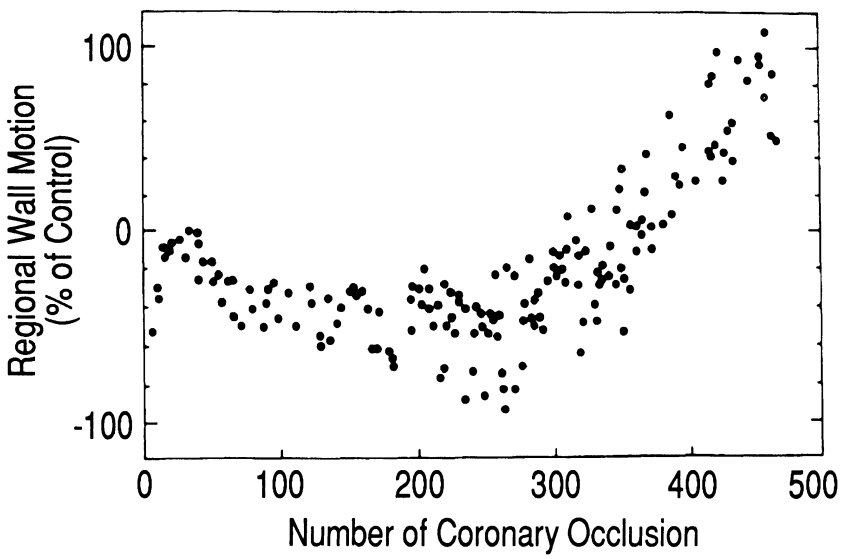

Fig. 14. The time course of collateral development with regard to changes in systolic shortening during temporary coronary occlusion.

collateral function was similar between the ameroid and repetitive occlusion models. These events suggest the importance of blood flow across anastomotic channels and the concept of increased wall tension or tangential shear stress as a necessary step in the proliferation in the arterial wall is given support (29). Increase in flow dilates the vascular caliber not only functionally by endothelium derived relaxing factor (30), but also structurally in case of chronic stimulation (31). Noma et al also noted the importance of native collaterals for the speed of later development of collateral function following ischemia-related stimuli (32). This would explain the basic mechanism of animal-to-animal variation in collateral development.

Unifying process in gradual stenosis and repetitive occlusions

Coronary reactive hyperemic responses have been used to evaluate the coronary reserve capacity (33). During the time course of collateral development, hemodynamics and non-cardiac disease states such as anemia and febrile illness remained stable. Accordingly, the extent of collateral growth determines the magnitude of the reactive hyperemic response. As described in the previous chapter, an inverse linear relation between regional shortening during temporary coronary occlusion and the debt repayment ratio during reperfusion was noted in either the ameroid or the repetitive occlusion model (Fig. 15). The collateral development affects the nutritional level of the perturbed area. Coronary stenosis was compensated for by the extra flow through the growing anastomosis between the LAD and LCX. If collateral growth is governed by the progression of myocardial ischemia, the presence of an irreversible myocardial damage means that the compensatory mechanism due to collateral growth cannot keep pace with the speed and severity of the ongoing myocardial ischemia.

Pharmacological modification of myocardial ischemia

Nitroglycerin, nitrate, Ca-antagonist, and β-blockade have widely been accepted as remedies for angina pectoris. Some of coronary vasodilators are occasionally deleterious in patients with significant coronary stenosis (34, 35). Measurement of regional myocardial blood flow or peripheral coronary pressure have been used to determine quantitatively the level of collateral contribution. However, these parameters did not provide information on how collateral channels do help cardiac pump performance, nor solve the role of collateral channels in amelioration of transient ischemia. As described in the previous chapter, regional wall motion of the area under perturbed perfusion of forward flow becomes dependent on the collateral flow.

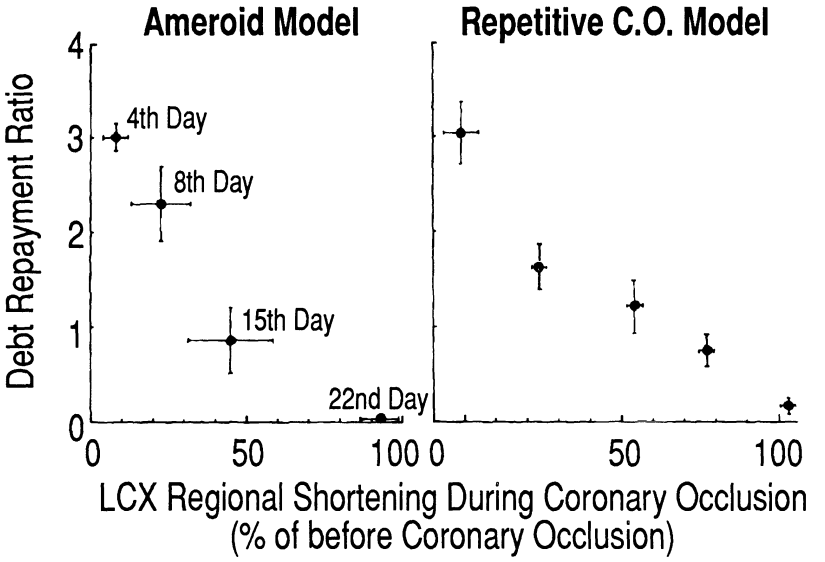

Fig. 15. Relationship between flow debt repayment during reactive hyperemia and % systolic shortening during coronary occlusion (C.O.) compared with each preocclusive value in the ameroid model (left panel) and repetitive occlusion model (right panel) (data from Tomoike et al (15) and Yamamoto et al (18)).

Effects of several coronary dilators on regional ischemia were examined functionally before and after collateral development. Such comparison in the same dog may uncover how well collateral function is modified pharmacologically. Accordingly, the same animal model as described in the previous chapter was used in conscious physiological condition. In this animal model, changes in left ventricular pressure, its dP/dt, arterial pressure and heart rate after the drug administration were similar before and after collateral development, in cases of nitroglycerin, isosorbide dinitrate, dipyridamole, propranolol and atenolol. For quantitative analysis during the period of collateral functioning, analogue data on segment lengths and left ventricular pressure were digitized at a rate of 250 points/sec and the end-diastolic and end-systolic points were determined before the beginning of dP/dt and 20 msec before the peak negative left ventricular dP/dt, respectively. Data on the end-diastolic (EDL) and end-systolic (ESL) lengths and % systolic shortening calculated by the formula ((EDL-ESL)/EDL x 100(%)) were then converted to analogue signals and recorded on a multi channel pen recorder. PDP 11/44 was used for data processing.

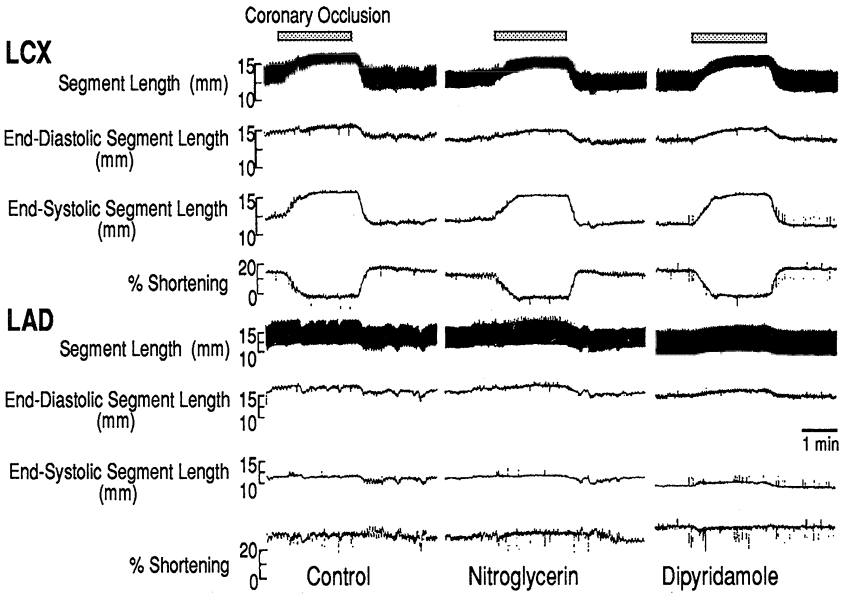

Fig. 16. Beat by beat domonstration of end-diastolic, end-systolic lengths and % shortening of segments in the LCX and LAD area of the same dog before collateral development (data from Nabeyama et al (36)).

Nitroglycerin and dipyridamole: Typical tracings of beat by beat determination of end-diastolic and end-systolic lengths and percentage systolic shortening at the LAD and LCX areas along with each original respective segment length before and after collateral development are shown in figures 16 and 17 (36).

Before collateral development, a 2 minute coronary occlusion was tested after 10 ml saline solution infusion (control), 3 min after intravenous administration of 20 μg/kg nitroglycerin and 5 min after intravenous administration of 0.5 mg/kg dipyridamole (Fig. 16). The sequence of these occlusions after saline or nitritroglycerin was randomly performed. The occlusion after a bolus administration of dipyridamole was made at the end of the study. Each intervention was separated by a 30 min reperfusion period. Regional dyskinesis and significant increases in end-diastolic length in the LCX area were similar after saline, nitroglycerin, and dipyridamole infusions.

After collateral development the same protocol was tested. As shown in figure 17, deterioration of regional wall motion during coronary occlusion was transient. This tendency continued after nitroglycerin but even more so after dipyridamole. Maximum

reduction in systolic shortening in the LCX area was seen 19.6, 15.6 and 93.2 sec after coronary occlusion in saline, nitroglycerin and dipyridamole infusions, respectively.

As an index of ischemic insult, changes in end-systolic length area was measured planimetrically before and after collateral development. Before collateral development end-systolic areas were 29.4, 26.4, and 29.7 cm·sec during saline, nitroglycerin and dipyridamole infusion, respectively. After collateral development, these were reduced from 4.1 to 2.2 cm·sec after nitroglycerin and increased from 4.1 to 14.9 cm·sec after dipyridamole. Effects of isosorbide dinitrate on collateral function were also beneficial as observed in case of nitroglycerin (37).

These results clearly demonstrated that nitroglycerin was beneficial for relieving the regional ischemia in the presence of collaterals. Further reduction of regional wall motion after dipyridamole represents coronary steal. These opposite effects of vasodilating substances on collateral function relate intimately to the site of coronary dilation. Dipyridamole mainly dilates arteriolar resistance vessels and nitroglycerin dilates mainly the proximal large epicardial arterial segments (14, 38). Accordingly,

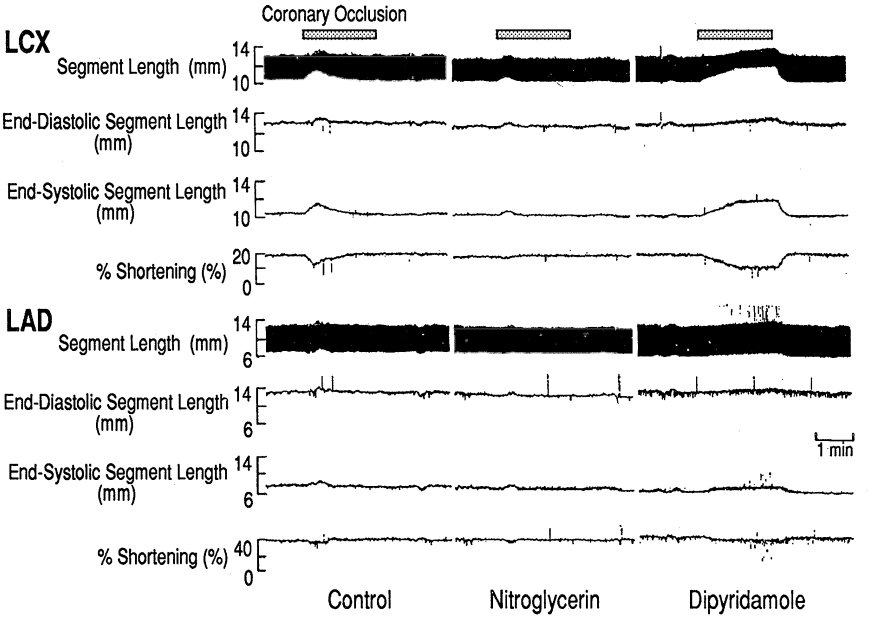

Fig. 17. Beat by beat demonstration of end-diasystolic and end-systolic lengths and % shortening of the LCX and LAD segments of the same dog in figure 16 after collateral development (data from Nabeyama et al (36)).

the presence of functionally well developed collaterals makes possible a coronary steal phenomenon when coronary resistance is decreased. To attenuate the steal phenomenon, selective and persistent dilation of the conduit vessel is mandatory.

β-adrenergic blockade: Beneficial effects of β-adrenergic blockade on regional myocardial dysfunction induced by coronary artery stenosis were studied in chronically instrumented dogs (39). However, intravenous atenolol (1-2 mg/kg i.v.) or propranolol (1-2 mg/kg i.v.) produced no beneficial effects on systolic shortening in the area rendered ischemic before collateral development (40). After collateral development by 125 to 465 consecutive 2-minute coronary occlusions, both atenolol and propranolol significantly improved the reductions of regional shortening by 19 and 18%, respectively ($p<0.05$ vs without β-blockade). These beneficial actions of β-blockade were also noted during atrial tachypacing at matched heart rates of 160 beats/min. Accordingly, such beneficial effects of β-blockade in the presence of well-developed collaterals cannot be totally accounted for by the reduction in heart rate or by cardioselectivity. A shift of coronary blood flow from the nonischemic region to the ischemic region may also be operative in the present model. To confirm this hypothesis, further studies regarding the transcollateral resistance are needed.

Perspective for future studies

Scheel eloquently commented on collateral growth as being not chaotic or undisciplined, as cancer growth is (41). The findings described above raise important questions that should pave the way to new concepts for the prevention and treatment of ischemic heart disease. We may be able to vision sound rules in inherent defensive measures, following stepwise analyses of the process in collateral development. There are chemical factors and cell-to-cell information systems specific to events linked to collateral growth. Namely,

1) What are the stimuli needed for collateral formation at the initiation, lag and acceleration phases ?
2) Why are the epicardial collaterals located so far away from the ischemic center ?
3) What is the optimal speed applied as a stimulus for collateral growth, or is there any governing mechanism in collateral development, as related to ischemic events and hemodynamic changes ?
4) What and when halts the formation of collaterals ?
5) Can one modify the level and speed of collateral formation pharmacologically ?

Acknowledgements: This work was supported in part by Grants-in-Aid for Scientific Research (No.04454261, 04237206) from the Ministry of Education, Science and Culture, Japan, and a grant from Uehara Memorial Foundation.

I thank Prof. Dr. M.Nakamura for critical comments and my co-workers Drs. T. Inou, K. Watanabe, H. Shimokawa, S. Nabeyama, H. Yamamoto, T. Inoue, K. Hisano, M. Mohri, M. Noma, H. Ando, and M. Nagano at the Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, for their participation in numerous experiments and publications, and Ms. M. Sasaki and Mr. E. Tsuchida at Yamagata University for secretarial services.

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9

EFFECT OF EXERCISE AND PHARMACOLOGIC INTERVENTIONS ON CORONARY COLLATERAL BLOOD FLOW

Robert J. Bache
Cardiovascular Division, Department of Medicine, University of Minnesota, Minneapolis, Minnesota, U.S.A.

INTRODUCTION

Coronary artery occlusion results in remarkably rapid development of an effective intercoronary collateral circulation. If occlusion proceeds gradually, sufficient collateral vessel recruitment and growth may occur to allow progression to total arterial occlusion with little or no infarction of the dependent myocardium. In this situation the collateral vessels are able to provide adequate arterial inflow to maintain myocardial integrity during resting conditions, but the ability to augment blood flow in response to exercise or other stress may be limited and develop only gradually. This chapter will consider studies of the coronary vascular system in the intact heart which contains a region of collateral dependent myocardium. Emphasis will be placed on studies in which the response of the coronary collateral system is integrated into the overall response of the heart to exercise.

CORONARY COLLATERAL BLOOD FLOW DURING EXERCISE

Several investigators have examined the ability of blood flow in collateral dependent myocardial regions to increase in response to exercise. These studies have commonly used the ameroid constrictor technique to induce collateral vessel growth (1). Coronary artery occlusion is produced by surgically implanting a ring of casein plastic material ("ameroid") around a proximal coronary artery. The hygroscopic ameroid material slowly swells as it comes in contact with tissue fluid; external expansion is prevented by a stainless steel backing incorporated into the ring, so the inward expansion of the material causes progressive arterial narrowing. The arterial narrowing and accompanying inflammation generally results in total arterial occlusion within 3-4 weeks, often in association with local thrombus formation during the final stages of occlusion. Since occlusion occurs gradually, sufficient growth of collateral vessels generally occurs to allow progression to total occlusion without producing infarct.

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

Fedor et al (2) measured myocardial blood flow with radioactive microspheres during moderate treadmill exercise 11-12 weeks after placement of ameroid constrictors on both the right and left circumflex coronary arteries of adult dogs. During resting conditions myocardial blood flow was similar in the normally perfused and collateral dependent regions. During moderate treadmill exercise blood flow in the collateral region increased normally in six of eleven dogs. In the remaining five animals blood flow in the subepicardial half of the collateral dependent region increased normally during exercise, but the increase in blood flow to the subendocardium was markedly impaired, resulting in a prominent decrease of the subendocardial/subepicardial flow ratio.

Since collateral vessel development is a time dependent process, the heterogeneous response to exercise might be related to differences in the rate of collateral vessel growth between animals. To examine this hypothesis, we studied the exercise induced increase in collateral blood flow relatively early (1 month) and late (8 months) after gradual coronary artery occlusion (3, 4). An ameroid constrictor was implanted on the proximal left circumflex coronary artery of adult mongrel dogs trained to run on a motor driven treadmill. A snare occluder was positioned about the artery immediately distal to the ameroid occluder to insure that total coronary occlusion was achieved at the time of study. Microspheres were administered into the left atrium for measurement of myocardial blood flow during resting conditions and during two levels of treadmill exercise which produced heart rates of approximately 180 and 230 beats/minute. Left ventricular myocardium from the collateral dependent region as well as a control region perfused by the anterior descending coronary artery was divided into 4 transmural layers from epicardium to endocardium for examination of the transmural pattern of perfusion.

During resting conditions, mean myocardial blood flow was similar in the collateral dependent and the normally perfused regions in all animals. Exercise resulted in similar increases in heart rate and mean arterial pressure in normal animals and in animals with coronary occlusion. One month after ameroid placement the response of myocardial blood flow to exercise in the collateral dependent region was highly variable (3). Examination of blood flow in the collateral dependent region revealed that the degree of collateral development could be grouped into three different patterns. Approximately one-third of the animals had sufficient collateralization to allow a completely normal response of mean myocardial blood flow during both light and heavy levels of exercise (Group 1). In a second group of animals (Group 2), mean myocardial

blood flow was normal at rest and demonstrated a normal increase during light exercise, but a further increase in exercise intensity resulted in no further increase in mean blood flow. In the third group of animals (Group 3) blood flow was normal at rest but failed to increase or underwent a subnormal increase even during light exercise with no additional change in response to a further increase of exercise intensity. Animals grouped according to the ability of mean blood flow in the collateral dependent region to increase in response to exercise are shown in figure 1.

The distribution of myocardial blood flow in four transmural layers from epicardium to endocardium is shown for the collateral dependent region; data from a group of normal animals undergoing similar exercise is shown for comparison. For animals in Group 1, not only was the mean blood flow rate normal in the collateral dependent region during both stages of exercise, but the transmural distribution of perfusion was also normal. For the animals in Group 2, the normal increase in mean blood flow in response to light exercise was associated with a normal transmural distribution of perfusion. However, when the exercise level was further increased, mean flow failed to increase and a prominent transmural redistribution of perfusion away from the subendocardium occurred. Finally, for the animals in Group 3, any exercise resulted in a prominent redistribution of perfusion, so that subepicardial flow increased, while blood flow to the inner half of the left ventricular wall did not change or decreased in comparison with resting values.

Minipigs studied 4-16 weeks after ameroid placement on the left circumflex coronary artery had responses to exercise similar to those of the dogs in Group 3 (5, 6). Exercise which increased heart rates to 245 beats/min caused a 198 percent increase in blood flow in the normal region, while mean blood flow to the collateral dependent region increased only 49 percent. The subnormal increase in blood flow to the collateral dependent region during exercise was associated with redistribution of perfusion away from the subendocardium, with the ratio of subendocardial/subepicardial flow falling to 0.39. The findings indicate that even in the pig in which coronary collateralization is notoriously poor, there is some ability to increase blood flow to the collateral dependent region during exercise relatively early after coronary occlusion. As in the dog, the limited blood flow in the collateral dependent region is delivered primarily to the outer myocardial layers, resulting in preferential underperfusion of the subendocardium.

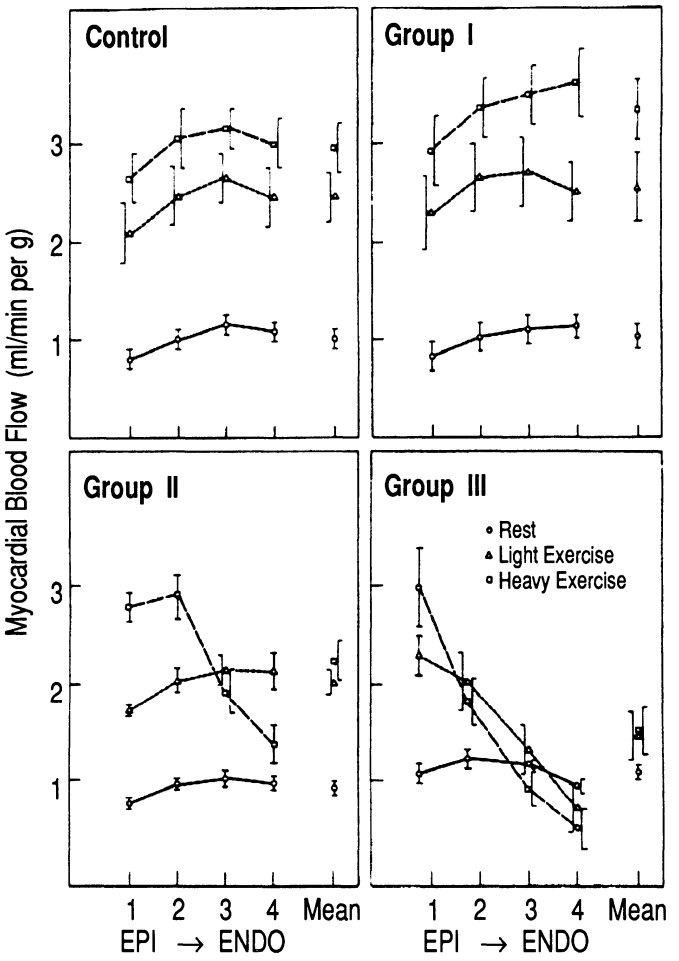

Fig. 1. Myocardial blood flow to four transmural layers from epicardium to endocardium at rest and during two levels of treadmill exercise data are shown for seven normal dogs (control), and for collateral dependent myocardium one month after placement of an ameroid constrictor on the left circumflex coronary artery of 14 dogs. Animals with coronary occlusion were divided into three groups. Group 1 represents animals with sufficient collateralization to allow a completely normal increase of mean myocardial blood flow during both levels of exercise. In Group 2 collateralization was sufficient to allow a normal increase in mean myocardial blood flow during light exercise, but a further increase in exercise intensity resulted in no further increase in mean blood flow. In Group 3 blood flow was normal at rest but underwent a subnormal increase during light exercise with no additional change during heavy exercise. (Reproduced from the American Journal of Physiology, 1983;245:H131-H138 by permission of the American Physiological Society).

The transmural distribution of blood flow away from the subendocardium which occurs when the collateral vessels are unable to provide an adequate increase in mean blood flow during exercise is similar to the subendocardial underperfusion which occurs when an arterial stenosis limits myocardial blood flow during exercise (7, 8). The ability to maintain a normal transmural distribution of perfusion is likely related to the degree of vasodilation of the resistance vessels within the collateral dependent region. When collateral vessels are poorly developed, the resistance vessels in the collateral dependent region must maintain a substantial degree of vasodilation even during resting conditions to compensate for the resistance of the collateral vessels. Poorly developed collateral vessels impose a limit beyond which flow cannot increase. When myocardial demands are below this limit the resistance vessels are able to maintain a normal transmural distribution of perfusion in the collateral dependent region. However, when myocardial demands exceed the capacity of the collateral vessels to deliver arterial inflow, intense vasodilation of the resistance vessels impairs their ability to maintain a normal transmural distribution of blood flow.

The observed wide variation in the ability of the collateral vessels to increase blood flow in response to exercise could be due to intrinsic differences between animals in the ability to develop collateral vessels, or differences in the rate of collateral vessel development between animals. If the latter were true, then allowing additional time after coronary occlusion would permit all animals to develop sufficient collateralization to undergo a uniform increase in blood flow during exercise. To examine this hypothesis, myocardial blood flow was measured at rest and during two exercise stages eight months after implantation of an ameroid constrictor on the left circumflex coronary artery in 6 dogs (4). As shown in figure 2, both the increase in mean myocardial blood flow and the transmural distribution of perfusion were normal at rest and during both levels of exercise in the collateral dependent region. Similar findings were reported by Longhurst et al (9) in dogs studied 2-3 months after ameroid occlusion of the left circumflex coronary artery. These findings demonstrate that the marked heterogeneity of perfusion in the collateral dependent region during exercise one month after ameroid implantation did not result from differences in the intrinsic ability for ultimate collateral vessel development, but rather from differences in the rate of collateral vessel development between animals. When enough time was allowed, all animals developed sufficient collateralization to permit a normal increase in blood flow in response to a moderate level of treadmill exercise.

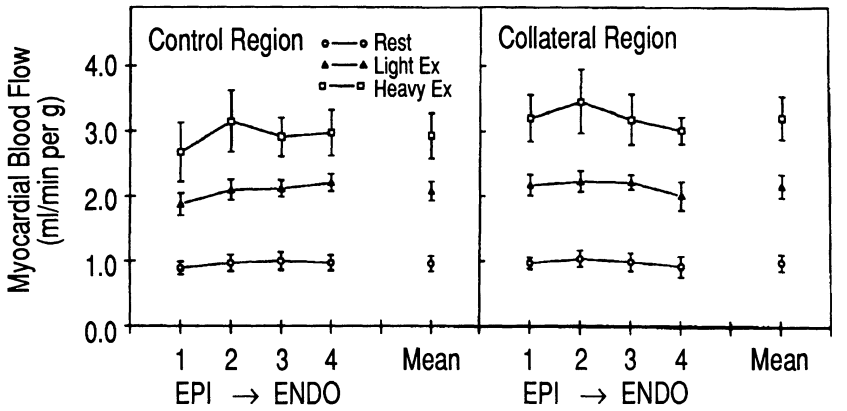

Fig. 2. Blood flow to four transmural myocardial layers from epicardium to endocardium at rest and during two levels of treadmill exercise in 6 dogs studied 8 months after ameroid occlusion of the left circumflex coronary artery. Blood flow in the collateral dependent region was not significantly different from flow to a normally perfused control region at rest or during either level of exercise. (Adapted from the Journal of Clinical Investigation, 1977; 59:127 by copyright permission of the American Society for Clinical Investigation).

The results of these studies can be summarized as follows. *If the coronary collateral vessels can conduct sufficient arterial inflow to meet the needs of the dependent myocardium, then the volume and distribution of blood flow will be determined by the normal autoregulatory responses of the microvasculature in the dependent myocardium. However, if the collateral vessels cannot deliver sufficient arterial inflow to meet the needs of the dependent myocardium, then the volume of flow will be determined by the conductance of the collateral system and the transmural distribution of perfusion will be similar to that observed when flow is limited by an arterial stenosis.*

Influence of collateral vessel tone

Studies using isolated vessel segments and *in vivo* studies performed in open chest animals have demonstrated that well developed collateral vessels are capable of active vasomotion (10, 11). Thus, in the intact animal collateral vessels may not behave as fixed conduits with a constant upper limit for conductance of blood to the dependent myocardium. It is consequently reasonable to speculate that vasomotor tone of collateral vessels could modulate the availability of blood to the dependent myocardium during exercise. This hypothesis can be tested by using an agent known to cause vasoconstriction of coronary collateral vessels, but which does not cause vasoconstriction of the epicardial coronary arteries, since constriction of arteries proximal to the origin of

the collateral vessels could also impair collateral blood flow. Arginine vasopressin is such an agent, since it causes contraction of isolated segments of collateral vessels (10), but does not cause contraction of epicardial coronary artery segments (10, 12).

A study was performed to test the hypothesis that active vasoconstriction of coronary collateral vessels can worsen hypoperfusion of the dependent myocardium during exercise (13). Dogs were instrumented with a Doppler flowmeter probe and hydraulic occluder on the proximal left circumflex coronary artery. An intraarterial microcatheter distal to the occluder allowed measurement of distal coronary pressure. Collateral vessel development was initiated using the repetitive coronary occlusion technique of Franklin et al (14), whereby one to two minute coronary artery occlusions are produced repeatedly for several weeks. Using this model, distal occlusion pressures gradually increase as collateralization occurs; when distal pressure increased to 35-40 mmHg, it was possible to permanently occlude the artery without causing infarct. Following permanent occlusion, distal coronary pressures increased even more rapidly, so that within one week mean distal pressure had increased to 75 mmHg. At this time myocardial blood flow was measured with radioactive microspheres at rest and during treadmill exercise which increased heart rates to approximately 190 beats/min. After completion of control measurements, arginine vasopressin was infused at a rate of 0.01 μg/kg per minute intravenously. Exercise was repeated during vasopressin infusion and myocardial blood flow was again measured with microspheres. At the conclusion of study, the animals were anesthetized and the left circumflex coronary artery was cannulated at the site of the previous coronary occlusion. The region of collateral dependent myocardium was identified using the shadow technique of Patterson and Kirk (15), whereby radioactive microspheres were injected into the left atrium while the left circumflex artery was perfused with nonradioactive blood from a reservoir pressurized to maintain cannula tip pressure 10 mmHg above mean aortic pressure. Collateral blood flow was determined as the sum of flow to all myocardial specimens identified by the shadow technique to be within the perfusion bed of the left circumflex coronary artery.

Myocardial blood flows to the normally perfused and collateral dependent regions at rest and during exercise are shown in figure 3. During resting conditions, blood flow was slightly but significantly lower in the collateral zone than in the normal zone. Exercise resulted in significant increases of blood flow in both the normal and collateral dependent regions, although the increase was subnormal in the collateralized region.

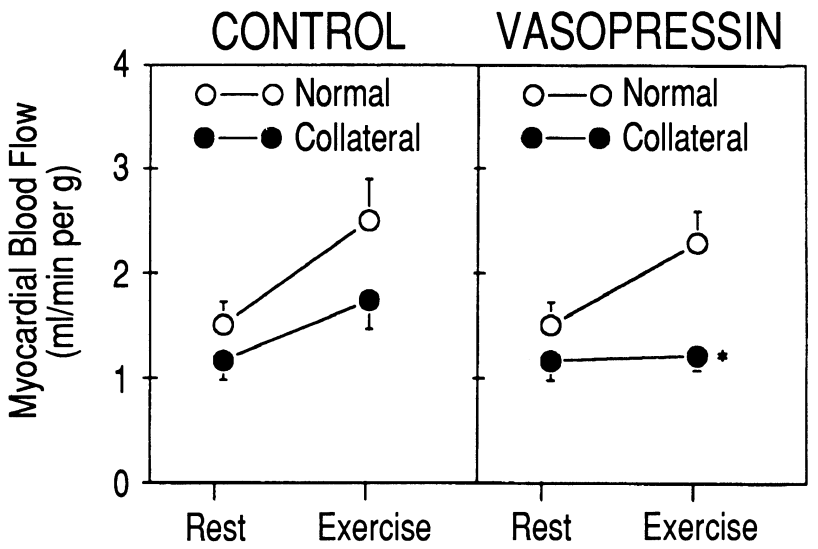

Fig. 3. Effect of vasopressin on myocardial blood flow to normally perfused and collateral dependent left ventricular regions at rest and during treadmill exercise. During control conditions exercise resulted in significant increases in blood flow in both the normal and collateral dependent regions, although the increase in flow was subnormal in the collateralized region. Infusion of vasopressin did not significantly alter the response of blood flow during exercise in the normally perfused region, but essentially abolished the increase in blood flow in response to exercise in the collateral dependent region (13).

When exercise was repeated during vasopressin infusion, the increase in blood flow in the normal zone in response to exercise was not significantly different from control. However, in the collateral zone vasopressin essentially abolished the ability to increase in flow in response to exercise. The impaired response of coronary blood flow during exercise in the presence of vasopressin resulted from vasoconstriction of both coronary collateral vessels and resistance vessels within the collateral dependent region. Thus, transcollateral resistance increased 48±14% in response to vasopressin, while small vessel resistance during exercise increased 40±9% in response to vasopressin. In contrast, vasopressin did not significantly increase coronary vascular resistance in the normally perfused region. The finding that vasopressin caused small vessel constriction in the collateral dependent region but not in the normal zone, suggests that small vessel responsiveness is altered in regions of myocardium perfused through collateral vessels. This finding is in agreement with the report of Sellke and associates (16) that endothelial function is abnormal in coronary resistance vessels perfused by collaterals. *These results indicate that the coronary collateral vessels do not act as fixed conduits. Rather,*

active vasoconstriction of both collateral vessels and coronary resistance vessels can worsen hypoperfusion of the collateral-dependent myocardium during exercise.

It should be noted that there is no evidence to suggest that vasopressin can normally limit collateral blood flow during exercise, since exercise does not cause a sufficient increase in vasopressin levels to have a significant vasomotor effect. Rather, vasopressin was used in the above study as a convenient agent to test the general hypothesis that active constriction of well developed coronary collateral vessels has potential for limiting perfusion of the dependent myocardium during exercise. Consequently, a dose of vasopressin was calculated to correspond to levels which cause collateral vasoconstriction *in vitro* but without causing substantial systemic effect (10). As predicted, plasma levels increased to far above those during physiologic conditions, being 2.1±0.64 pg/ml during control conditions and increasing to 98±34 pg/ml during vasopressin infusion. However, it is of interest that individual vasopressin plasma levels were within the range reported to occur in response to smoking two cigarettes or during hemorrhage of moderate degree (17, 18). *Thus, although plasma vasopressin levels are normally far less than would influence collateral flow during physiologic conditions, it is possible that sufficient elevations of vasopressin levels can be achieved during pathologic conditions to cause constriction of coronary collateral vessels in vivo.*

Influence of infarct in the collateral region

Collateral flow during resting conditions: Gradual application of a coronary occlusion to produce a region of collateral dependent myocardium without infarct allows study of the collateral circulation uninfluenced by infarcted myocardium. In the clinical setting, however, collateral dependent regions frequently include areas of infarcted myocardium. In this situation, blood flow to the collateral dependent region is influenced not only by the structure and function of the collateral vessels, but also by abnormalities resulting from the presence of infarcted tissue. In both human subjects suffering acute myocardial infarction and experimental canine models of abrupt coronary occlusion, the area of infarcted myocardium is smaller than the region of tissue normally perfused by the coronary artery ("risk region") (19). *Survival of a portion of the myocardium normally perfused by an occluded artery is dependent upon delivery of a limited inflow of arterial blood by preexisting coronary collateral vessels. The fraction of myocardium surviving acute coronary*

occlusion is quantitatively related to the volume of collateral blood flow available early after coronary occlusion (20).

Several reports have examined blood flow to collateral dependent myocardium which included regions of infarct studied approximately 2 weeks after acute coronary artery occlusion (21, 22, 23). Regional myocardial blood flow was measured with radioactive microspheres in multiple myocardial specimens; the percent infarct in each myocardial specimen was determined using quantitative microscopy. *When blood flow to collateral dependent myocardial specimens was plotted against percent infarct during resting conditions, an inverse linear relationship was found, with myocardial blood flow decreasing in direct proportion to the fraction of infarct within a myocardial specimen.*

Collateral flow during exercise: The above studies indicate that during resting conditions blood flow to collateral dependent regions which include infarct is

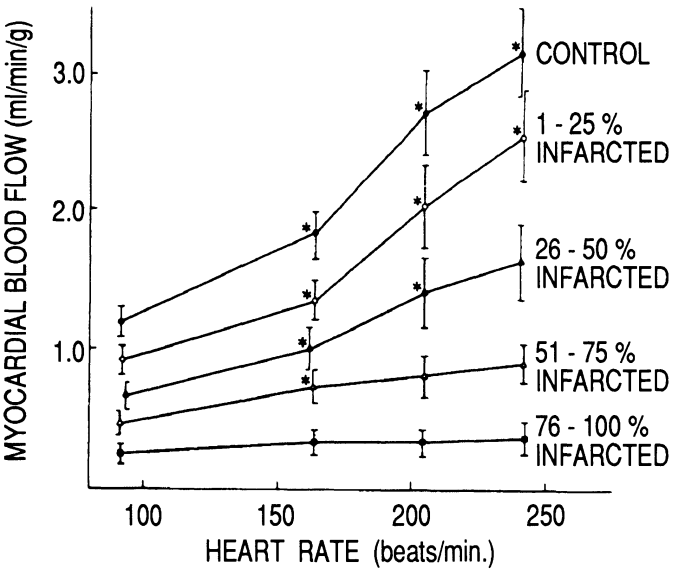

Fig. 4. Effect of the presence of infarcted myocardium on the increase in myocardial blood flow in the collateral dependent region during three levels of treadmill exercise. In regions which contained less than 50% infarct, blood flow during resting conditions was depressed in proportion to the degree of infarct, but the relative increase in blood flow during exercise was not different from the normally perfused control region. However, when more than 50% of a myocardial region was occupied by infarct, both resting blood flow and the percent increase in flow during exercise were decreased. (Reproduced from Circulation Research, 1980; 47:59-68 by permission of the American Heart Association).

proportional to the volume of residual viable myocardium. The response of blood flow to exercise could be influenced by the characteristics of the collateral vessels as well as by changes related to the presence of infarct. To examine this question, myocardial blood flow was measured during exercise in chronically instrumented dogs two weeks after abrupt total occlusion of the left anterior descending coronary artery had resulted in a collateral-dependent region containing some degree of infarct (21). Myocardial blood flow was measured during three levels of treadmill exercise which increased heart rates to 164, 205 and 242 beats/min. As shown in figure 4, the degree of infarcted myocardium importantly influenced the response of blood flow to exercise. In specimens which contained less than 50% infarct, blood flow during resting conditions was depressed in direct proportion to the degree of infarct, but the proportionate increase in blood flow in response to exercise was not different from the normally perfused control myocardial region. *Thus, the presence of infarct did not impair the increase in blood flow which occurred in the collateral dependent noninfarcted myocardium.* However, when more than 50% of a myocardial specimen was occupied by infarct, not only was resting flow depressed, but the percent increase in flow during exercise was also reduced. It is likely that impairment of perfusion in regions containing more than 50% infarct resulted from the high resistance to blood flow offered by poorly developed collateral vessels. Regions with the largest percent infarct were those with the sparsest native collateral vessels; even two weeks after coronary occlusion collateral vessel development was insufficient to allow a normal increase in flow to the residual viable myocardium during exercise. In addition, the presence of infarct might directly influence blood flow to the residual viable myocardium, or metabolic alterations in the residual myocardium (such as hibernation) might blunt the increase in oxygen demands during exercise (24).

Effect of β-adrenergic blockade: Using isolated rings of well-developed canine coronary collateral vessels, Feldman and associates (25) demonstrated that β-adrenergic stimulation resulted in vasorelaxation. Similarly, Maruoka et al (26) found that norepinephrine infusion caused a decrease in collateral resistance in dogs studied 2-3 months after ameroid constriction implantation on the left circumflex coronary artery. These findings suggest that β-adrenergic blockade might decrease collateral flow during exercise by blocking the β-adrenergic vasodilator influence resulting from sympathetic activation during exercise. In dogs in which acute coronary artery

occlusion had resulted in a collateral dependent myocardial region containing infarct, myocardial blood flow was measured with microspheres at rest and during treadmill exercise before and after non-selective β-adrenergic blockade with timolol (4 μg/kg iv) (22). During resting conditions, β-adrenergic blockade caused no significant change in blood flow to either normally perfused or collateral dependent myocardium, indicating minimal adrenergic activity at rest. However, during exercise timolol caused significant decreases of myocardial blood flow which were in large part related to the negative chronotropic effect of β-adrenergic blockade. To correct for changes resulting from alterations of myocardial oxygen demand, results were compared at similar rate-pressure products before and after timolol. At comparable rate-pressure products, timolol significantly decreased blood flow in both the subepicardium and subendocardium of normally perfused myocardial regions. In the collateral dependent region, timolol caused a reduction of subepicardial blood flow which was comparable to that in the normally perfused region. However, in the subendocardium of the collateral dependent region (in which blood flow during exercise was substantially less than normal), β-adrenergic blockade did not cause a further reduction of blood flow.

β-adrenergic blockade could have decreased blood flow in the collateral dependent region during exercise by causing vasoconstriction of either the collateral vessels or the resistance vessels within the collateral zone. The previously described studies have demonstrated that limitation of arterial inflow by the collateral vessels is accompanied by preferential underperfusion of the subendocardium (2, 3). Consequently, the finding that β-adrenergic blockade caused a reduction of blood flow in the subepicardium, but not in the subendocardium, does not support limitation of blood flow by constriction of the collateral vessels. This conclusion is supported by the finding that the decrease in subepicardial flow of the collateral dependent region in response to β-adrenergic blockade was proportionately similar to the decrease in flow which occurred in the normally perfused region. *Thus, decreased subepicardial blood flow in the collateral dependent region in response to β-adrenergic blockade likely resulted from vasoconstriction of the coronary resistance vessels rather than from constriction of the collateral vessels.*

Effect of α-adrenergic blockade: α-Adrenergic vasoconstrictor influences can restrain the increase in coronary blood flow which occurs in response to the increased myocardial oxygen demands during exercise (27, 28). This vasoconstriction occurs in the normal heart, but also has been demonstrated to occur in the presence of a coronary stenosis and

during myocardial hypoperfusion (29, 30, 31). A study was performed to determine whether α-adrenergic vasoconstriction can limit blood flow to collateral dependent myocardial regions during exercise (23). Dogs underwent acute occlusion of the left circumflex coronary artery to produce a collateral dependent region which included a variable degree of subendocardial infarct. Myocardial blood flow was measured with radioactive microspheres approximately two weeks after coronary occlusion at rest and during two levels of treadmill exercise. The increase in blood flow in response to exercise was impaired in the collateral dependent region. This occurred because subendocardial flow underwent a subnormal increase while the increase in subepicardial blood flow was not significantly different from that in the normally perfused region.

During resting conditions, selective α_1-adrenergic blockade with prazosin caused no significant change in either mean blood flow or the transmural distribution of perfusion in either the normal or the collateral dependent regions. At comparable levels of exercise, however, prazosin caused a 27% increase in mean blood flow in the normally perfused region and a 35% mean increase in blood flow in the collateral dependent region ($p<0.01$). The increase in blood flow in the collateral dependent region produced by prazosin occurred in all transmural layers from epicardium to endocardium, although the absolute increase in flow was most marked in the subepicardial layers.

The greater increase in myocardial blood flow in response to exercise in the normally perfused region is similar to the effect of α-adrenergic blockade reported in the normal heart (27, 28). The increase in blood flow in the collateral dependent region could have resulted from interruption of α-adrenergic vasoconstrictor activity in either the collateral vessels or the resistance vessels within the collateral dependent region. Since previous studies have failed to document substantial α_1-adrenergic vasoconstriction of coronary collateral vessels studied either *in vitro* or *in vivo*, it is unlikely that the increase in blood flow to the collateral dependent region produced by prazosin was the result of interruption of α_1-adrenergic vasoconstriction in the collateral vessels (10, 11). It is more likely that α_1-adrenergic blockade increased blood flow in the collateral dependent region by interrupting vasomotor tone of the resistance vessels. Until recently it had generally been assumed that myocardial hypoperfusion resulted in intense ischemic vasodilation which would render the resistance vessels unresponsive to vasoconstrictor stimuli. However, recent studies have demonstrated that reduction of coronary perfusion pressure sufficient to decrease coronary blood flow and depress regional systolic function does not result in maximal vasodilation of the resistance

vessels in the ischemic region (29, 30, 31, 32). Thus, even when a flow-limiting coronary stenosis results in myocardial ischemia during exercise, the resistance vessels retain residual tone, since intracoronary administration of adenosine causes an increase in blood flow to the ischemic region with no change in distal coronary pressure (33). This residual vasomotor tone of the resistance vessels in the ischemic region appears to be mediated principally by α-adrenergic influences, since α_1- or α_2-adrenergic blockade has been reported to result in significant increases in blood flow to the ischemic region (31, 34). It appears that α-adrenergic vasoconstriction of the resistance vessels also limits blood flow in the region perfused by coronary collateral vessels, since α_1-adrenergic blockade significantly increased blood flow to the collateral dependent region.

In summary, adrenergic nervous system activity can influence blood flow to collateral dependent myocardium during exercise. *The available data suggest that the influence of the adrenergic nervous system on blood flow in the collateral dependent region results principally from altered vasomotor activity of the microvasculature within the collateral dependent region rather than from direct vasomotor effects on the collateral vessels.*

Effect of pharmocologic interventions on coronary collateral flow

In 1964 Fam and McGregor (35) reported that nitroglycerin caused an increase in retrograde blood flow from a coronary artery cannulated at the site of a previous chronic occlusion. This finding demonstrated that collateral vessels do not act as fixed conduits, but are capable of active vasomotor activity. Several investigators have subsequently examined vasomotor responses in isolated rings of epicardial collateral vessels obtained from dogs with prolonged coronary occlusion, as well as the effects of vasoactive agents on intracoronary collateral blood flow in the intact animal. This chapter will consider studies which have examined the responses of coronary collateral blood flow to vasoactive agents in the intact heart.

Response to direct vasodilators: Nitroglycerin is a potent vasodilator of well-developed coronary collateral vessels, generally causing a 30 to 50 percent increase in retrograde blood flow from the cannulated collateralized coronary artery (11, 35). Some, but not all, investigators have reported that nitroglycerin also causes a small increase in blood flow through the native coronary collateral vessels which exist at the time of acute coronary artery occlusion (36, 37).

Atrial natriuretic peptide causes vasodilation of well developed coronary collateral vessels (38). Collateral vessel dilation produced by atrial natriuretic peptide was sufficiently potent so that the subsequent addition of nitroglycerin did not cause a further increase in collateral blood flow (38). Nitroglycerin and atrial natriuretic peptide likely act through a common mechanism, since both agents are known to stimulate soluble guanylate cyclase (39, 40).

Calcitonin gene related peptide (CGRP), which is known to dilate coronary arteries but not coronary resistance vessels, has been reported to cause vasodilation of well-developed coronary collateral vessels in the dog (41).

Effect of vasoconstrictors on collateral flow: In dogs in which occlusion of the anterior descending coronary artery had resulted in well developed coronary collateral vessels, vasopressin (0.01-0.03 μg/kg per minute administered into the left main coronary artery) resulted in a 22±7% decrease in retrograde blood flow from the cannulated left anterior descending coronary artery (11). This is in agreement with *in vitro* studies in which isolated rings of epicardial coronary collateral vessels have also been shown to undergo contraction in response to vasopressin (10). In contrast, vasopressin causes minimal contraction or even relaxation of isolated rings of normal epicardial coronary artery (10, 12). This finding indicates that the decrease in retrograde flow in response to vasopressin could not be attributed to vasoconstriction of the conduit arteries proximal to the origin of the collateral vessels.

Angiotensin II in doses of 0.03-0.06 μg/kg per minute infused into the left main coronary artery caused a 22±6% decrease in retrograde blood flow in dogs with well-developed coronary collateral vessels (11). Angiotensin II is known to cause constriction of epicardial coronary arteries, so that constriction of the conduit artery proximal to the origin of the collateral vessels could have contributed to the observed decrease in retrograde blood flow (42). However, *in vitro* studies of isolated epicardial coronary collateral vessel segments have demonstrated contraction in response to angiotensin II, thus supporting a direct effect of angiotensin II on the coronary collateral vessels.

Ergonovine, 0.2-0.8 μg/kg per minute, infused into the left main coronary artery of dogs with well-developed coronary collateral vessels resulted in a 22±4% decrease in retrograde blood flow (43). This dose of ergonovine caused no change in systemic hemodynamic variables. Interestingly, ergonovine has been reported to cause only weak contraction of isolated epicardial collateral vessels. This finding, in conjunction with

the observation that ergonovine causes diffuse vasoconstriction of epicardial coronary arteries, suggests that at least part of the decrease in collateral flow produced by ergonovine may be the result of vasoconstriction of the donor arteries proximal to the origin of the collateral vessels.

Alpha-adrenergic stimulation: Although α-adrenergic stimulation causes vasoconstriction of both epicardial arteries and coronary resistance vessels, the effect on coronary collateral vessels is equivocal. Harrison, et al (10) reported that neither the selective α_1-adrenergic agonist phenylephrine nor the selective α_2-adrenergic agonist clonidine, caused contraction of isolated rings of epicardial collateral vessels obtained from dogs with chronic coronary artery occlusion. Similarly, Bache et al (43), in studies of dogs with chronic occlusion of the left anterior descending coronary artery, failed to

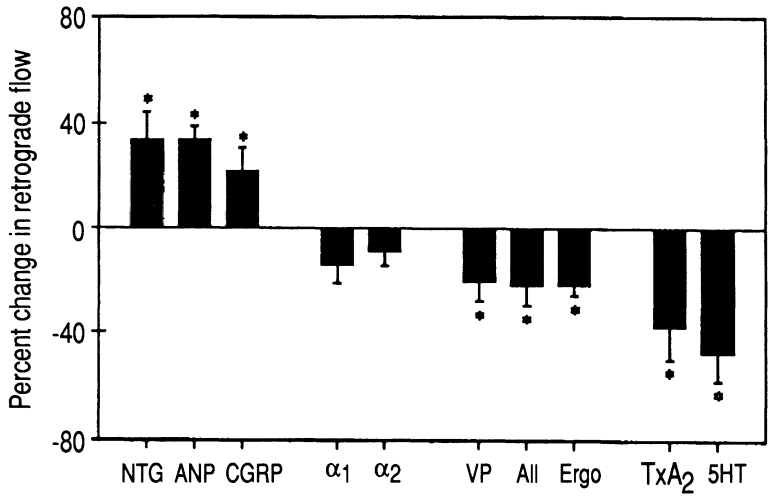

*Fig. 5. Summary of percent change in retrograde blood flow from the cannulated collateralized left anterior descending coronary artery of dogs in response to vasoactive agents. Agents tested were infused into the left main coronary artery to reach collateral vessels originating from the left coronary arterial system. Significant increases in retrograde flow occurred in response to nitroglycerin (NTG; n=14), atrial natriuretic peptide (ANP; n=9) and calciotonin gene related peptide (CGRP; n=9). Neither phenylephrine (α_1; n=10) nor the selective alpha$_2$-adrenergic agonist B-HT 933 (α_2; n=9) caused a significant change in retrograde flow. Significant reductions of collateral flow were observed in response to vasopressin (VP; n=10), angiotensin II (AII; n=11) and ergonovine (Ergo; n=11). Similarly, the platelet products thromboxane A_2 (TxA_2; n=7) and serotonin (5HT; n=5) caused significant decreases of collateral (11, 38, 41, 43, 44). *P<0.05 for change in retrograde flow in comparison with the control measurement prior to drug infusion.*

find a significant effect of either the α_1-adrenergic agonist phenylephrine or the α_2-adrenergic agonist, B-HT 933, on retrograde flow from the cannulated collateral dependent anterior descending coronary artery. In addition, neither selective α_1-adrenergic blockade with prazosin nor selective α_2-adrenergic blockade with rauwolscine caused a significant change in collateral blood flow. Maruoka, et al (26) reported that after β-adrenergic blockade with propranolol, norepinephrine did not alter coronary collateral resistance, while the selective α_2-adrenergic agonist B-HT 920 caused a 24% decrease in blood flow to the collateral dependent region. Bache et al (43) found that although the selective α_2-adrenergic agonist B-HT 933 did not alter retrograde flow, this agent did cause a decrease in continuing tissue flow in the collateral dependent region during the retrograde flow collection. These findings suggest that α_2-adrenergic activation may decrease tissue flow within the collateral dependent region by causing constriction of either small intramural collateral vessels or coronary resistance vessels within the collateral-dependent region. *Taken together, the findings do not support a major role for α_1-adrenergic vasoconstriction in modulating coronary collateral blood flow, although it is possible that α_2-adrenoceptor activation may influence coronary collateral flow in some circumstances.*

Platelet products: Aggregating platelets release thromboxane A_2 and serotonin, both of which are potent vasoconstrictors of coronary arteries (45). Administration of serotonin (50 μg/min) into the left main coronary artery of dogs with well-developed collateral vessels produced by chronic occlusion of the anterior descending coronary artery resulted in a 48±11% decrease in retrograde flow from the cannulated left anterior descending artery (44). The computed coronary blood level achieved with this dose of serotonin was within the range previously reported to occur during platelet aggregation *in vivo*. Administration of the thromboxane A_2 analog, U46619, in a dose of 0.01 μg/kg per minute caused a 38±13% decrease in retrograde blood flow. Although measurements of local thromboxane A_2 concentrations in coronary artery blood during platelet aggregation *in vivo* have not been reported, the computed concentrations were less than those which produce maximal vasoconstriction of isolated coronary artery segments *in vitro* (44). Figure 5 summarizes the data obtained with different pharmacological interventions. *The findings suggest that serotonin and thromboxane A_2 released during platelet aggregation could cause significant vasoconstriction of collateral vessels arising from the artery in which platelet aggregation occurred.*

Endothelial mediated responses

Products of cyclooxygenase metabolism: Although coronary arterial vessels are capable of metabolizing arachidonic acid into vasoactive prostanoids, most evidence does not support an important role for the prostaglandin system in control of the normal coronary circulation (46). In contrast to normal arteries, collateral vessels which develop in response to coronary occlusion demonstrate prominent subintimal hyperplasia and endothelial cell proliferation (47). Since vascular arachidonic acid metabolism occurs principally in the endothelium, the finding of endothelial cell hyperplasia suggests that arachidonic acid metabolism might be enhanced in collateral vessels.

To assess the contribution of endogenous prostanoid production, the effect of cyclooxygenase blockade on collateral blood flow was examined (48). Studies were performed in dogs in which an occluding plug was embolized into the left anterior descending coronary artery. Collateral blood flow was studied 4-6 months after coronary embolization by cannulation of the anterior descending artery at the site of occlusion for measurement of retrograde blood flow. Radioactive microspheres were administered simultaneously with the retrograde flow collection to allow measurement of continuing tissue flow. During control conditions, retrograde flow from the cannulated anterior descending coronary artery was 41±7 ml/min. Cyclooxygenase blockade with indomethacin (5 mg/kg) decreased retrograde flow 40±7% ($p<0.01$), while continuing tissue flow in the collateral dependent region was decreased by 20±10% ($p<0.05$). These changes in coronary collateral flow in response to indomethacin occurred with no change in systemic hemodynamics and with no change in blood flow to the normally perfused myocardial region. *These findings suggest that endothelial cell hyperplasia in coronary collateral vessels is associated with increased production of vasodilator prostaglandins which act to maintain tonic vasodilation of the collateral vessels.*

Endothelium derived relaxing factor: Since Furchgott and Zawadski (49) first observed an obligatory role of the endothelium in producing relaxation of isolated arterial rings in response to acetylcholine, numerous reports have demonstrated that responses to a number of vasodilator agents are dependent upon intact endothelial function. Vasodilation results from production and liberation of endothelial derived relaxing factor (EDRF), which appears to be nitric oxide or a related compound produced by endothelial cells from L-arginine (50). In normal coronary arterial vessels EDRF mediates the vasodilator responses to agonists such as acetylcholine, substance P,

bradykinin and ATP, as well as flow mediated vasodilation in response to the increased sheer stress exerted on endothelial cells during increased blood flow rates (51). Endothelial release of EDRF also restrains and modulates coronary vasoconstriction produced by agents such as norepinephrine, vasopressin and angiotensin II (51).

Endothelial-dependent vasodilator responses have been studied *in vitro* in well-developed coronary collateral vessels. Using isolated rings of preconstricted epicardial collateral vessels obtained from canine hearts after chronic coronary artery occlusion, several investigators have reported that the endothelial-dependent relaxation produced by acetylcholine, bradykinin and substance P are intact or slightly enhanced in comparison with normal epicardial arterial segments of similar size (52, 53). These responses were blunted by pretreatment with N^G-monomethyl-L-arginine, which inhibits nitric oxide synthesis from L-arginine. In the intact heart in which collateral growth had been stimulated by chronic occlusion of a coronary artery, retrograde flow from the previously occluded cannulated left anterior descending artery increased significantly in response to the endothelial-dependent vasodilators acetylcholine and bradykinin (48, 54). The increase in collateral flow was similar to that produced by nitroglycerin which appears to cause maximal collateral vessel dilation *in vivo. These findings indicate that EDRF-dependent vasodilator mechanisms are intact in well developed coronary collateral vessels. Endogenous production of EDRF can produce potent vasodilation similar in magnitude to that resulting from exogenous nitroglycerin.*

SUMMARY

The coronary collateral system embodies a dynamic network of interarterial vessels which can undergo both long and short-term adjustments that are capable of modulating blood flow to the dependent myocardium. Long-term adjustments including recruitment and growth of collateral vessels in response to arterial occlusion are time dependent and determine the maximum blood flow rates available to the collateral dependent vascular bed during exercise. Rapid short-term adjustments result from active vasomotor activity of the collateral vessels. Mature coronary collateral vessels are responsive to vasodilators such as nitroglycerin and atrial natriuretic peptide, and to vasoconstrictors such as angiotensin II, vasopressin, serotonin and thromboxane A_2. Alterations in collateral vasomotor tone can modify maximum collateral conductance, thereby influencing the blood supply to the dependent myocardium. Finally, vasomotor activity in the coronary resistance vessels of the

collateral perfused vascular bed can importantly influence the volume and distribution of blood flow within the collateral dependent myocardium.

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FUNCTION AND DEVELOPMENT OF CORONARY COLLATERAL VESSELS

John C. Longhurst, J. David Symons
University of California, Davis, Department of Internal Medicine, Division of Cardiovascular Medicine,
Davis, California, U.S.A

INTRODUCTION

It is well recognized that coronary collateral vessels help to preserve jeopardized myocardium in regions distal to a high grade stenosis (1, 2). However, there is substantial variation between species in their ability to develop collateral vessels. The dog in particular responds by developing these vessels to a substantial degree (3). Other species, such as humans and swine, have a much less well developed collateral circulation following chronic gradual coronary occlusion (4, 5). The purpose of this chapter is to examine the function and development of the coronary collateral circulation in dog and swine, with attention to underlying mechanisms.

Ameroid constrictor model of coronary collateral development

A number of animal models have been developed to study coronary collateral vessel function. Gradual occlusion of one or more of the main epicardial coronary arteries by an ameroid constrictor has been one of the most common models employed. These constrictors swell over a two to three week period and provide the stimulus for development of the coronary collateral circulation, generally without occurrence of significant infarction in the risk (i.e. collateralized) region (3, 6, 7). During development of the collateral vessels it is possible to estimate collateral vascular resistance at rest, e.g. in anesthetized preparations during studies on the mechanisms of collateral vasoconstriction (8) and during both unsedated rest and exercise-induced stress in animals instrumented chronically with indwelling coronary catheters (7). We have employed the ameroid constrictor model extensively in our studies.

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

Regulation of coronary collateral vascular resistance by β- and α-adrenoceptors in dogs

Sympathetic coronary vasoconstriction is recognized as an important mechanism that competes with metabolic vasodilation during both static and dynamic exercise and likely contributes to the favorable redistribution of blood flow towards the subendocardium and away from the subepicardium (9, 10). Although sympathetic nerve terminals have been observed in the walls of mature collateral vessels in both

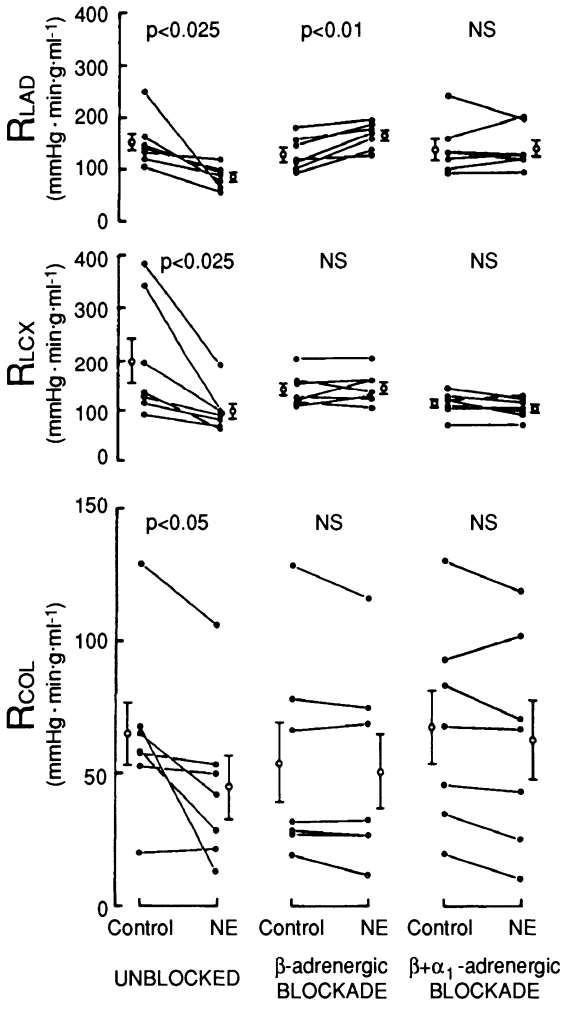

Fig. 1. Vascular resistances (R) in native left anterior descending (LAD) and collateral-dependent left circumflex (LCX) coronary artery regions of ameroid-constricted dogs before and during norepinephrine infusion in unblocked, β-adrenoceptor blockade, and β+α_1-adrenoceptor blockade conditions. Comparisons are made between control and norepinephrine (NE) infusion periods during each condition. P=mean pressure, Q=flow. Resistances were calculated as PLAD/QLAD for LAD region, PLCX/QLCX for LCX region and (PLAD-PLCX)/QLCX for collateral circulation. Circles with bars show means±SE. These data indicate that vascular smooth muscle of the LAD and collateral circulation is capable of relaxing in response to norepinephrine through stimulation of β-adrenoceptors. (From Maruoka et al (8), by permission of the American Physiological Society)

dogs (3, 11) and pigs (5), some studies have suggested that there is an absence of functioning α-adrenergic receptors (12). Therefore, we reexamined the importance of the α-adrenoceptor system in anesthetized dogs instrumented with an ameroid constrictor placed around the proximal left circumflex coronary artery two to three months prior to study (8). A Gregg cannula was inserted and wedged into the left main coronary artery and the circumflex artery distal to the ameroid constrictor was cannulated. This procedure allowed isolated perfusion of the entire left coronary circulation with a servo-controlled constant pressure pump as well as measurement of both native and collateral vascular resistance. Norepinephrine or B-HT 920, a selective α_2-adrenoceptor agonist, was infused in the unblocked condition, following β, β+ selective α_1, or β+ selective α_2-adrenoceptor blockade. We found that norepi-nephrine decreased collateral resistance in the unblocked condition (Fig. 1) and elicited α_2 but not α_1-adrenoceptor-mediated vasoconstriction (Figs. 1 and 2). We believe that the observed vasoconstriction originated, at

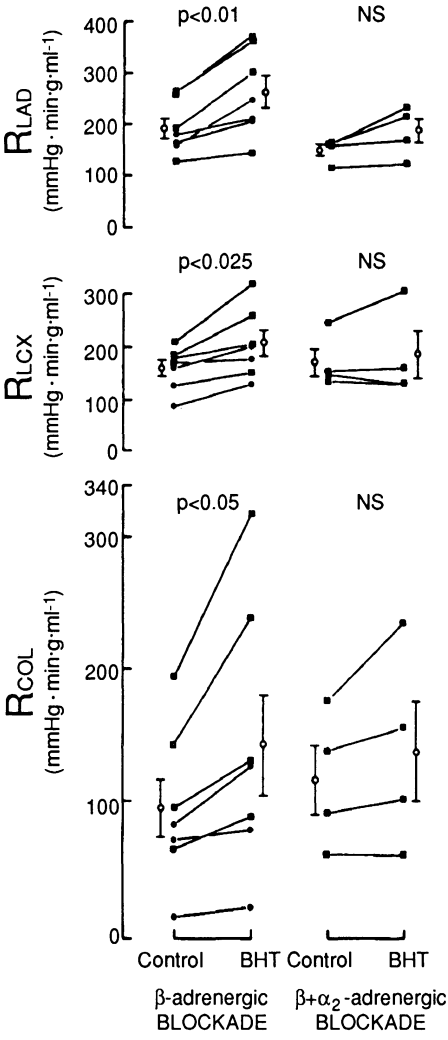

Fig. 2. Vascular resistances (R) in native left anterior descending (LAD) and collateral-dependent left-circumflex (LCX) coronary artery regions of dogs before and during B-HT 920 (BHT) infusion in β-adrenoceptor and β+α_2-adrenoceptor blockade conditions. These data are taken from the same study as represented in figure 1. Comparisons were made between control and BHT infusion periods during each condition. R were calculated by using the same formula as explained in figure 1. Circles with bars show means±SE. These data imply that the increased vascular resistance in response to BHT following β–adrenoceptor blockade is mediated by α_2 receptors. (From Maruoka et al (8), by permission of the American Physiological Society)

least in part, from smooth muscle contraction in the collateral vessels rather than in the upstream segment of conductance vessels included in our calculation. In this regard, other investigators have demonstrated that constriction in large coronary arteries is not proportionally greater than that observed in the total coronary circulation (13). Furthermore, large conductance vessels are regulated mainly through stimulation of α_1- rather than α_2-adrenoceptors (14), which is in opposition to our results in coronary collateral vessels. Our findings differ from a previous study (12), possibly because the isolated fibrillating heart preparation and/or the background of adenosine-induced vasodilation used in the other study may have overwhelmed the α_2-mediated constriction of collateral vessels. *We conclude, therefore, that the vascular smooth muscle of the collateral circulation of dogs is capable of relaxing in response to norepinephrine through stimulation of β-adrenoceptors as well as vasoconstricting during selective α_2-adrenoceptor stimulation in animals whose β-adrenoceptors have been blocked.*

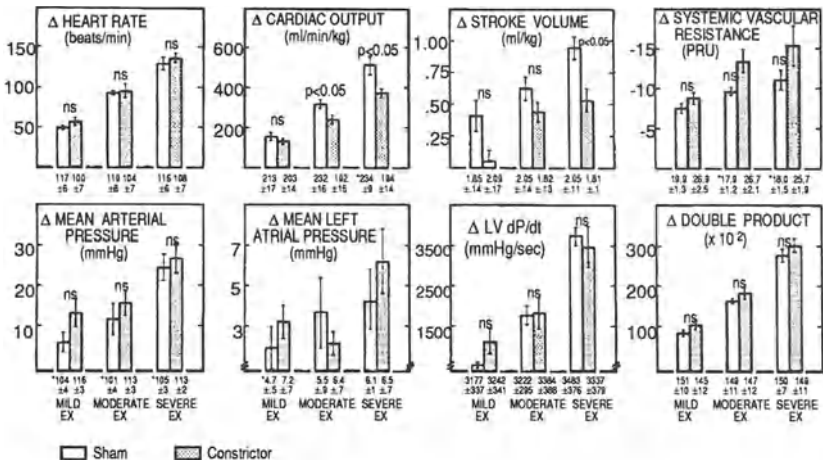

Fig. 3. Comparison of hemodynamic responses between sham-operated and left circumflex ameroid occluded animals during mild, moderate, and severe exercise (EX) in dogs instrumented with an ameroid constrictor around the left circumflex coronary artery. Numbers below each bar represent control or resting values (means±SE) taken just before exercise; heights of bars indicate means; brackets represent means±SE; NS indicates that changes were not significantly different between groups. These data indicate that the cardiac function responses to severe exercise are compromised in ameroid constricted compared to sham animals. (From Longhurst et al (16), by permission of the American Physiological Society)

Function of coronary epicardial conductance vessels in ameroid constrictor model in dogs

Early studies (15) have demonstrated normal perfusion of the collateral region during moderate exercise in dogs with mature collateral vessels, 6-10 months after constriction of the coronary circulation with an ameroid occluder. These studies, however, did not consider the possibility that there may be abnormalities of flow during severe exercise or that there may be a global derangement in flow occurring in both collateralized and native regions of myocardium. To examine this condition more carefully, we studied coronary flow responses and ventricular function in dogs 2 months after application of an ameroid constrictor. These animals were studied during mild, moderate and severe treadmill exercise and their hemodynamic responses were compared

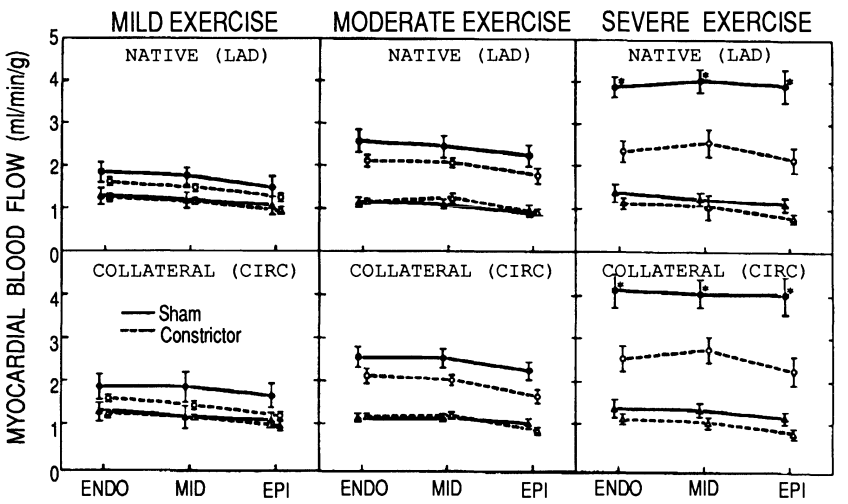

Fig. 4. Transmural left ventricular blood flow responses to mild, moderate, and severe exercise in the same group of dogs as represented in figure 3. Symbols indicate means and brackets the SE. Comparisons of blood flow were made at each rest (triangles) and each exercise (circles) between sham (closed symbols) and left circumflex ameroid occluded (open symbols) animals in native and collateralized regions of myocardium. Native regions in sham animals consisted of areas supplied by both left anterior descending (LAD) and circumflex (CIRC) vessels. Native region in constrictor animals consisted of area supplied by LAD and collateral region included of area supplied by CIRC. Asterisks indicate significant differences in blood flow during exercise between sham and constrictor animals. These data suggest that during severe exercise the absolute increases in blood flow to the native and collateral regions were attenuated in left-circumflex coronary artery constricted animals compared to sham operated animals. (From Longhurst et al (16), by permission of the American Physiological Society).

to a group of sham-instrumented control animals (16). Heart rate increased to approximately 160, 200 and 245 beats/min during the three levels of exercise (Fig. 3). Regional myocardial blood flow, measured with radioactive microspheres, increased similarly in the collateral and native regions of myocardium during each of the three exercise levels, indicating that the hydraulic resistance imposed by the collateral circulation did not limit blood flow (Fig. 4). However, during severe exercise we found that the absolute increases in flow were significantly reduced in the collateral and native regions in the constricted animals compared to the sham group (Fig. 4). The decreased flow was accompanied by a reduction of the increase in cardiac stroke volume and cardiac output during severe exercise (Fig. 3). These responses were not the result of a reduction in myocardial oxygen requirements, nor was there a reduction in coronary perfusion pressure (Fig. 3). It also was unlikely that the flow reserve of the collateral vessels was exceeded since Scheel et al (17) have demonstrated equal or greater flow under conditions of constant perfusion. Although the mechanism(s) underlying the global reduction in flow and the resulting compromise in cardiac performance are uncertain, we believe that the vasodilatory reserve of the arterial system supplying flow to both native and collateral regions (i.e. proximal left anterior descending, septal and right coronary arteries) was exhausted or that there was inappropriate vasoconstriction of resistance vessels in collateralized and noncollateralized regions. *This study implies that, in species such as the dog, which develop an extensive collateral circulation in response to gradual coronary occlusion, the flow reserve of conduit vessels supplying blood to the entire left ventricle can be exhausted during conditions of high stress (e.g. severe exercise) such that ventricular performance is compromised. However, in species such as pigs and humans who develop a sparse collateral circulation, it is less likely that the flow through the proximal circulation would be as high as in dogs and the vasodilatory reserve in these vessels therefore probably would not be exceeded.*

Development of a pig ameroid constrictor model to study coronary collateral development and function

Following our earlier studies of the function and development of dog collateral vessels, we became increasingly concerned that the dog may not be representative of the collateral circulation of humans. We chose the pig as a more suitable model of humans since, like humans, this species has a sparse innate collateral circulation consisting of a fine anastomotic network of endomural vessels (3, 18), and because we suspected that

they would respond with modest collateral vessel development during gradual occlusion of a coronary artery. Our interest was in defining the response of the pig's collateral circulation to acute exercise as well as physical training using exercise. Previous studies on dogs from our laboratory (19, 20) and on pigs from others (21, 22) had demonstrated that, in contrast to both the acute and chronic exercise responses in dogs, the physiological responses of pigs are much more analogous to those manifested in humans. Thus, the oxygen consumption, cardiac output and work load capacity of pigs, like humans, is much more limited than those of dogs (19, 20, 23). We therefore have completed a series of studies designed to define the natural history of coronary collateral development during and following ameroid constriction, to determine the responses to chronic physical conditioning with exercise and to investigate mechanisms that might underlie our observations. Finally, we have begun studies on the regulation of collateral blood flow during exercise in pigs.

A major obstacle was the finding in previous studies by others that ameroid occlusion of the coronary circulation in pigs leads to extensive myocardial infarction and a high mortality (24, 25, 26). This problem was solved by placing the constrictor on the proximal left circumflex rather than the left anterior descending or right coronary arteries. The circumflex circulation has greater collateral blood flow than the left anterior descending coronary artery and therefore constriction of this former vessel results in relatively less ischemia and infarction (27). Also, the region of myocardium supplied by the left circumflex artery in pigs is smaller than that supplied by either the right or left anterior descending coronary arteries (28). Proportionally, however, the collateral supply is greater so that presumably there is less ischemia and less infarction resulting from ameroid constriction. Thus, we routinely observe <10%, and generally ~5% infarction in the left circumflex ameroid constriction model in miniswine, even in animals that have undergone chronic physical conditioning (5, 29).

Initially we defined the natural history of collateral vessel development in miniswine over a 16 week period following placement of an ameroid constrictor around the circumflex artery (4, 5). We found that blood flow through collateral vessels, estimated by the radioactive microsphere method, and regional myocardial function, measured as the change in systolic wall thickening with sonomicrometer dimension gauges, was similar to blood flow and regional function at rest in the non-collateralized region of the left ventricle. However, during moderate exercise to a heart rate of 230 beats/min or severe exercise to a heart rate of 280 beats/min there was a smaller

increase in flow in the collateral compared to the non-collateral regions of myocardium. For instance, eleven weeks after placement of the constrictor the ratios of blood flows in the collateral region compared to the non-collateral region during moderate exercise were 0.37 and 0.87 in the subendocardium and subepicardium, respectively (Fig. 5). The relative under-perfusion of the collateral region suggested the presence of ischemia, a fact that was confirmed by demonstrating a decrease in wall thickening from 41 to 16% in the collateral region compared to the non-collateral region in which the function was unchanged (39 vs. 36%, rest vs. exercise, respectively) (Fig. 6). *Thus, like humans (30), pigs develop a network of endomural collateral vessels that provide normal flow and support normal myocardial function at rest, but allow insufficient flow and hence cause ischemic myocardial dysfunction during moderate and severe exercise.*

Regulation of coronary collateral blood flow in pigs by vasopressin

After establishing that the model of coronary collaterals in pigs resembles that in

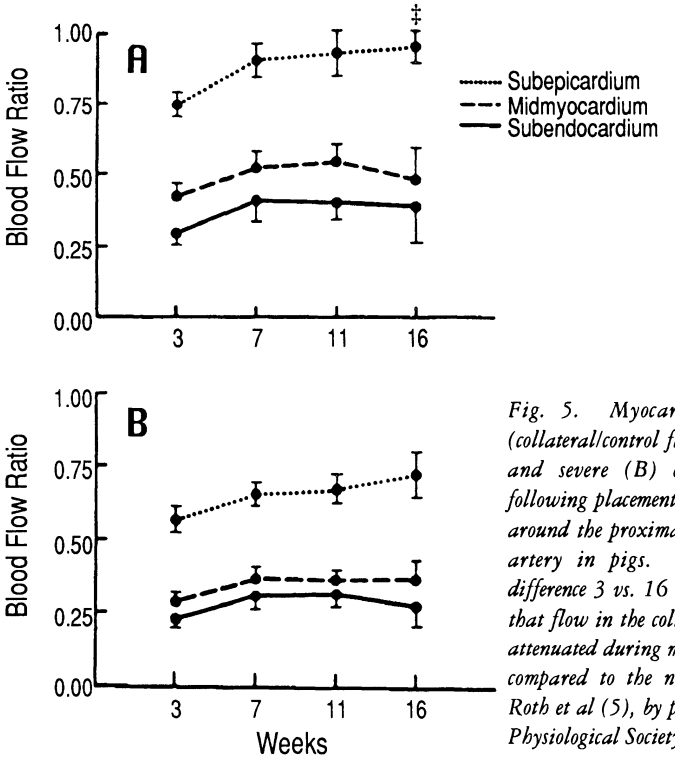

Fig. 5. Myocardial blood flow ratios (collateral/control flow) during moderate (A) and severe (B) exercise over 16 weeks following placement of an ameroid constrictor around the proximal left circumflex coronary artery in pigs. ‡ indicates significant difference 3 vs. 16 weeks. These results show that flow in the collateral-dependent region is attenuated during moderate and severe exercise compared to the native circulation. (From Roth et al (5), by permission of the American Physiological Society).

humans, both anatomically and physiologically, we have begun to study the function of these vessels. Previous investigations have suggested that these vessels are particularly sensitive to vasopressin. In this regard, collateral vessels demonstrate significantly more vasoconstriction in response to the exogenous administration of vasopressin than native or non-collateral vessels of dogs (12, 31, 32). Furthermore, during exercise, exogenous vasopressin has been shown to be capable of limiting blood flow to the collateral-dependent myocardium (33). However, we have been concerned that the concentrations of vasopressin infused in these studies may exceed those that could be produced during normal physiologic and most pathophysiologic circumstances. Therefore, we examined the hypothesis that vasopressin produced during prolonged treadmill exercise (80% of heart rate reserve above rest for 25 min, average heart rate = 223 beats/min) would

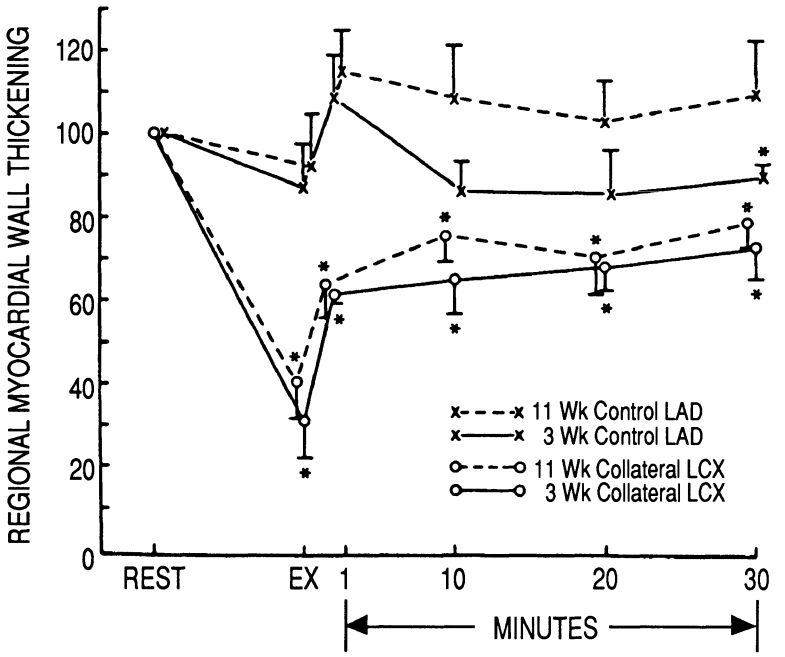

*Fig. 6. Regional myocardial wall thickening in pigs measured with sonomicrometer dimension gauges in collateral-dependent left circumflex (LCX) and control left anterior descending (LAD) regions normalized to resting values of 100. Data in this figure are taken from the same study as described in figure 5. Values are means±SE, at rest, exercise (EX), and during 30 min of recovery from exercise. * indicates significant difference between rest vs. exercise or rest vs. recovery. It can be seen that systolic wall thickening in the left-circumflex region is significantly attenuated during exercise, implying myocardial ischemia in the collateral region. (From Roth et al (5), by permission of the American Physiological Society).*

attenuate collateral blood flow and regional function (34). This form of exercise was selected since previous studies have shown relatively large increases in plasma vasopressin in the pig with this degree of exertion (34). Animals were studied 10 weeks after placement of an ameroid constrictor around the left circumflex coronary artery during prolonged exercise before and after V_1 receptor blockade with d(CH_2)$_5$Tyr(Me) arginine vasopressin (10-12 mg/kg) administered intravenously. This concentration of antagonist significantly attenuated the pressor response to intravenous vasopressin. We found that there was no alteration in either regional collateral flow or function after administering the vasopressin antagonist, suggesting that the concentration of vasopressin produced during this intensity of exercise is not capable of competing with the metabolic coronary collateral vasodilation. Certain pathological situations such as hemorrhage and dehydration may be associated with higher concentrations of vasopressin than have been observed during prolonged high intensity exercise (35, 36, 37). It is possible that these higher concentrations of vasopressin can compete successfully with metabolic vasodilation to cause collateral vasoconstriction. *However, our data suggest that vasopressin is not an important factor in causing coronary collateral vasoconstriction and therefore does not limit collateral flow and myocardial function during prolonged and relatively intense exercise in ameroid-constricted miniswine.*

Response of pig coronary collateral vessels to chronic exercise training

Recently we studied growth and development of coronary collateral vessels in ameroid-constricted miniswine during physical conditioning. Previous investigations of exercise training had yielded mixed results with some demonstrating a beneficial effect (17, 38, 39, 40, 41, 42) while others have not shown any significant influence (43). In addition, although some studies have demonstrated enhanced development of the coronary collateral circulation with chronic exercise training, very few have measured the extent of ischemia in the collateral-dependent region, and those that did were unable to demonstrate any improvement (40). One investigation that was unable to demonstrate any improvement in collateral flow following physical conditioning used a two constrictor dog model (43). Following a period of exercise training, hearts were studied in vitro in a blood-perfused Langendorff preparation during maximum vasodilation with adenosine. We believe that the use of dogs and the techniques employed in this study may have influenced its outcome. As noted above, this species develops a very dense network of both epicardial and endomural collateral vessels with

ameroid constriction alone, even in the absence of physical conditioning. It is possible that little further ischemia results during exercise training, even with two vessels occluded. It is also possible that there is little capacity for collateral vessels in this species to further enlarge in response to repetitive exercise. Furthermore, the use of maximum vasodilation in conjunction with the isolated heart preparation does not allow examination of coronary collateral function under physiological conditions of exercise and potentially could mask a subtle improvement in collateral functional capacity resulting from an altered responsiveness of collateral vascular smooth muscle in physiologically trained animals during acute exercise. Therefore, we reexamined this question in pigs (29), a model which we knew from prior studies would develop ischemia during exercise, and a model which has a similar exercise capacity as humans. Our goal was to allow the collaterals to mature for approximately four weeks after

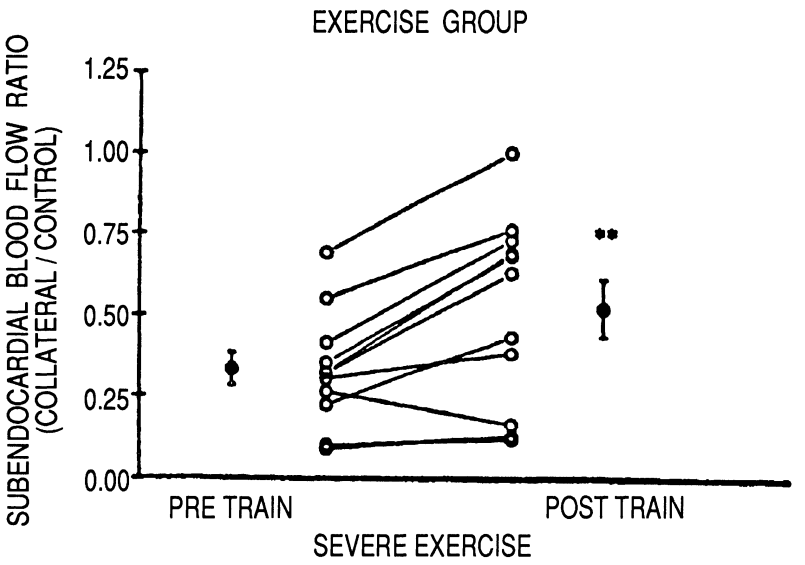

*Fig. 7. Ratios of myocardial blood flow in the subendocardium of the collateral-dependent left circumflex region to flow in the nonoccluded control regions (left anterior descending and right coronary perfused regions of the left ventricle) in individual exercise-trained pigs during severe exercise. Values are shown from the pretraining exercise test (PRE TRAIN) and the post-training exercise test (POST TRAIN) accomplished after 5 weeks of physical conditioning. ** indicates $p<0.01$ PRE TRAIN vs POST TRAIN. These data suggest that long-term exercise training is capable of elevating collateral-dependent blood flow by enhancing collateral vessel development. (From Roth et al (29), by permission of the American Heart Association).*

application of the ameroid occluder, measure the collateral blood flow and regional myocardial function during severe exercise, train the animals five days/week for 30 - 50 min/day for another five weeks using a motor driven treadmill, then restudy the animals at rest and during exercise to determine whether or not there was improvement in the functional capacity of the collateral circulation and lessening of ischemia in the collateral-dependent region. The pigs achieved a target heart rate of 210 - 220 beats/min during each period of exercise. They averaged 25 days of exercise training for a total of 930 min. A group of sedentary pigs served as controls. Following the period of training we observed significant increases in the workload achieved during severe exercise (710 vs. 867 kg·m/min) at a heart rate of 265 beats/min, suggesting a training adaptation. Comparing blood flow ratios (collateral/non-collateral flow), there were significant increases in subepicardial (0.33 vs. 0.52) and subendocardial flow ratios

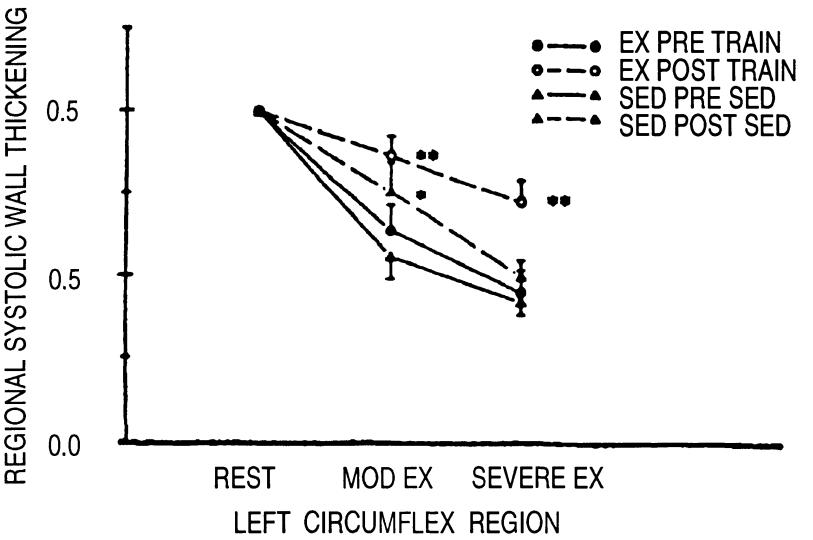

*Fig. 8. Regional systolic wall thickening in the collateral-dependent left circumflex region of pigs in the exercise trained (EX) and sedentary groups (SED) normalized to a preexercise rest value of one. The data were obtained in the study as depicted in figure 7. Values are shown before and after training in the exercise-trained group and the control sedentary group at the beginning (PRE SED) and end of the study (POST SED) at rest, moderate exercise (MOD EX) and severe exercise (SEVERE EX). * indicates $p<0.05$ PRE SED vs POST SED; ** indicates $p<0.01$ PRE TRAIN vs POST TRAIN. These data demonstrate that, following a period of physical conditioning, the elevated blood flow to the collateral-dependent myocardium (Fig. 7) limits dysfunction in this region. (From Roth et al (29), by permission of the American Heart Association).*

(0.79 vs. 1.02, Fig. 7), pre vs. post-training during severe exercise. The improvement in collateral flow was accompanied by attenuation of the abnormal systolic wall thickening during exercise in the animals following training compared to either their pretraining responses or compared to the sedentary controls (Fig. 8). *Thus, chronic physical training in this miniswine model increased collateral blood flow during exercise and lessened the extent of exercise-induced ischemia, as reflected by systolic myocardial function in the collateral region.*

Importance of ischemia in coronary collateral development in pigs

The observation that exercise training improves collateral blood flow and regional myocardial function does not provide an explanation of the underlying mechanism(s) responsible for this observation. In this regard, although a number of both chemical and mechanical hypotheses have been advanced to explain the development of coronary collateral vessels, the actual mechanism(s) have not been defined. Myocardial ischemia has been proposed as one factor that may be important in causing collateral development (44, 45, 46, 47, 48). Mitogenic factors produced during ischemia may be responsible for increasing the coronary collateral network (49). Studies have suggested that both the duration and intensity of ischemia are determinants of collateral vessel growth (50). The actual importance of hypoxia in stimulating myocardial capillary growth (i.e. angiogenesis) has been both supported and refuted (51, 52). However, the process of growth and enlargement of pre-existent coronary collaterals may be quite different from the angiogenic process associated with the formation of new capillary vessels, and therefore these studies may not have examined comparable phenomena. Thus, the overall importance of ischemia in collateral development is uncertain. Therefore, we recently completed a study examining the importance of ischemia. The left circumflex ameroid-constricted miniswine preparation was used to test the hypothesis that reduction of both the intensity and duration of ischemia with long-term suppression of β-adrenoceptor activity with propranolol would attenuate collateral and capillary vessel development (53). To accomplish this study we developed a biotelemetry system which allows continuous beat-to-beat measurement and analysis of cardiovascular hemodynamic variables including heart rate, blood pressure and regional wall motion in both normal and collateral regions over prolonged periods (up to 18 hours continuously, repeated 2-3 days/week for five weeks) (54, 55). Using this technology we have demonstrated that 320 mg propranolol (Inderal LA), administered

twice daily, significantly reduced the daily number, individual duration, and severity of events representing myocardial dysfunction, which we believe indicated ischemia (Fig. 9). Ischemic events were measured as a decrease, relative to baseline, in regional collateral thickening compared to simultaneously measured regional thickening in the non-collateral region and at a time when myocardial oxygen demand (estimated by the double product) was increased. When an event lasted for at least 30 s, it was felt to represent ischemia. Compared to a group of unblocked animals, we found

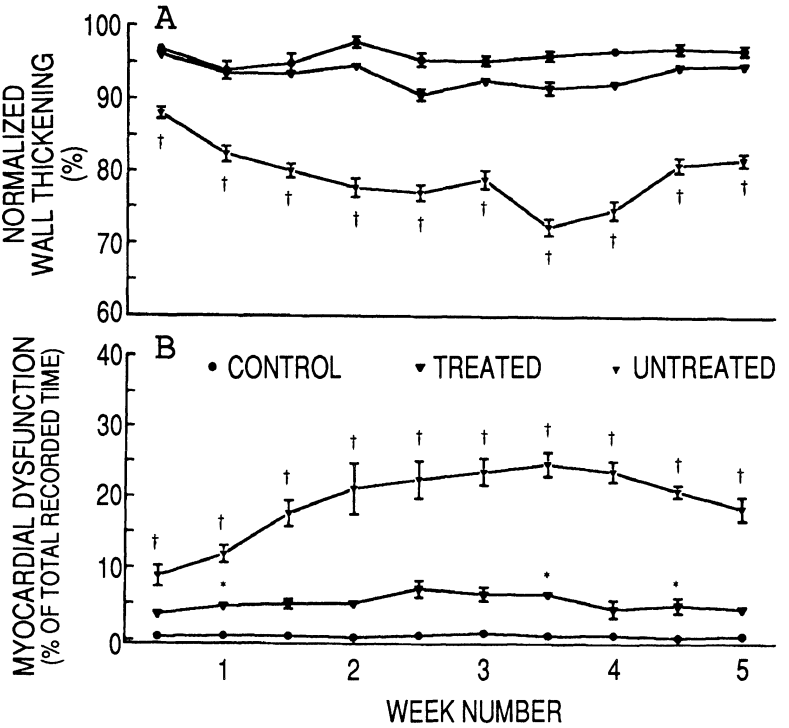

Fig. 9. Biotelemetry data demonstrating extent (Panel A) and duration (Panel B) of myocardial dysfunction. Left circumflex regional wall motion, normalized to control values, is shown in Panel A. 100% represents the control value obtained during a particular recording period and decreases from 100% indicate dysfunction. Values are means±SEM for all events of dysfunction during a particular recording. Duration of myocardial dysfunction is shown in Panel B. The total duration of dysfunction during an individual recording period was divided by the total duration of the recording period. Duration of dysfunction is expressed as a percentage of total recorded time. Data for both Panel A and B were obtained twice per week, for five weeks, from four sham animals (no ameroid constrictor), five treated animals (320 mg propranolol, BID), and six untreated animals. (From Symons et al (53), by permission of the American Heart Association).

that ischemia in the β-adrenoceptor-blocked animals was greatly attenuated and occurred only slightly more than a group of sham control animals that had not been instrumented with an ameroid constrictor. However, despite the almost complete reduction in ischemia we were unable to confirm our hypothesis. Instead we found that collateral blood flow and regional myocardial function at rest and during moderately severe exercise (heart rate = 240 beats/min) were not different in the untreated and β-blockade groups (Figs. 10 and 11).

We conclude, therefore, that growth and development of the coronary collateral circulation in ameroid-constricted miniswine are not closely related to the extent and duration of myocardial

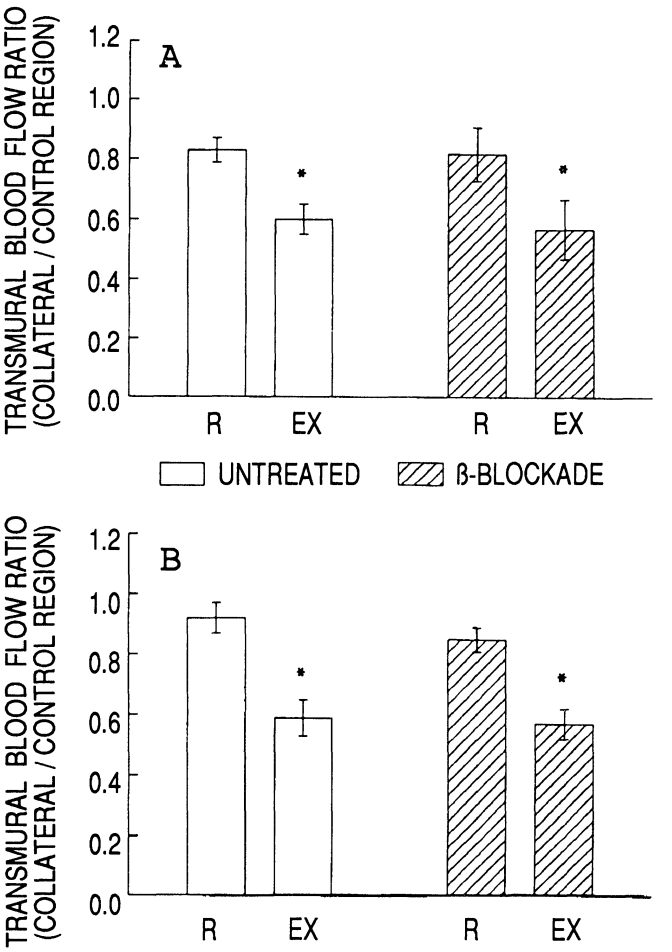

*Fig. 10. Ratios of transmural flow at rest (R) and during exercise (EX) for both propranolol treated and untreated groups at five weeks (Panel A) and eight weeks (Panel B). Data were obtained after treated animals had been withdrawn from propranolol for at least 72 hours. Values are means±SEM. *p<0.05 rest vs exercise. (From Symons and Longhurst (53), by permission of the American Heart Association).*

ischemia, or, that a very small amount of ischemia is capable of stimulating collateral development.

Effect of mechanical stress on coronary collateral development in pigs

Since we have not been able to confirm that ischemia is a major determinant of coronary collateral development in miniswine, we have examined the hypothesis that mechanical stimulation augments the growth of these vessels (56). A previous theoretical study suggested that mechanical stress may be required for collateral development during gradual coronary artery occlusion (44). The importance of mechanical stimuli is suggested by the observation that collateral vessel growth

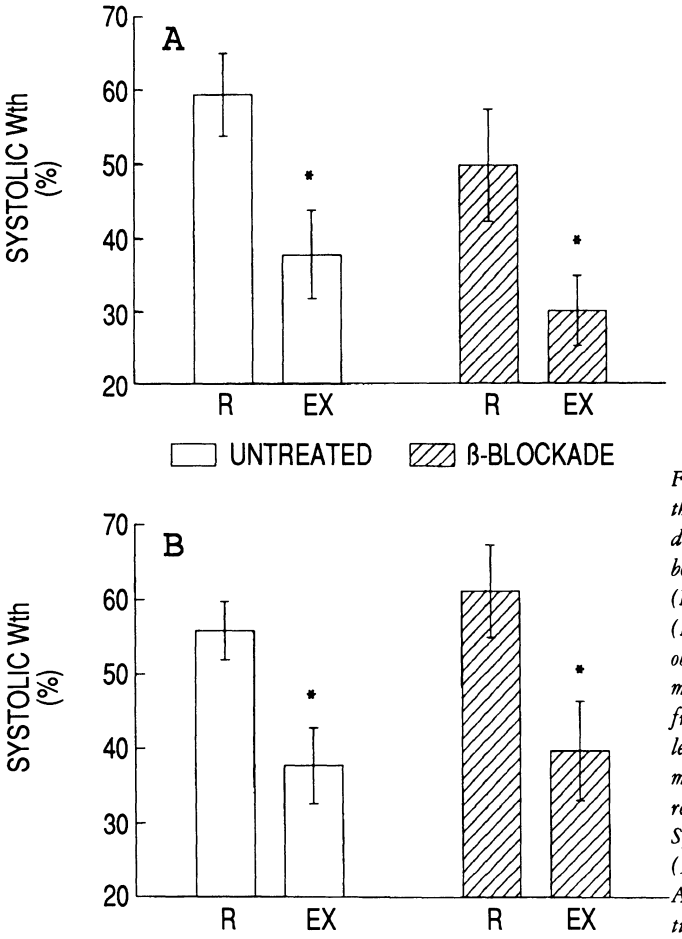

*Fig. 11. Systolic wall thickening at rest (R) and during exercise (EX) for both groups at five weeks (Panel A) and eight weeks (Panel B). Data were obtained after treated animals had been withdrawn from propranolol for at least 72 hours. Values are means±SEM. *$p<0.05$ rest vs exercise. (From Symons and Longhurst (53), by permission of the American Heart Association).*

continues even after ischemia is no longer present in the ameroid or other constrictor models (57, 58). To test the hypothesis that repeated mechanical stimulation augments collateral growth, we have infused dipyridamole intravenously 90 min/day, 5 days/week for 8 weeks in miniswine instrumented with a left circumflex ameroid constrictor. Dipyridamole increases collateral blood flow in this model (4), by preventing the uptake of adenosine (59, 60) a potent coronary vasodilator. At the termination of the study, collateral blood flow and regional myocardial function were measured during exercise to a heart rate of 240 beats/min. Compared to a non-infused control group and a group infused with tartaric acid, the vehicle control, we determined that there were smaller decreases in the collateral/non-collateral flow ratio and regional systolic wall thickening in the collateral region during exercise. *We conclude, therefore, that the increase in coronary blood flow and presumably collateral vascular wall stress caused by repeated dipyridamole administration enhances collateral blood flow and attenuates myocardial dysfunction in the collateral region during exercise in miniswine. Thus, we have supported our hypothesis that mechanical factors contribute to growth and development of coronary collateral vessels.* However, further studies will be necessary to determine whether adenosine has a direct effect separate from its coronary vasodilator properties in inducing collateral vessel development.

CONCLUSIONS

In summary, the dog forms a rich network of both epicardial and intramural collateral vessels, which do not limit blood flow into the collateral region during either rest or exercise. However, the large increase in collateral flow that does occur in dogs appears to overwhelm the capacity of proximal native vessels to supply adequate flow to both non-collateral and collateral regions. Exceeding the vasodilatory capacity of these conductance vessels causes a global reduction in flow and function in the whole dog heart during severe exercise. However, our studies provide evidence that, relative to humans with severe coronary artery disease, the pig is a better model than dogs for studying the function as well as growth and development of the coronary collateral circulation. Like humans, pigs develop a sparse intramural network of these vessels, which supply adequate blood flow at rest but insufficient flow during exercise so that myocardial ischemia and dysfunction in the collateral-dependent myocardium results. Studies from other investigators suggest that exogenously administered vasopressin causes collateral vasoconstriction. Our studies in pigs using prolonged intense exercise,

however, suggest that the vasopressin concentrations are insufficient to overcome the metabolic vasodilation associated with this physiologic stress. Although the effect of physical training on the collateral circulation of dogs is controversial, we have been able to demonstrate that chronic repetitive exercise in pigs increases collateral blood flow and reduces the ischemic dysfunction that is normally observed during acute exercise. The mechanism underlying the improved flow and function may not be the result of repeated ischemia, although our studies employing chronic suppression of ischemia, to date, have only examined sedentary animals. Repetitive mechanical stimulation, however, does appear to be capable of augmenting coronary collateral growth and reducing myocardial ischemia. Future studies will be necessary, however, to definitively conclude that such mechanical stimuli are important determinants of the improvement seen during physical conditioning.

Acknowledgements: We gratefully acknowledge the technical assistance of Elaine Wilkins, Deborah Frank, Roberta Holt and Steven Rendig in these studies. Dr. Colin Bloor and Mr. Francis White are thanked for their critical comments on the manuscript. These studies were funded, in part, by NIH grant HL39114 and the Rosenfeld Heart Fund.

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11

THE ROLE OF GROWTH FACTORS IN COLLATERAL DEVELOPMENT

Ellis F. Unger
National Institute of Health, National Heart, Lung, and Blood Institute,
Bethesda, Maryland, U.S.A.

INTRODUCTION

At least eleven angiogenic peptides have been characterized during the last decade, including seven members of the fibroblast growth factor (FGF) family (1), transforming growth factor α (2), transforming growth factor β (3), angiogenin (4), platelet derived endothelial cell growth factor (5), and vascular endothelial growth factor (6, 7, 8). Diverse in their biochemical and biological characteristics, these peptides exert a number of effects on vascular target cells. Acting in concert on endothelial cells, smooth muscle cells, and fibroblasts, they are believed to modulate blood vessel homeostasis under normal conditions. In addition, an important role has been ascribed to these peptides in embryonic development, (9, 10, 11, 12, 13, 14), and in pathologic conditions such as angiogenesis associated with wound healing (15, 16) and tumor formation (17). These peptides are described in various chapters of this book, and are the subject of several recent reviews (1, 18, 19, 20, 21, 22, 23). It is likely that growth factors play a significant role in coronary collateral formation as well; however, their interrelationships and functions *in vivo* remain obscure, and a satisfactory mechanism has yet to be advanced to explain their actions (24, 25).

GROWTH FACTORS IN THE HEART

Several investigators have extracted and purified acidic and basic FGF's from the heart (26, 27, 28, 29, 30). More recently, FGF's have been localized in neonatal (27, 31) and adult (32) cardiac myocytes *in vitro*. Thus, their localization in the heart is due at least in part to their presence in myocytes, and is not merely a reflection of their existence in vascular smooth muscle cells, endothelial cells, pericytes, interstitial cells, or blood-borne elements. Using immunohistochemical techniques, transforming growth factor β has been localized to ventricular myocytes as well (33). The mere presence of peptide growth factors in the cardiac myocyte proves little, but suggests that this cell might participate importantly in myocardial vascular homeostasis, or the adaptation to pathological states such as myocardial hypertrophy or infarction. Such a

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

hypothesis is tenable, given that the myocyte is more sensitive to the stress of ischemia than the endothelium and/or components of the vascular wall.

Altered expression of growth factors has been observed in settings of myocardial ischemia, infarction, and *ex vivo* myocardial necrosis. Increased mitogenic activity found in the lysate of explanted rat heart first provided the suggestion that myocardial necrosis might cause the release of an angiogenic growth factor (34). In a pig model of ameroid-induced coronary artery occlusion, mRNA for endothelial cell growth factor β (a precursor of acidic FGF) was found in the collateral-dependent zone, but was undetectable in the normal zone. *In situ* hybridization localized the mRNA transcripts to the walls of blood vessels (35). This provided the first evidence in favor of a widely held (but unproven) hypothesis: that ischemia causes the synthesis and/or release of growth factors that in turn leads to collateral development. Subsequently, enhanced expression of transforming growth factor β mRNA was observed in collateral-dependent myocardium of pigs, with localization to histologically intact myocardium and the Purkinje system (36). Transforming growth factor β was noted to increase in border zone myocytes in the setting of myocardial infarction, but the peptide became undetectable within myocytes in the infarct area proper (37). These examples, demonstrating an association between altered growth factor expression and myocardial ischemia and/or infarction, imply a functional role for these peptides in the vascular adaptation to these pathological conditions. Given the profound effects of these growth factors on angiogenesis *in vitro* and *in vivo* (see below), it would be imprudent to dismiss their expression in these situations as mere epiphenomena.

Acidic and basic FGF lack the signal peptide sequence associated with extracellular secretion. Thus, it has been suggested that these peptides function primarily as intracrine hormones (38), with extracellular release only upon cellular injury or death (39). Some modality for extracellular transport must exist, however, because FGF is sequestered by the extracellular matrix (31, 40). In fact, restriction of FGF activity is thought by some to result from the interaction between FGF and the extracellular matrix (41). The mechanism of FGF transport and release is obviously a fertile area of research.

EFFECT OF EXOGENOUS GROWTH FACTORS ON THE HEART

In our laboratory, we have studied the effects of growth factors on the myocardium, both to gain insight into their role in coronary collateral formation, and to

assess their potential as pharmacologic agents to enhance collateral blood flow to ischemic myocardium. For the patient with occlusive coronary artery disease, existing pharmacologic therapies ameliorate the balance of myocardial oxygen supply and demand, but do not alter the underlying pathological substrate. Mechanically-based therapies (coronary artery bypass surgery and percutaneous transluminal coronary angioplasty) immediately improve anatomic determinants of myocardial perfusion; however, they are inappropriate for patients with only mild symptoms (and who are at low risk of sudden death), they are "invasive", and significant limitations are associated with their use. Inadequate myocardial perfusion is the fundamental problem in patients with obstructive coronary disease: pharmacologic facilitation of coronary collateral growth could potentially provide a unique and important therapeutic modality for the majority of such patients.

From the standpoint of potential therapy, the pathology of coronary artery disease can be divided broadly into two anatomic classes: 1) a circulation in which at least one coronary artery is relatively free of obstruction (i.e., feeder vessel *present*); and 2) a circulation in which there are high grade obstructions of all major coronary vessels (feeder vessel *absent*). If a patent feeder vessel is present, the facilitation of inter- and intra-coronary collateral development can lead to improved ischemic zone blood flow, with perfusion driven by a transcollateral pressure gradient. In the absence of such a vessel, the development of coronary collaterals is problematic. Chilian et al (42) have demonstrated that myocardial ischemia, even in the absence of a pressure gradient, can provide an adequate stimulus for collateral growth. When high grade proximal obstructions are present in all major coronary arteries, however, there is no source from which to recruit collateral flow, and no pressure gradient to drive it. Short of surgically bypassing or mechanically dilating the obstructed segments, the development of a vascular supply from an extracardiac source is necessary to improve collateral blood flow in such instances.

We have developed experimental models based on these two anatomic conditions: 1) development of extracardiac to coronary anastomoses when a feeder vessel is *absent*; and 2) facilitation of inter- and intracoronary collaterals when a feeder vessel is *present*.

Feeder vessel *absent*

Vineberg pioneered a myocardial revascularization procedure in which a systemic artery (internal mammary artery, IMA) was tunnelled directly into the myocardium

with its branches actively bleeding (43). In a series of innovative studies, Vineberg and his collaborators demonstrated that intramyocardial implantation of the IMA engendered the formation of systemic to coronary anastomoses, potentially providing collateral blood flow from the IMA to the ischemic myocardium. Vineberg was convinced that atherosclerosis of the epicardial coronary arteries was inexorably progressive, and that the coronary microcirculation was consistently spared from obstructive disease. He advocated IMA implantation, therefore, based on the contention that anastomoses between the IMA and coronary microcirculation would continue to provide adequate collateral flow in the face of progressive epicardial atherosclerosis. Many patients underwent such operations in the 1950's and 1960's; however, the relative merits of the procedure remained controversial, and its efficacy was never established with certainty (44).

Assuming that collaterals *did* develop between the systemic artery and the ischemic myocardium, the success of the procedure would be critically dependent on myocardial angiogenesis, because no intrinsic connections existed between the two vascular systems. Thus, we developed a model of myocardial angiogenesis (45) based on

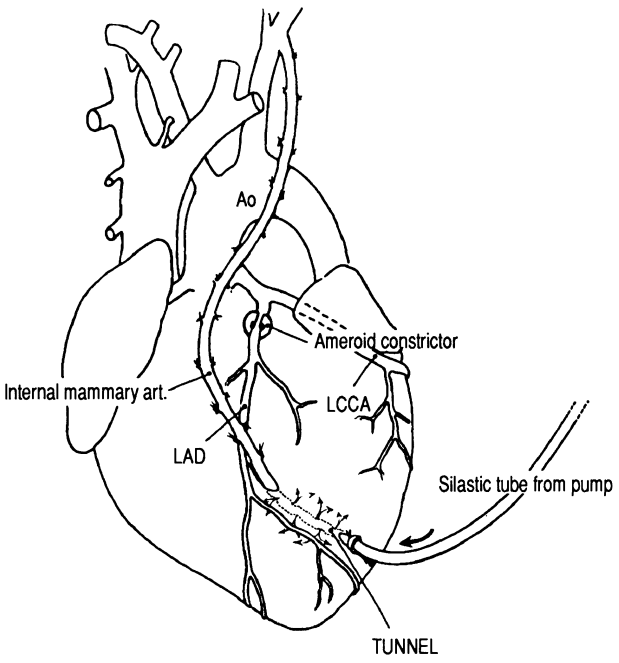

Fig. 1. Model of myocardial angiogenesis based on the formation of collaterals between an extracardiac artery and the coronary circulation. An ameroid constrictor has been applied to the LAD and the IMA has been implanted in the myocardium of the LAD territory. Anastomoses formed between the IMA and the ischemic zone result from an angiogenic process, and their contribution to collateral flow can be determined with radiolabeled microspheres. The silastic tube permits chronic infusion of heparin or growth factors (reprinted from ref. 47, with permission from the American Physiological Society).

an adaptation of this procedure. Ameroid constrictors (46) are devices used to cause gradual arterial occlusion in experimental animals. The ameroid constrictor consists of hydrophilic "ameroid" material encased in a C-shaped stainless steel ring. Once the device is placed around an artery, the ameroid material swells as it absorbs water, gradually compressing the vessel from the outside as it expands. This is actually an oversimplification, because the constrictor elicits a foreign body response as well, and the resulting fibrotic contraction contributes to progressive vascular compromise. Extraluminal compression is generally followed by thrombotic occlusion, with the entire process requiring 2 to 3 weeks. Thus, in this experiment, ameroids were placed on the left anterior descending coronary artery (LAD) of dogs, and the left IMA was implanted into the myocardium of the LAD territory during the same operative procedure (Fig. 1).

The vessel was dissected free from the surrounding connective tissue and the intercostal branches were transsected at their point of egress from the IMA; immediately following these procedures, the bleeding vessel was tunnelled into the myocardium. Thus, the perfusion territory of the LAD was rendered collateral-dependent over several weeks, during which time collaterals would potentially develop between the IMA and the myocardium. After an interval of 8 weeks, microsphere blood flow studies demonstrated that the anastomoses created between the IMA and the coronary circulation provided significant nutritive blood flow in 7 of 12 dogs. In these dogs, IMA-associated collaterals supplied 30.0±2.5% of total LAD zone collateral blood flow under conditions of maximal vasodilatation. Thus, we confirmed that angiogenesis can occur within the myocardium, evidenced by the development of collaterals between an extracardiac artery and the coronary circulation.

As a next step, we assessed the potential of heparin to enhance the formation of IMA to coronary collaterals in this preparation (47). Again, ameroid constrictors were placed on the LAD and the IMA was implanted in the LAD territory. Retrograde infusions were established into the distal IMA: heparin (15 or 150 IU/hr) or placebo were injected directly to the site of neovascularization on a continuous basis, and treatment was continued for eight weeks. Heparin was selected as the subject of the investigation because of its important interactions with FGF, properties suggesting a consequential role in angiogenesis (48, 49, 50, 51, 52, 53, 54, 55, 56). Using radiolabeled microspheres to quantify maximal collateral blood flow, the proportion of IMA-derived collateral flow was more than doubled in heparin-treated dogs, relative to

controls. Thus, continuous heparin infusion promoted extracardiac to coronary collateral formation in this preparation.

These results were consistent with earlier data of Fujita et al (57). This group used repeated, brief (two minute) occlusions of the left circumflex coronary artery (LCX) to engender the formation of collaterals to the LCX territory in dogs. Regional left ventricular function was assessed during LCX occlusion with piezoelectric dimension gauges. Initially, LCX occlusion was associated with regional left ventricular dysfunction, due to limited collateral blood flow. With succeeding LCX occlusions, there was progressive improvement of collateral flow; the eventual absence of a contraction abnormality during LCX occlusion was taken as evidence of adequate collateral flow. Dogs treated with systemic heparin required approximately 40% fewer occlusions than controls in order for ischemic zone contraction to remain normal during the two minute LCX occlusion. The reduced number of occlusions necessary for disappearance of the LCX zone functional abnormality in heparin-treated dogs was taken as evidence of heparin-induced acceleration of collateral growth.

Heparin promotes the angiogenic activity of the FGF's through a number of mechanisms; however, it is not angiogenic by itself. We hypothesized that acidic FGF, a peptide that is directly angiogenic *in vitro* and *in vivo*, would enhance coronary collateral formation to a greater extent than heparin. Thus, using the IMA implant model, we compared the effect of acidic FGF plus heparin to that of heparin alone. Heparin was added to the acidic FGF to potentiate its mitogenic activity and prolong its biological half-life (53, 54). Dogs were randomized to receive acidic FGF 300 μg/week with heparin 6800 IU/week, or heparin alone. The peptide and/or heparin was delivered from an implanted pump as a constant infusion directly into the distal IMA. The extent of IMA-derived collateral blood flow was evaluated with radiolabeled microspheres. The results of this study (unpublished) were disappointing: IMA-derived collateral flow was no better in dogs treated with acidic FGF plus heparin than in dogs treated with heparin alone.

Thompson and associates reported that gelatin sponges or collagen-coated Gore-tex sponges, treated with acidic FGF, were angiogenic when implanted in the rat peritoneum (58, 59). We were intrigued by the possibility that these sponges could be used to engender the formation of collaterals between an extracardiac source and the ischemic myocardium. Again, we created a collateral-dependent zone in dogs by placing ameroid constrictors on the LAD (60). Three weeks after ameroid placement,

an IMA pedicle was placed *on the epicardium* of the LAD territory, and a sponge saturated with acidic FGF was interposed between the vascular graft and the epicardium (Fig. 2). This was done in lieu of implanting the IMA directly into the myocardium as we had done previously (45). Using an indwelling catheter for access, IMA angiography was performed weekly during the next four weeks; however, significant IMA to coronary anastomoses were not evident in any dog.

Although myocardial revascularization was not achieved, an interesting and unexpected finding was observed in virtually all of the acidic FGF-treated dogs but in none of the controls. Striking vascular smooth muscle cell (SMC) hyperplasia was evident in arterioles and small arteries, exclusively in areas of subendocardial infarction (Figs. 3 and 4). Smooth muscle cell hyperplasia was extreme, causing obliteration of vascular lumina in a number of areas, and whorls of SMC's took on an almost neoplastic appearance at some locations. Marked SMC proliferation occurred in the subendocardium despite the fact that the peptide-soaked sponge was placed on the epicardium, and despite the fact that the peptide was administered only once. The effect was independent of dose over a four log range (0.03 to 40 μg/kg) and the type of sponge used (collagen or Gore-tex). In all dogs, the vasculature in viable myocardium appeared normal, including the epicardium of the LAD territory: an area intimately exposed to the acidic FGF-treated sponge. Thus, concomitant ischemic injury and exposure to exogenous aFGF were necessary to trigger vascular SMC proliferation. Considering the route of administration (an *epicardial* "reservoir") and the marked

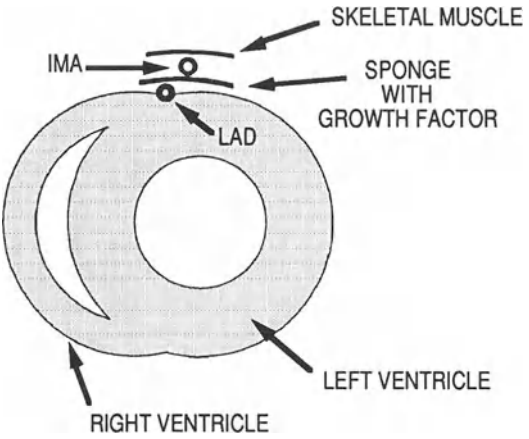

Fig. 2. Cross section of the heart showing the relative locations of the left ventricular myocardium, LAD, peptide-treated sponge, IMA, and associated skeletal muscle of the IMA pedicle. An ameroid applied to the LAD 3 weeks before placement of the IMA pedicle and sponge rendered the LAD territory collateral-dependent (reprinted from ref. 60, with permission from the American Heart Association, Inc.).

proliferation observed in the *subendocardium* at all doses used, it is likely that these doses were extreme, well out of the physiologic range.

Thus, on the basis of this study, caution must be exercised in speculating about a *physiologic* role of acidic FGF in myocardial injury. It could be concluded, however, that acidic FGF had no effect on quiescent vascular SMC's in uninjured areas of myocardium. The proliferative effect observed in areas of injury suggests that injury induces upregulation of FGF receptor(s), or the expression of growth factors acting in synergism with acidic FGF.

Feeder vessel *present*

In patients with at least one patent feeder vessel, angiogenic therapy would be of great value to enhance the development of inter- and intra-coronary collaterals. With this concept in mind, we have developed a canine model of single coronary occlusion in which to test the potential effects of angiogenic peptides (61, 62).

In dogs, ameroid-induced occlusion of a single major coronary artery induces the development of collaterals (63). The major improvement in collateral blood flow occurs during the first 2-3 weeks after ameroid placement, coinciding with the period of active

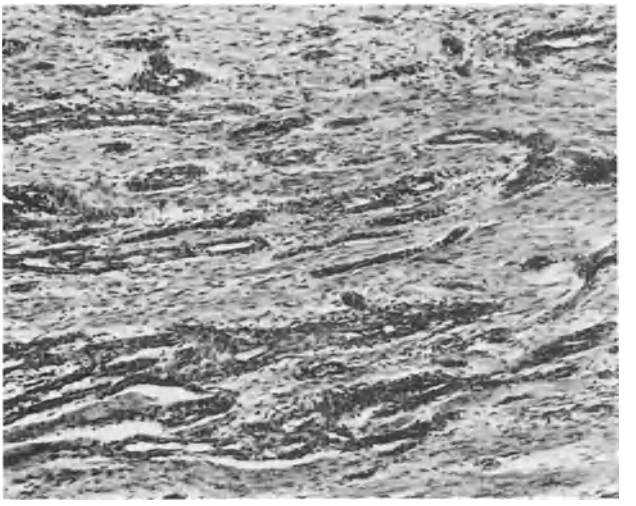

Fig. 3. Marked vascular smooth muscle cell hyperplasia in an area of subendocardial infarction in an acidic FGF-treated dog. Magnification, X80.

coronary narrowing; some additional improvement occurs during the next 9-10 weeks. In our experience, approximately 20% of dogs with LCX ameroids will die suddenly as a result of abrupt coronary occlusion: an event that occurs generally by the third week following ameroid placement. The majority of dogs will survive, however, with variable degrees of patchy non-transmural infarction in the LCX territory. The extent of infarction ranges from none to replacement of the entire endocardial half of the left ventricular wall by scar. Most commonly, there is patchy fibrosis or no infarction at all. Once substantial collateral development has occurred, resting blood flow in the collateral-dependent zone generally returns to the near-normal range. Increased metabolic demand or the stress of pharmacologically-induced coronary vasodilation, however, elicits a disparity between myocardium that is normally perfused and myocardium that is collateral-dependent, because vasodilator reserve is limited in the collateral-dependent zone.

Using a number of variations of this single-ameroid model, we have tested the effects of acidic FGF, basic FGF, and vascular endothelial growth factor (VEGF) on collateral development in the canine heart. The mode of peptide delivery and timing of administration have varied; however, the experimental design has been consistent. These studies share in common a unified hypothesis: that exposure of the myocardium

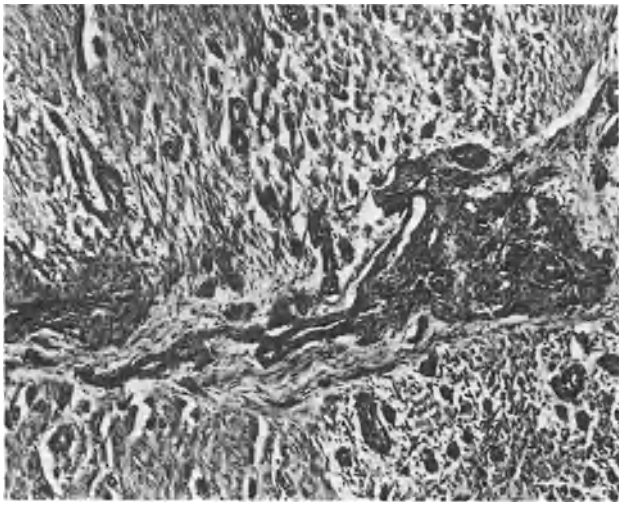

Fig. 4. Extreme smooth muscle cell hyperplasia with neoplastic appearance with in the subendocardium of another dog treated with acidic FGF. Magnification, X80.

to exogenous growth factors could accelerate the development of coronary collaterals. In all of these investigations, the LCX has been subjected to ameroid-induced coronary occlusion, and improvement in collateral blood flow as a function of time has been utilized as the primary endpoint. As noted above, collateral flow progressively increases during and following ameroid-induced arterial occlusion, such that resting blood flow returns to near-normal within a few weeks of ameroid placement. Therefore, resting blood flow provides an index of collateral function that is both insensitive and inadequate. Thus, we assessed collateral blood flow during pharmacologically-induced maximal vasodilatation, and expressed collateral function as the ratio of ischemic zone/normal zone (IZ/NZ) blood flow.

In an investigation using acidic FGF in this model (unpublished), we placed ameroid constrictors on the LCX in dogs. Five weeks later, after substantial collateral development had ensued, dogs were randomized to receive acidic FGF with heparin (30 μg/h and 30 IU/h, respectively) or placebo with heparin (30 IU/h). The drugs were delivered as continuous infusions into the *left main coronary artery*. Treatment was begun

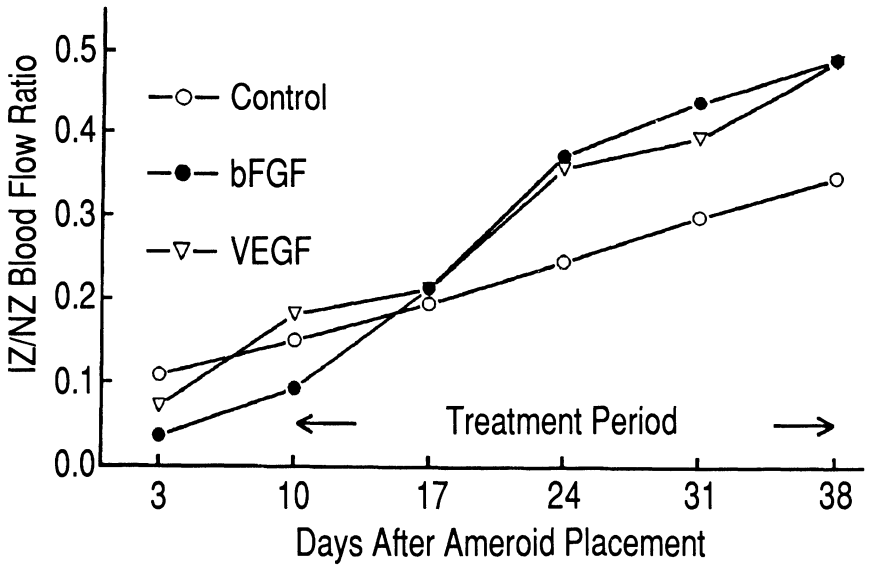

Fig. 5. Collateral blood flow as a function of time for basic FGF-treated (●), VEGF-treated (▽), and control dogs (○). Collateral flow is expressed as the ischemic zone to normal zone (IZ/NZ) ratio during maximal vasodilatation. Randomization to treatment group was done following blood flow measurements on the tenth day, and treatment was begun at this time. Administration of either peptide was associated with an increase in collateral flow of approximately 40% after 28 days of treatment.

5 weeks after ameroid placement and maintained for 4 weeks. Collateral blood flow was quantified with microspheres during maximal coronary vasodilatation at the beginning and end of treatment. As expected, the IZ/NZ blood flow ratio increased over the course of treatment. In acidic FGF-treated dogs, the IZ/NZ ratio increased by 28%; however, similar improvement occurred in the control group. The marked vascular SMC proliferation observed in the previous investigation, when acidic FGF was delivered via an epicardial sponge, was absent in this study.

In contrast, positive results were obtained using basic FGF and vascular endothelial growth factor (VEGF), using a modification of this experimental design (61, 62, 64, 65). Again, ameroid constrictors were placed on the LCX coronary artery; however, treatment with peptide or placebo was begun 10 days after ameroid placement, during the active phase of collateral development. The agents were administered daily, as bolus injections into the distal LCX. Treatment was continued for 4 weeks, from day 10-38. Using intravenous chromonar to elicit maximal coronary vasodilatation, collateral blood flow was assessed weekly: 3, 10, 17, 24, 31, and 38 days following ameroid placement. During the initial 3 weeks after ameroid placement, throughout the period of gradual LCX occlusion, collateral flow was quantified during temporary *complete* occlusion of the LCX (accomplished by inflating a hydraulic balloon occluder on the LCX). Thus, residual antegrade LCX flow was transiently arrested during collateral blood flow measurements, permitting determination of collateral flow exclusively. After 14 days of treatment, the IZ/NZ ratio in basic FGF and VEGF-treated dogs exceeded that of controls: final IZ/NZ blood flow ratios were 0.49±0.13, 0.46±0.12, and 0.34±0.08 in the basic FGF, VEGF and control groups, respectively ($p<0.05$, Fig. 5). Thus, basic FGF and VEGF enhanced IZ blood flow to a comparable extent; increasing collateral flow by approximately 40% after 4 weeks of treatment.

Proliferation of vascular cells constitutes an integral part of the angiogenic response. Therefore, we have attempted to quantify the number of cells undergoing DNA synthesis as a primary endpoint of these studies. Bromodeoxyuridine (BrdU) is a synthetic thymidine analogue that can be used in lieu of tritiated thymidine to label cells undergoing DNA synthesis. Like thymidine, BrdU is incorporated into the DNA of S-phase cells; however, the compound can be detected in tissue sections using immunohistochemistry (Fig. 6). The major advantage of this method is its compatibility with the radioactive microsphere technique. Using autoradiography, it is not feasible to detect tritiated thymidine (a low energy β-emitter) in tissue sections in

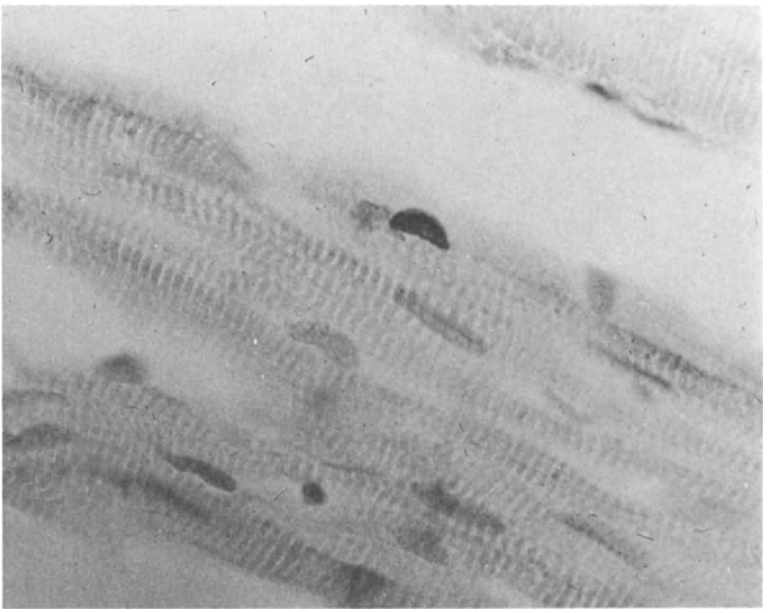

Fig. 6. Positive immunostaining of an endothelial cell nucleus within viable myocardium following incorporation of bromodeoxyuridine, a synthetic thymidine analogue incorporated into cells during the S-phase of the cell cycle. Approximately 4 such cells are located within each mm² of normal myocardium.

the presence of γ-emitting microspheres of much higher energy; however, the BrdU technique is completely compatible with the use of radiolabeled microspheres. In normal myocardium, only rare nuclei stained positively for BrdU, regardless of whether the dogs received basic FGF, VEGF, or placebo. However, treatment with basic FGF was associated with a four-fold increase in the number of nuclei staining positively for BrdU in the IZ. VEGF treatment, on the other hand, had no effect on IZ cellular proliferation.

Basic FGF and VEGF induced similar improvement in collateral blood flow; however, increased IZ cellular proliferation was observed exclusively in basic FGF-treated dogs: VEGF had no such effect. How can these results be reconciled? By light microscopy, the labeled cells in the IZ of basic FGF-treated dogs appeared to be predominantly fibroblasts and SMC's, although a double labeling technique or electron microscopy would be required to confirm this. Basic FGF is a mitogen for all

mesodermally-derived cells, whereas VEGF has relative specificity for vascular endothelial cells (6, 7, 8). Thus, the disparate biological characteristics of these peptides provide a plausible explanation for the dissimilar effects observed on IZ cellular proliferation, assuming that these cells are largely fibroblasts and SMC's. If true, it follows that exogenous basic FGF could have important consequences in infarct healing/remodeling, whereas VEGF would be less likely to exert a significant effect. The relationship between basic FGF-induced fibroblast proliferation, infarct strength, infarct expansion, and residual left ventricular function is a matter of conjecture, however, and not readily predictable from the data at hand. Basic FGF could have a positive effect, due to accelerated neovascularization and increased collagen deposition; alternatively, the presence of exogenous FGF could interfere with a process that is already optimized. Given the comparable effects of basic FGF and VEGF on collateral blood flow, it also follows that selective stimulation of endothelial cells (as by VEGF) may provide a sufficient catalyst for angiogenesis. Stimulation of SMC's and fibroblasts (in addition to endothelial cells) would appear to be unnecessary. These hypotheses are worthy of future testing.

An explanation is also required to account for the fact that VEGF increased IZ collateral blood flow without affecting IZ cellular proliferation. Epicardial collaterals constitute the principal vascular connections between disparate vascular beds of the canine coronary circulation, and the development of such anastomoses occurs predictably as a result of gradual coronary occlusion (66, 67). We believe that it is these epicardial collaterals that respond to the angiogenic stimulus of basic FGF or VEGF, although this is purely speculative at this point. Our analyses focused on intramyocardial vessels: epicardial collaterals were substantially overlooked by our analyses. This provides a plausible explanation for the ability of VEGF to enhance collateral flow in the apparent absence of increased cellular proliferation.

It should be noted that the negative results obtained with acidic FGF are not directly comparable with those obtained with basic FGF and/or VEGF, because of differences in the timing and/or route of peptide administration. Acidic FGF was infused continuously into the left main coronary artery, towards the area of normally perfused myocardium from which collaterals would potentially *originate*. In subsequent studies, basic FGF and VEGF were given as bolus injections into the ischemic zone myocardium, into the area that would potentially *receive* collateral flow. Induction of angiogenesis may require a concentration gradient favoring the ischemic zone,

consistent with the concept that *ischemia* causes the elaboration or release of growth factor(s), presumably from the IZ itself. Another important difference between the studies was the timing of drug administration. All three peptides were given for a duration of 28 days; however, basic FGF and VEGF were given beginning 10 days after ameroid placement, much earlier in the course of LCX constriction than acidic FGF was administered. In fact, acidic FGF was not begun until 35 days after ameroid placement, coinciding with the time at which studies with basic FGF or VEGF were concluded. As can be seen from figure 5, there is still potential for improved collateral flow after day 35; however, this is not a period of rapid collateral development. Based on these studies, it appears that peptide-induced enhancement of collateral formation is most likely to occur during the period of most intense ischemia, when collateral growth is most active.

In accordance with prior studies using acidic FGF (60), myocardial infarction appears to prime the surviving vessels, increasing responsiveness to the proliferative effects of FGF. If ischemia is a mild form of injury, then all of these investigations lead to a common hypothesis: growth factors exert a synergistic effect with ischemia and/or injury, leading to a proliferative response. Upregulation of FGF receptors as a result of injury would account for this enhanced responsiveness to FGF, and would also explain why FGF has no apparent effect on vasculature in non-ischemic myocardium.

During the last decade, a number of angiogenic peptides has been characterized, and numerous models of experimental myocardial ischemia and infarction have been available during this period. Nevertheless, the published studies of the relationship between growth factor expression and myocardial ischemia or infarction can be counted on one hand. Investigations of the effects of exogenous growth factors on normal and ischemic myocardium are similarly limited. Thus, we are only beginning to unravel the biology of collateral development. The role of growth factors in the maintenance of vascular homeostasis and adaptation to disease states is extremely complex, and its elucidation will provide an important area of research for many years to come.

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12

INTERACTIONS OF THE CORONARY AND COLLATERAL CIRCULATIONS

Konrad W. Scheel, Howard Mass, and James T. Gean
Texas College of Osteopathic Medicine, Department of Physiology,
Fort Worth, Texas, U.S.A.

INTRODUCTION

In this chapter we examine the blood flow interactions between the coronary and collateral circulations and analyze the implications of these interactions as they change or modify the characteristics of the individual circulations. To accomplish this, we will present experimental evidence and use a computer model simulation to explain and enhance our understanding of the operative mechanisms involved.

Based on recent work from our laboratory, we will reexamine the pressure-flow relationships of the *coronary* and *collateral* circulations, present evidence supporting the contention that intramural collaterals dominate the collateral circulation in the native dog heart, explore the hemodynamics of collateral blood flow in hearts with mature collaterals, and consider the influence of *venous* pressure (right atrial or coronary sinus) on the coronary and collateral circulations. We have chosen to highlight the mechanism(s) involved rather than attempt to explore the subject matter exhaustively. For additional details the reader can avail himself of the underlying referenced publications.

It is important to recognize that our experimental observations were conducted on an isolated heart with severed neural connections, during steady state conditions that exclude capacitance effects, and during relatively constant metabolic demands by allowing the heart to pump against atmospheric pressure by venting the ventricles. We would like to emphasize that certain fundamental mechanisms operate in the intact and/or isolated heart preparation. It is these fundamental mechanisms that are the focus of this chapter. The isolated heart preparation allows the researcher greater latitude in controlling extrinsic factors that may have confounding effects due to interactions of the heart with the peripheral circulation. The experiments that have led to the observed results would be practically impossible to carry out without the isolated heart preparation.

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

We recognize that our knowledge of the coronary and collateral circulations is incomplete, and since our model simulations are based on our current knowledge and can not be better than what is known, they too will be incomplete. The model, however, allows certain predictions and extrapolations that require further experimental explorations for confirmation.

The retrograde flow measurement

Recognition of the interactions between coronary and collateral blood flow probably has its beginnings with the experiments by Anrep and Hausler (1). These investigators occluded one of the coronary arteries, cannulated the vessel distal to the occlusion, allowed it to bleed against atmospheric pressure, and measured the amount of effluent. This procedure is known as the *retrograde flow method* and is shown in figure 1. From these experiments they concluded that the source of retrograde blood flow had to be from anastomotic connections between the occluded vessel and other coronary vessels. Because the blood collected from the occluded vessel was well oxygenated, it was presumed that the anastomotic connections originate from arteries of donor vessels, artery - to - artery connections.

Early investigators recognized two important questions: 1) Does the collected retrograde flow represent the entire flow through the anastomotic connections? 2) Is the collected retrograde flow representative of the flow that would be available to the coronary artery during coronary occlusion? To answer the first question, one must consider that during a retrograde flow measurement, flow through the collaterals is impeded by the resistance of the proximal coronary artery, Rcp in figure 1, on which the collaterals terminate and the coronary resistance Rc2. These resistances can be viewed as being in parallel with each other during a retrograde flow measurement because each terminates at a common point namely atmospheric pressure (if the right atrial pressure is considered to be 0 mmHg). If Rc2 is much larger than Rcp, and Rcp is much smaller than the collateral resistance, Repi, the retrograde flow can be considered to be a good representation of the potential collateral flow. The following discussion will show that this condition does not always exist and furthermore, that the conditions change. Thus, the question whether retrograde flow represents the entire flow through the anastomotic connections, overestimates (2, 3, 4), or underestimates (5, 6, 7, 8) collateral flow has been the subject of much controversy.

To answer the second question, one must consider that during coronary occlusion, collateral flow is impeded by the resistance of the coronary vessel, Rc2 in figure 1, assuming right atrial pressure is fixed at 0 mmHg. Therefore, whether retrograde flow overestimates or underestimates the blood flow available to the coronary artery depends in large measure on whether the magnitude of the resistance Rcp is greater or less than Rc2. Because the coronary resistance, Rc2, changes under various hemodynamic conditions, there is no simple answer.

The dynamics of the coronary resistance (Rc2)

It can be argued that during coronary occlusion, Rc2 is very small, specifically, equal to the resistance measured during maximal vasodilation, because coronary occlusion produces myocardial ischemia leading to vascular dilation and minimum vascular resistance. Experimental support for this supposition can be sought in the

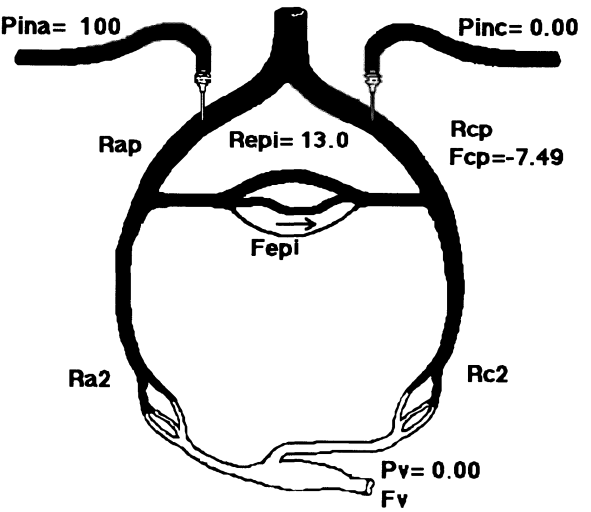

Fig. 1. The retrograde flow measurement. The left side represents the left anterior descending coronary artery, subscripted by "a". The right side represents the circumflex coronary artery, subscripted by "c". Pin = Input pressure. The capital letters R, F, and P indicate resistance, flow, and pressure, respectively. Repi represents the resistance of epicardial collaterals. The subscript "v" represents the venous circulation. When the numerical value for flow, F, is preceded by a negative, (-), sign, this indicates that the flow is retrograde rather than antegrade. The subscript "p", as in Rap and Rcp, represents the proximal or large coronary vessels.

observation that when the occlusion is released, coronary flow is very high, as demonstrated by the hyperemic response.

However, it has been demonstrated microscopically that microvessels, after coronary occlusion or at low perfusion pressures, neither collapse nor maintain a diameter consistent with maximum vasodilation. Instead, they are partially constricted, perhaps due to the influence of myocardial tissue pressure (9). We used the model to gain a better understanding of the problem.

In figure 2 the resistance Rc2 was given the value calculated at maximum vasodilation. For collateral resistance, Repi, we substituted a value obtained from retrograde flow measurements between the left anterior descending and circumflex coronary arteries (10, 11). A coronary occlusion was simulated by substituting a large value for the proximal coronary resistance, Rcp=9999. The model calculated a pressure distal to the occlusion, P3, of 2.95 mmHg. This value is not consistent with measurements obtained by other investigators (12, 13, 14) or in our laboratory.

To test whether the collateral resistance value was incorrect, we changed Repi until P3 became equal to the experimental results generally obtained. Figure 3

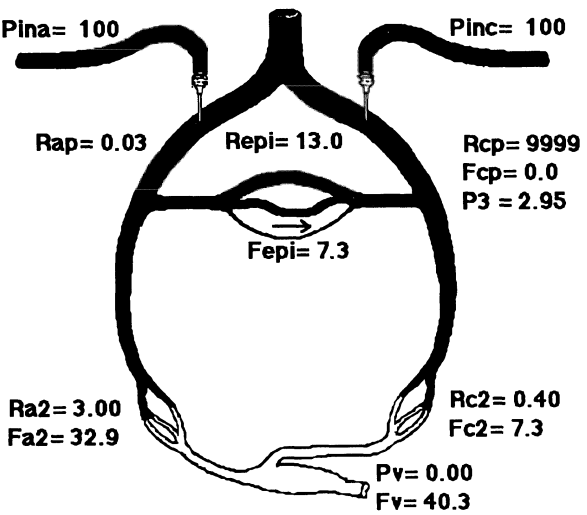

Fig. 2. Model simulation for the determination of peripheral coronary pressure, P3, with the assumption that coronary resistance, Rc2, assumes the resistance at maximum vasodilation. The model demonstrates that a peripheral coronary pressure of 2.95 mmHg is inconsistent with experimentally obtained results.

demonstrates the result of this simulation. We note that the collateral resistance necessary to produce the experimentally observed peripheral coronary pressure of 16.3 mmHg is so low that flow through the coronary vessel became 40.8 ml/min., a value equal to or greater than that seen in a normal, resting, autoregulating dog heart. This would imply that coronary occlusion does not produce ischemia, another inconsistency. We concluded that the resistance of the coronary artery during coronary occlusion had to be larger than the resistance at maximal vasodilation to produce results consistent with experimental observations.

This conclusion was verified in our experiments in which we measured coronary inflow at various coronary perfusion pressures during maximal vasodilation and calculated the resistance. We found that coronary resistance was small and constant over a wide range of perfusion pressures, but increased exponentially at pressures less than about 20 mmHg (15). This resistance did not become infinite at 0 mmHg perfusion pressure, but reached a maximum value of 3 mmHg/(ml/min). The results suggested that during maximum vasodilation the coronary resistance, Rc2, is a function

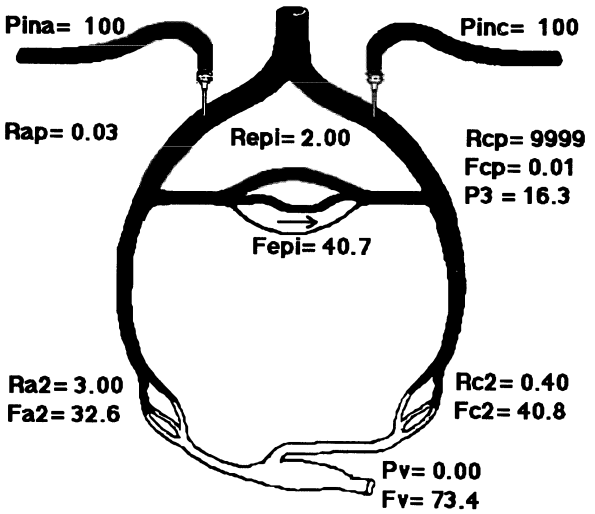

Fig. 3. Model simulation for the determination of peripheral coronary pressure, P3, after adjusting the collateral resistance, Repi, to produce a peripheral coronary pressure consistent with experimentally obtained results, while maintaining the assumption that coronary resistance is maximally vasodilated during coronary occlusion. We note that the coronary flow, Fc2=40.8 ml/min, is inconsistent with flow measurements obtained with coronary occlusion in dog hearts with native collaterals.

of the perfusion pressure in the low pressure range. The reason for this phenomenon could be attributed to an interaction between the pressure within the vasculature and myocardial tissue pressure which is unmasked at low perfusion pressures.

Figures 2 and 3 demonstrate that, following coronary occlusion, the pressure P3, often referred to as the *peripheral coronary pressure or PCP*, is not only dependent on the coronary resistance Rc2, but also dependent on the collateral resistance. In the native dog heart collateral resistance is high and P3 is small because the ratio between coronary resistance/collateral resistance is relatively small. In a well collateralized dog heart, P3 is high and can be near the value of the perfusion pressure. When the collateral resistance is small, collateral flow in a resting dog can be sufficient to maintain normal coronary perfusion. And, with very well developed collaterals, the coronary resistance, Rc2, can be high and become the flow limiting element in the collateral dependent circuit because of autoregulation in the resting heart. When the metabolic demand on the myocardium is increased, as during the stress of exercise, Rc2 can become small and the collateral resistance, once again, becomes the flow limiting element. Parenthetically it may be noted that coronary reserve, when collateral dependent, can never be identical to non-collateral dependent perfusion (flow through the original vessel) unless collateral resistance is 0.

In summary, coronary resistance, Rc2, changes dynamically during autoregulation, during vasodilation, and after acute or chronic coronary occlusion.

The dynamics of the coronary collateral resistance (Repi)

The preceding section focused primarily on the variability of the coronary resistance, Rc2. In the following discussion we would like to examine the changes that can occur in the collateral vasculature and the interactions between the coronary and collateral vessels.

In the dog heart with native collateralization no vasomotor activity is ascribed to the collateral circulation because true collateral vessels consist of a single layer of endothelial cells and lack a muscular coat. With collateral growth, the architecture of a collateral vessel changes and the vessel more closely resembles that of a normal coronary artery. The influences to which mature collateral vessels respond are covered in greater detail in the chapter authored by Dr. Harrison. In our discussion the collaterals will be viewed as conduit vessels serving the purpose of supplying the microcirculation of the collateral dependent bed. These conduit collateral vessels exhibit vascular growth (16)

by either de novo generation of vessels or by increasing the diameter of preexisting collateral vessels. The latter mechanism has been confirmed and collateral vessels can assume diameters as large as those of normal coronary arteries. The resistance of the collateral vessels between the left circumflex and left anterior descending coronary artery can decrease from an average of 13 to 0.3 mmHg/(ml/min) in dogs with mature collaterals. The stimulus for growth of collaterals is largely thought to be associated with myocardial ischemia (10, 17, 18, 19, 20), though the contribution of blood flow velocity to collateral growth has not been thoroughly investigated (18).

Under non-pathological conditions, the average pressure at both ends of the collateral bed is similar, although pressure gradients between the two ends can develop during the cardiac cycle giving rise to a small back-and-forth flow through the collateral vessels. However, with coronary occlusion, a net flow in the direction of the occluded vessel can be observed. In this situation the collateral vessels become one element in a series circuit consisting of the collateral resistance and the coronary resistance. And, blood flow through a series circuit must be the same in each element of the circuit. In a series circuit (often referred to as voltage divider network) the pressure (voltage) between the two series elements is proportional to the resistance ratio of the two elements. From figures 2 and 3 it can be observed that the pressure between the series elements can be calculated as P3 = Pina x (Rc2/(Repi+Rc2)). From the equation it can be seen that P3 depends on the magnitude of the collateral resistance and the resistance of the vessel distal to the occlusion. The peripheral coronary pressure, PCP or P3 in the model, was thought to be a good index of collateralization (13, 21), because it was assumed that the myocardium distal to the occlusion was ischemic, the arterioles maximally dilated, and Rc2 was small and constant. In the preceding discussion it was pointed out that Rc2 changes dynamically and non-linearly, and hence, the two variables (peripheral coronary pressure and collateral resistance) are not linearly related.

The influence of collaterals on the coronary pressure-flow (P-F) relationship

The function of the collateral resistance as part of a voltage divider network has been largely ignored in determinations of coronary pressure-flow, P-F, relationships. In this procedure, the input pressure to a coronary vessel was lowered while inflow to the vessel was measured on the proximal coronary artery. With decreasing inflow pressure,

it was found that coronary inflow was zero at a positive perfusion pressure. This point on the pressure-flow relationship was termed *pressure at zero flow or PZF* and attributed to the "Waterfall" phenomenon (22), which states that myocardial compressive forces acting on coronary vessels exceed the pressure within these vessels, particularly in the deeper layers of the myocardium, causing a cessation of coronary blood flow below a certain critical pressure. Coronary P-F, experiments were performed in our laboratory in a similar manner to those by other investigators. However, instead of lowering the pressure on a single coronary artery, the pressure to all coronary arteries was lowered simultaneously while inflow was measured on the proximal coronary artery.

The P-F relationships obtained in this manner exhibited no positive pressure intercept at zero flow, but instead intercepted the pressure axis at its origin (see "No

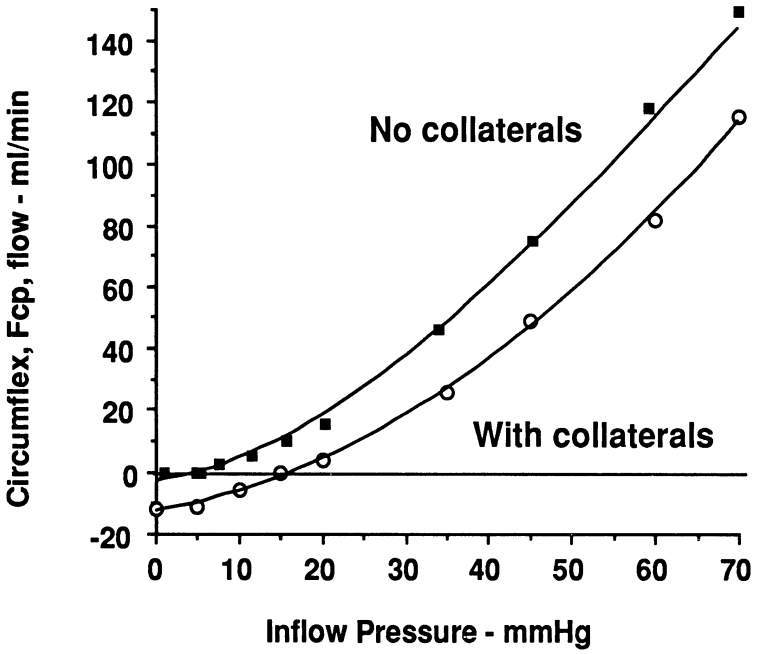

Fig. 4. Experimentally obtained pressure-flow relationship of the left circumflex coronary artery. The graph marked "No collaterals" was obtained while the perfusion pressure to all coronary vessels was changed simultaneously. The graph marked "With collaterals" was obtained when the perfusion pressure to only the left circumflex was changed while the pressure to the remaining vessels was maintained constant. In the latter graph, inflow was negative (retrograde) at pressures less than about 16 mmHg.

collaterals" in Fig. 4). When these experiments were repeated by changing the inflow pressure on only a single vessel while maintaining a constant pressure on the remaining vessels, the P-F relationship displayed a positive pressure intercept of about 16 mmHg (see "With collaterals" in Fig. 4). It occurred to us that in the first situation, when the pressure to all coronary vessels was reduced simultaneously, the role of the collateral circulation was practically eliminated because there was no pressure gradient across the collaterals. In the second situation, when the pressure to only a single vessel was reduced, the role of the collateral circulation was optimized and a pressure gradient existed between the origin of the collaterals (other vessels were perfused at constant input pressure) and the vessel on which the P-F relationship was obtained. These experiments led us to the conclusion that the collateral circulation can be involved in an apparent positive pressure intercept (15). (Parenthetically it should be noted that when, because of technical difficulties, the heart became edematous and myocardial tissue pressure was elevated, the pressure-flow relationship was shifted to the right. That is, the pressure intercept became non-zero).

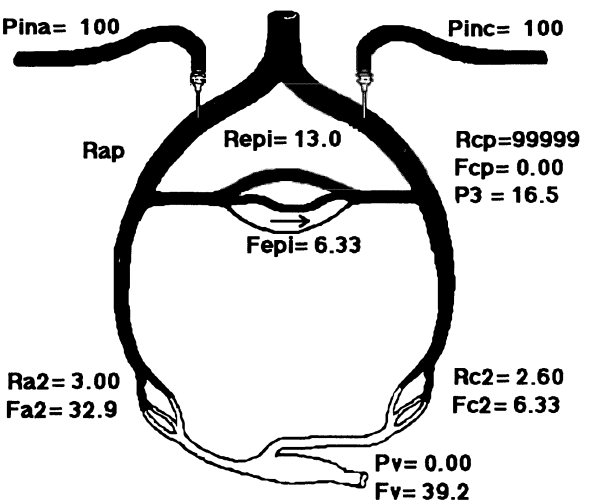

Fig. 5. Model simulation of a coronary occlusion, Rcp=9999 mmHg/(ml/min). The model demonstrates that proximal coronary vessel flow, Fcp=0, while flow through the microcirculation of the same coronary vessel Fc2= 6.33 ml/min.

The model can provide further insight into an understanding of the mechanisms involved. In figure 5, proximal coronary occlusion is simulated by allowing the circumflex resistance Rcp to become very large (9999 mmHg/(ml/min)). We note that the entire collateral flow, Fepi=6.33 ml/min, is diverted antegrade through the coronary artery (see Fc2=6.33). The pressure P3=16.5 is the peripheral coronary pressure due to the voltage divider properties of the collateral resistance in series with the coronary resistance. We note that circumflex inflow is zero, Fcp=0, but flow through the microcirculation of the left circumflex coronary artery or venous out-flow is not zero.

When a pressure-flow procedure is performed on the circumflex coronary artery and input pressure is lowered to 16.5 mmHg, as shown in figure 6, we note the following: 1) When the input pressure is equal to the voltage divider pressure produced by the series connection of the collateral and coronary vasculature, the proximal circumflex flow, Fcp, becomes zero. 2) The flow through the microcirculation and venous flow from the circumflex is, however, not 0, but equal to the collateral flow seen with coronary occlusion, demonstrated in figure 5. The condition common to coronary

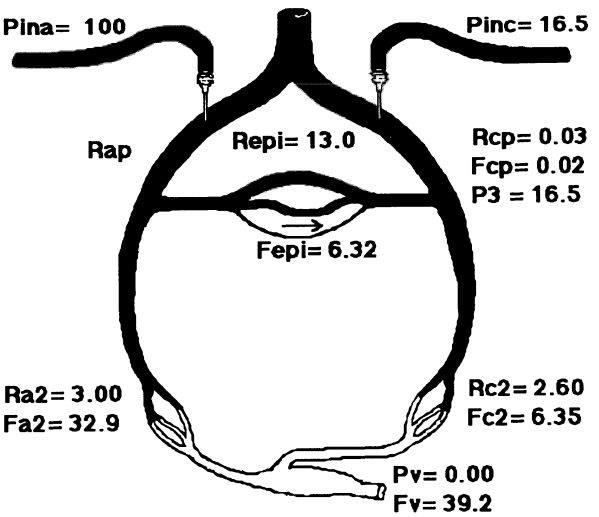

Fig. 6. Model simulation of a coronary pressure-flow relationship when perfusion pressure, Pinc=16.5 mmHg, the "voltage divider" pressure. Proximal coronary vessel flow, Fcp=0, while flow through the microcirculation of the same coronary vessel is 6.3 ml/min. These results are identical to those of figure 5 although the conditions are different.

occlusion (Fig. 5) and a pressure-flow determination when inflow pressure is 16.5 (Fig. 6) is that inflow is 0 ml/min. Thus, the peripheral coronary pressure, PCP, is identical to the pressure at zero flow, PZF. This fact, though without explanation, was observed experimentally by Messina (23).

In the computer model, we can remove the influence of the collateral circulation by replacing Repi with a very large resistance (Repi=9999 mmHg/(ml/min) in Fig. 7). We note that now the inflow, Fcp, becomes non-zero and equals the flow through the microcirculation, Fc2. Collateral flow, Fepi, is also 0. Although it is not possible to remove all collateral vessels experimentally, collateral flow can be essentially eliminated by maintaining the inflow pressure to all coronary vessels identical. Under those conditions, there would be no pressure gradient across the collateral vessels and collateral flow would be 0. This was the experimental procedure we followed with the results shown in figure 4 (no collaterals).

In summary: In the determination of the coronary pressure-flow relationship we were able to reproduce a positive pressure intercept of about 16 mmHg, as seen by other investigators (23, 24, 25, 26), when the pressure-flow procedure was performed on a

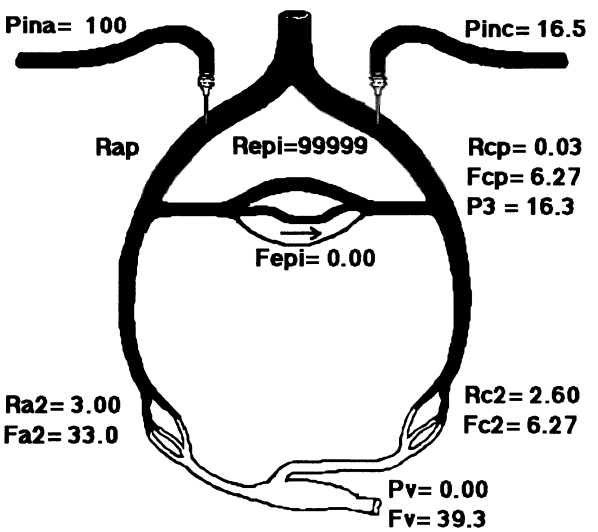

Fig. 7. Model simulation of removal of epicardial collaterals, Repi=9999 mmHg/(ml/min). Removal of collaterals results in a non-zero inflow, Fcp and Fc2=6.27 ml/min, while the remaining conditions are the same as those shown in figure 6.

single vessel while the inflow pressure on the remaining vessels was maintained constant at 100 mmHg. This condition emphasizes the influence of the collateral circulation on the coronary pressure-flow relationship by producing a pressure gradient, hence collateral flow, to the coronary circulation. However, we were also able to demonstrate that the coronary pressure-flow relationship passes through the origin of the axis when the influence of the collaterals was removed by eliminating the pressure gradient across the collaterals.

The role of coronary venous pressure

A strong counter-argument to our conclusion can be raised when one considers the P-F experiments conducted during long diastoles (26, 27). Under such conditions, it can be argued, that the pressure to all coronary vessels was lowered simultaneously and coronary flow also ceased at a positive pressure.

However, additional experiments from our laboratory have shown that the P-F relationship intercepts the pressure axis at almost precisely the pressure of the coronary sinus (28). In other words, the coronary sinus pressure can determine the pressure intercept of the P-F relationship shown in figure 8. This graph demonstrates that the P-F relationship is shifted to the right when coronary sinus pressure is raised from 0 to 20 mmHg. When the experiments with long diastoles are considered in light of our experiments, it can be argued that although cardiac output during long diastoles is zero, venous return continues, at least transiently, and increases the right atrial pressure. The P-F relationship, in this case, would demonstrate a positive pressure intercept, not because of the collateral circulation, but because of the elevated right atrial or coronary sinus pressure.

Although our experiments were performed in the absence of vasomotor tone, we would like to address its possible role in modifying the pressure intercept of the coronary P-F relationship. With sympathetic stimulation coronary resistance Rc2 can increase from 20 to 70% (29, 30, 31). Under the "Waterfall" concept, which is based on the assumption that vessels collapse, the P-F intercept would be shifted to the right. It has been shown that vessels do not collapse (9), not even when the coronary sinus pressure is as low as -40 mmHg (32). The most likely reason that coronary vessels do not collapse is that they are tethered to the myocardium (33). Thus, an increase in coronary resistance only would not affect the pressure intercept, but change the slope of the line. This leaves the question why Bellamy et al (27) observed a high pressure

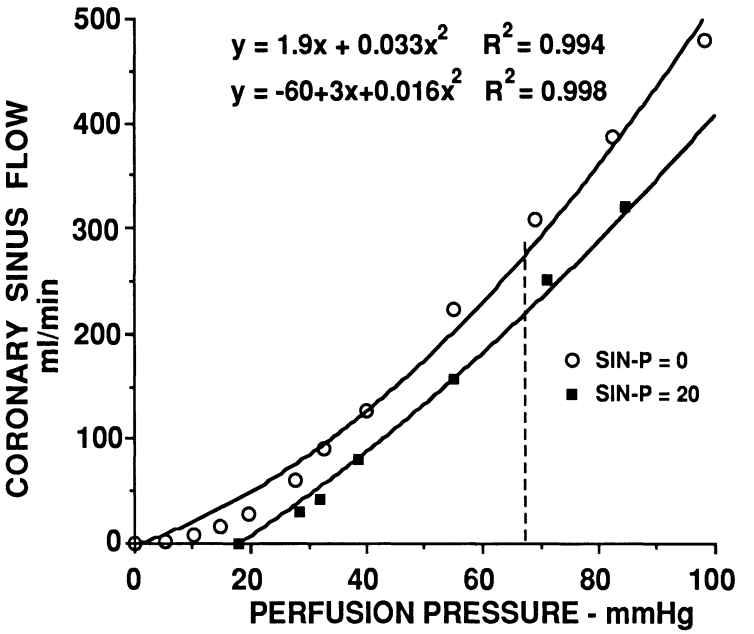

Fig. 8. Experimentally obtained pressure-flow relationship of the left circumflex coronary artery when coronary sinus pressure was 0 mmHg (open circles) and when coronary sinus pressure was 20 mmHg (squares). When coronary sinus pressure was 20 mmHg the zero-flow pressure intercept occurred at 20 mmHg. Also shown in this graph is that, for any given perfusion pressure (about 68 mmHg on graph), coronary flow is reduced when coronary sinus pressure is 20 mmHg compared to when coronary sinus pressure is 0 mmHg.

intercept in P-F experiments during long diastoles. If a decreasing aortic pressure would activate sympathetic stimulation, then central venous pressure would not only increase because venous return continues, but *venous* constriction could further add to an elevation in central venous pressure, thus increase the pressure intercept of the P-F relationship.

It has been reported that when it is more difficult for coronary blood flow to drain into the atrium than the ventricle, it drains into the ventricle (34). Thus, Thebesian drainage could be considered a "venous drainage by-pass" during elevated right atrial pressure. If the Thebesian drainage would constitute a large proportion of coronary drainage, then a venous back-pressure would not develop to reduce coronary flow during elevated right atrial pressure. Since 70 to 80% of left coronary inflow drains into the coronary sinus, hence the right atrium (28, 35), the Thebesian system could, at

best, drain 20% of coronary inflow and reduce the venous back-pressure by that same amount. However, it has been shown by Gregg that the Thebesian system does not play a major role in venous drainage (35). If the Thebesian system assumed a major role in coronary drainage, then we would not have been able to obtain such a close correlation between coronary sinus pressure and the pressure intercept of the P-F relationship in our experiments (28). We estimate that Thebesian drainage by-pass could reduce the venous back-pressure by about 1 mmHg. In the intact dog, it is unlikely that there would be much pressure difference between the right atrium and right ventricle during diastole, hence, the Thebesian contribution could be presumably even smaller.

In summary: Depending on the method used to examine the coronary pressure-flow relationship, a positive pressure P-F intercept can be obtained when the collateral resistance is not taken into account, the right atrial pressure is greater than 0 mmHg, or when the heart is edematous.

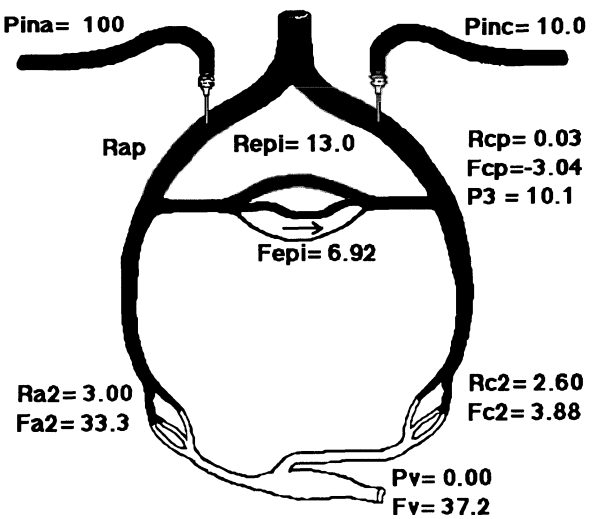

Fig. 9. Model simulation of the back pressure method, Pinc=10 mmHg/(ml/min), for the determination of the collateral pressure-flow relationship. With this method a large portion of the collateral flow, Fepi=6.92 ml/min, is diverted antegrade, Fc2=3.88 ml/min.

The influence of the coronary vasculature on the collateral pressure-flow relationship

To study the pressure-flow relationship of the collateral circulation, the following protocol was used by other investigators (14, 25). The retrograde outflow tubing was adjusted to different heights, changing the height of the column of blood in the tubing, thus, the back-pressure to retrograde flow. Retrograde flow was determined at various back-pressures. This method is reproduced on the model shown in figure 9. In the model, the back-pressure is assumed to be 10 mmHg. We note that the retrograde flow, Fcp, is -3.04 ml/min, while the actual collateral flow is 6.92 ml/min. Closer observation indicates that a portion of the collateral flow is diverted through the circumflex coronary artery, Fc2=3.88 ml/min. From previous discussions it is clear that when the back pressure is raised to 16.5 mmHg, collateral flow will stop (Fcp=0 ml/min) because the condition is identical to that used to study the pressure-flow relationship of the circumflex coronary vessel (Fig. 6). Again, it is the voltage divider mechanism that dictates the condition when retrograde flow will become 0 ml/min. Figure 4 (with collaterals) illustrates that during a pressure-flow protocol

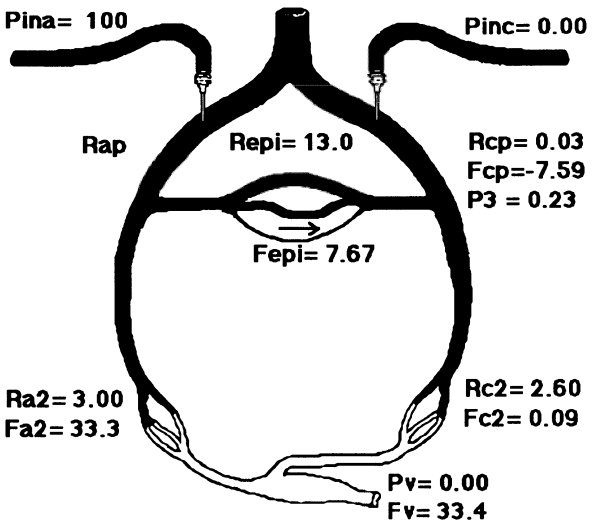

Fig. 10. Model simulation of the method to determine the collateral pressure-flow relationship in which antegrade flow diversion of collateral flow was largely eliminated, Fc2=0.09. ml/min

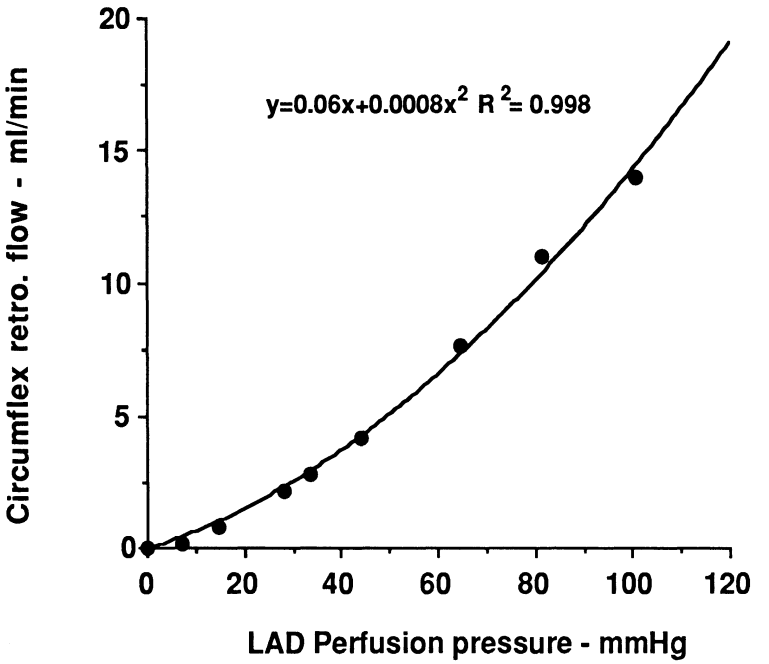

Fig. 11. Experimental pressure-flow relationship of the total coronary collateral vasculature. The zero-flow pressure intercept occurred at 0 mmHg. LAD = left anterior descending coronary artery.

when the inflow pressure was lowered to a value less than 16 mmHg (or less than the pressure dictated by the voltage divider network), the forward flow changed direction and became retrograde flow (15).

To avoid this problem and isolate the influence of the coronary circulation on the collateral circulation to determine its intrinsic characteristics, we used the following method. Retrograde flow was measured on the circumflex coronary artery while the back pressure was maintained at 0 mmHg, as shown in figure 10 (Pinc=0 mmHg). This pressure was maintained constant and it can be seen that the pressure to the peripheral coronary vessel was very low (P3=0.23 mmHg) and flow through the circumflex coronary artery was Fc2=0.09 ml/min. This procedure, in essence, isolated the collateral circulation from that of the coronary circulation. The inflow pressure to all coronary vessels, Pina, was lowered in steps while the retrograde flow was determined by timed collections. The resulting pressure-flow relationship of the collateral circulation is shown in figure 11. From the equation shown in the graph it can be seen that the curve can be fitted with a second degree polynomial with a small

non-linear component. Collateral flow was 0 ml/min at 0 mmHg pressure. With the back-pressure method "collateral" flow would have been 0 ml/min at a back-pressure of 16.5 mmHg (Fig. 6).

Intramural collaterals in the native dog heart

The existence of intramural collaterals at various levels in the native dog heart was described by Bellman and Frank with histological methods (36). In their report they conclude that most of the intramural collaterals are of microvascular dimensions (40 - 60 μm diameter) with occasional anastomoses in the 100 to 200 μm range. Thus, as epicardial collaterals can be of macrovascular, defined as vessels >100 μm in diameter, or microvascular dimensions (<100 μm), intramural collaterals can be similarly categorized (37). However, the functional significance of intramural collateral vessels to contribute blood flow to an occluded coronary artery was not demonstrated until 1973 (38). Although the work of Cibulski et al suggested the dominance of intramural collateral blood flow compared with epicardial collateral flow in the native dog heart, the experiments were performed on only a single diagonal branch of the circumflex artery. In their experimental procedure, epicardial collaterals in the perimeter of this

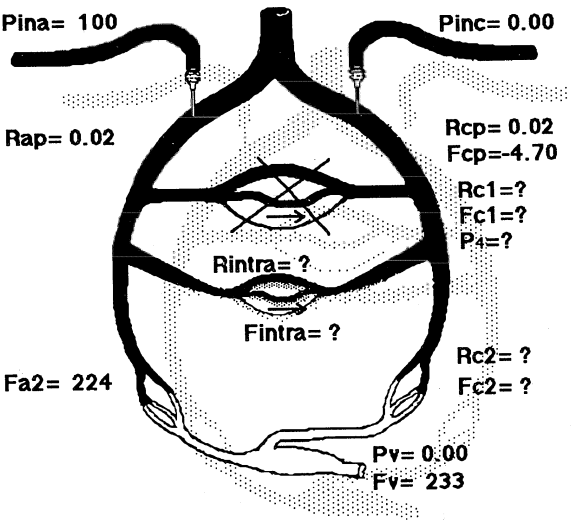

Fig. 12. Model simulation of the method for determining intramural collateral flow. Epicardial collaterals were removed by cauterizing the epicardial surface along the border of the left circumflex coronary perfusion territory. Retrograde flow, Fcp, was measured before and after cautery.

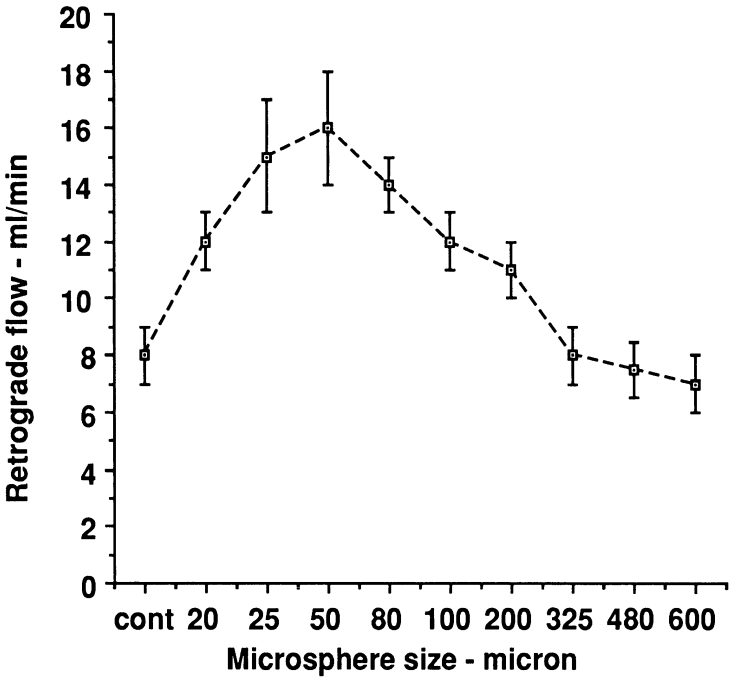

Fig.13. Experimentally obtained retrograde flows after embolization of the circumflex coronary artery with plastic microspheres of various sizes. Peak retrograde flow occurred with 50 μm spheres.

branch were ligated with superficial sutures and the elimination of epicardial collateral flow was verified by cineangiography. Because of the limited resolution of angiography, the possibility of microvascular epicardial collateralization could not be excluded.

In a recent work from this laboratory, the epicardial boundaries for the entire left anterior descending, LAD, right, and circumflex, LCX, coronary artery perfusion territories were cauterized to eliminate *all* epicardial interconnections (macro- and micro vascular anastomoses). Retrograde flow measurements were performed on the LCX before and after cautery during maximum vasodilation, and total intramural collateral contribution from all coronary vessels to the LCX were determined. We found that more than 50% of the retrograde flow was still present after cauterizing epicardial collateral vessels (39). This procedure is simulated in figure 12. We note that at the junction of intramural collaterals with the LCX, the intramural collateral flow separates into two directions, one portion is collected as retrograde flow (Fcp=-4.7 ml/min), the other component flows in the antegrade direction through the micro-circulation of the LCX (Fc2). These results raised several new questions, for

instance: What is the flow through the intramural collaterals, Fintra ? What is the resistance of intramural collaterals, Rintra, compared to epicardial collateral resistance ? What are the resistance values Rc1 and Rc2; and how much of the intramural collateral flow is diverted antegrade through the coronary circulation, Fc2 ?

Our first objective was to determine the magnitude of the entire intramural collateral flow. We reasoned that if the antegrade flow component of intramural flow could be blocked, it would be directed retrograde and could be collected during a retrograde flow measurement. It was recognized that in an embolization procedure a sphere size had to be chosen that would block the antegrade flow component without interfering with the retrograde flow component. To accomplish this objective, multiple sphere sizes were chosen for the embolization procedure (40). The results are shown in figure 13 and illustrate that retrograde flow increased during embolization with smaller microspheres and declined with larger sphere sizes. Maximum retrograde flow was obtained with 50 μm diameter spheres. The data further demonstrate that embolization with 50 μm spheres resulted in a doubling of retrograde flow. In the model shown in figure 14, the values identified with a question mark in figure 12 were adjusted in such a manner as to produce consistent results obtained by the two experimental procedures

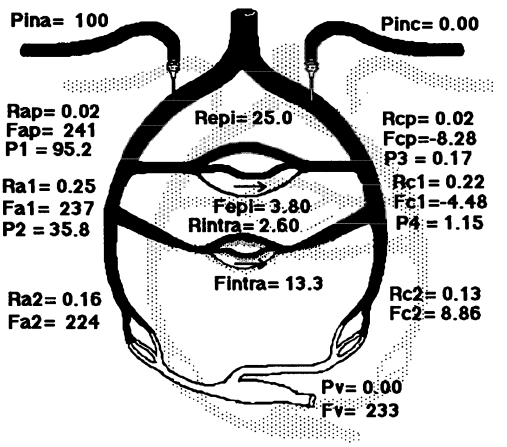

Fig. 14. Model simulation of a retrograde flow measurement demonstrating the distributions of epicardial and intramural collateral flows to retrograde and antegrade flow. Resistances proximal (Rc1, Ra1) and distal (Rc2, Ra2) to the junction of intramural collaterals with the parent vessels are also shown. Note that intramural collateral resistance, Rintra, is almost 1/10 that of epicardial collaterals, Repi. However, because the pressure at the origin of intramural collaterals is relatively small (P2=35.8 mmHg), its collateral flow is only 3.5-fold larger than epicardial flow during a retrograde flow measurement.

(cauterization of epicardial collaterals and embolization experiments).

Figure 14 illustrates the distribution of resistances proximal and distal to the origin of intramural and epicardial collaterals in a heart with maximum coronary vasodilation during a retrograde flow measurement. There are several interesting observations: 1) The pressure at the origin of the epicardial collaterals (P1 or stem-pressure) is about 95 mmHg, 2) The pressure at the origin of intramural collaterals is about 36 mmHg, 3) Epicardial collateral resistance in the native dog heart is 25 mmHg/(ml/min), and 4) Intramural coronary collateral resistance in the native dog heart is 2.6 mmHg/(ml/min), almost 10-fold smaller than that of epicardial collateral resistance. However, because the pressure at the origin of intramural collaterals is small, its collateral flow is only 3.5fold larger than epicardial flow during a retrograde flow measurement. 5) The entire epicardial collateral flow component is represented in the retrograde flow measurement. 6) Intramural collateral flow contributes 54% (Fc1 = -4.48 ml/min) to total retrograde flow, 7) 67% of the intramural collateral flow is diverted in the antegrade direction (F_{c2} = 8.86 ml/min) through the microcirculation of the LCX. 8) Retrograde flow constitutes about 1/2 of the total collateral flow (8.28 ml/min in the retrograde direction and 8.86 ml/min in the antegrade direction). Summing these two flow components results in the doubling of the retrograde flow observed with embolization in figure 13.

Because the role of intramural collaterals in the native dog heart is controversial, we would like to address a recent work in which it was concluded that "The contribution of microvascular anastomoses to total collateral flow is likely small" (41). The investigators did not measure retrograde flows, as was done in our experiments, but the micro-circulation of the LCX was embolized with 25 μm spheres and during each embolization step, the peripheral coronary pressure, PCP, was measured. Because, total embolization of the circumflex with 25 μm spheres could not be achieved, the data were extrapolated to what PCP would have been, if total embolization had occurred. This maximum PCP pressure was equated with the pressure at the origin of the collaterals (stem pressure). Based on the observation that the measured PCP was 65 mmHg and the extrapolated PCP was 74 mmHg and that this pressure was very close to their perfusion pressure of 80 mmHg, the investigators concluded that the origin of collateral had to be on coronary vessels > 200 μm in diameter.

The above experimental conditions were simulated on the model shown in figure 15. In the model, Rc2 was given a very large value to indicate total embolization

to reflect the extrapolated data. Rcp was also given a large number to simulate coronary occlusion to allow the measurement of peripheral coronary pressure, P3. However, the only way the peripheral coronary pressure seen in their results could be obtained on the model was to consider the possibility that during the embolization of the LCX, injection of 25 μm spheres caused the spheres to travel through the collaterals, which are thought to have an average diameter of 40 μm (13), and partially embolized the LAD (Ra2=3.0 mmHg/(ml/min) in Fig. 15). This condition raised the stem pressure for the epicardial collaterals on the LAD (P1=79.5 mmHg) to near the perfusion pressure Pina=80 mmHg. Under this condition we note that the collateral stem pressure, P1=79.5 mmHg, is very near the peripheral coronary pressure P3=74 mmHg in figure 15. We note that the flow through intramural collaterals, Fintra, is in the opposite direction from that shown in figure 14. Thus, in this case, the peripheral coronary pressure formed a voltage divider network in which the epicardial and intramural collaterals formed the series elements in the circuit. Indeed, we note that the flow through epicardial, Fepi=0.2 ml/min, and intramural collaterals, Fintra=0.2 ml/min, is identical.

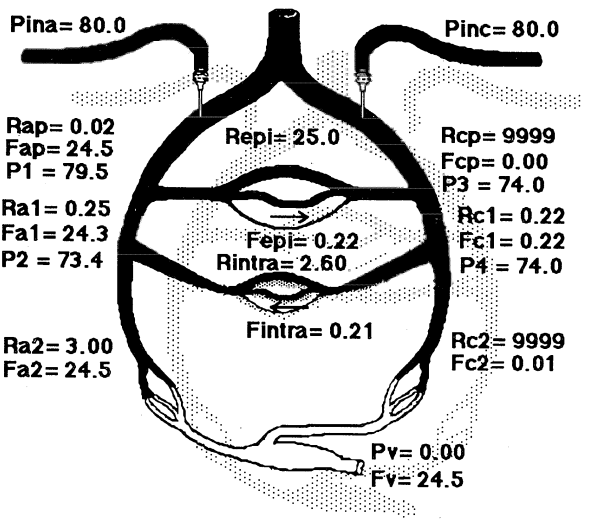

Fig. 15. Model simulation of the method in which the coronary vasculature, Rc2, was embolized and peripheral coronary pressures were measured for the determination of intramural collateralization (Harrison et al (41)). The simulation is based on the assumption that the LAD was inadvertently embolized (Ra2=3).

Embolization of other coronary vessels during microsphere injections could be immediately detected in our preparation because blood flows through all coronary vessels were monitored simultaneously. To avoid embolization of other vessels the following precautionary measures were taken: 1) Prior to embolizing a coronary artery a clamp was applied to the proximal LCX and flow was reduced to 1/2 of control. This favored the collateral flow in the direction of the vessel to be embolized. 2) Based on the observation that the peripheral coronary pressure, PCP, rises with embolization of the microcirculation (41), we reasoned that when all vessels of a given diameter were embolized, PCP would plateau. Since this indeed occurred experimentally, the criteria for injecting the next larger size spheres was established and avoided forced injection of spheres. We termed this method the "*pressure plateau method*". 3) Multiple sphere size injection had the further advantage of providing a more complete embolization of the vessel to about 3% of the control flow. (Both, Harrison's (41) and our laboratories share the experience that complete embolization cannot be accomplished with 20 μm or 25 μm diameter spheres). With these criteria for microsphere injections, the problem of embolization of other vessels was largely avoided.

Mature collaterals

The computer model was used to predict the epicardial and intramural collateral hemodynamics during coronary occlusion in dog hearts with mature collaterals (Ameroid occlusion of 3 months duration). The following assumptions were made: 1) Intramural collateral resistance, Rintra, was reduced to 1/4 of the resistance in native dog hearts. This assumption was based on our observations that collateral flow from the septal artery (intramural) to the LCX increased 4-fold with chronic LCX occlusion. 2) Total retrograde flow to the LCX was about 106 ml/min with 3 months Ameroid occlusion (11). Thus, epicardial collateral resistance was adjusted to 0.93 mmHg/(ml/min). The model predicts that retrograde flow underestimates total collateral flow by about 30% under those conditions. This percentage is smaller than that for native collaterals, but consistent with the impression that epicardial collateral flow dominates intramural collateral flow after chronic coronary occlusion.

To test our assumptions, the model was used to simulate the experiments of other investigators. Based on the assumption that control flow through the LCX is 45 ml/min, the model shows that, during a retrograde flow measurement, LCX flow is 71% of control flow (Fc2=32.5 ml/min in Fig. 16). This value is similar to 67%

observed experimentally by Downey et al in dogs with mature collaterals under similar conditions (42). Cibulski et al measured the retrograde flow on dogs instrumented with an Ameroid occluder for 2 months. The retrograde flow after ligating visible epicardial collaterals between the LCX and LAD was reduced to 23% (43). When their experiment was simulated in our model, the retrograde flow dropped to about 16%. Thus, the assumptions underlying our model attribute a larger retrograde flow contribution to epicardial collaterals compared to intramural collaterals. This assumption may be justified considering that our data were taken from dogs with 3 months Ameroid occlusion.

Some predictive aspects of the model

Although these could be numerous, we would like to focus our attention on the following presentation because of the potential clinical implications. In all of the preceding discussions we have assumed that right atrial or coronary sinus pressure was 0 mmHg. However, it is important to realize that coronary occlusion can lead to heart failure with a concomitant increase in right atrial pressure to as high as 40 mmHg (44, 45, 46). In the following discourse we will examine possible implications of increased

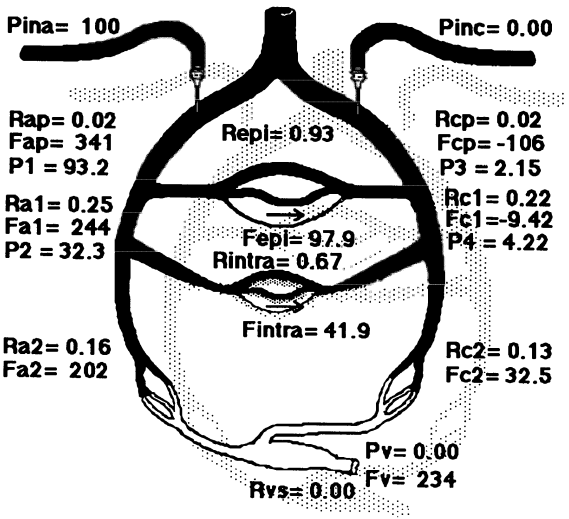

Fig. 16. Model simulation of collateral blood flow distributions during a retrograde flow measurement in dog hearts with mature *collaterals.*

right atrial pressure on coronary collateral flow.

In the model shown in figure 17 a coronary occlusion was simulated by substituting a large value for Rcp. Based on pilot studies, we assumed the venous resistance, Rvs, to be 0.01 mmHg/(ml/min). This results in a coronary sinus pressure of 0.39 mmHg during autoregulating flow conditions (this pressure would be about 5 mmHg with vasodilation). The right atrial pressure, Pv, was allowed to assume the value of 0 mmHg. We note that flow through the microcirculation of the LCX, is the sum of the epicardial and intramural collateral flows, Fc2=11.9 ml/min. We also note that flow through the LAD is Fa2=27.6 ml/min.

In figure 18 we assumed that congestive heart failure resulted in an elevation of right atrial pressure to 20 mmHg (Pv=20). We note that flow through the microcirculation of the LCX decreased to 9.5 from 11.9 ml/min, a 20% decrease. Thus, the model suggests that an increased right atrial pressure of 20 mmHg results in a 20% reduction in flow to the collateral dependent myocardium. Furthermore we note that the elevated right atrial pressure decreased coronary flow of the LAD from 27.6 ml/min (Fa2 in Fig. 17) to 22.1 ml/min (Fa2 in Fig. 18). This is also a 20% reduction in coronary flow.

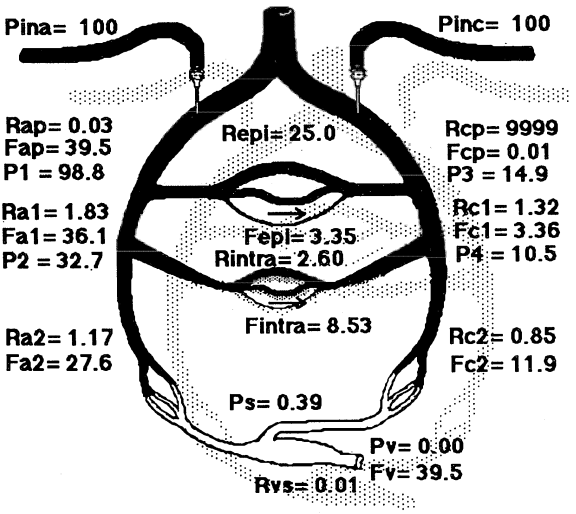

Fig.17. Model simulation of left circumflex coronary occlusion , Rcp=9999 mmHg/(ml/min). In this simulation it was assumed that coronary sinus resistance, Rvs=0.01 mmHg/(ml/min), right atrial pressure, Pv, was 0 mmHg.

Although we have shown that coronary flow decreases with increased venous pressure in the maximally vasodilated heart, the above result appears to be inconsistent with our expectations in an autoregulating heart. In accord with the metabolic theory for autoregulation one would expect a reduction in flow to result in an accumulation of metabolic vasodilator end-products with a subsequent return of coronary flow to control. However, elevation of venous pressure could result in an elevation of arteriolar pressure, from P2=32.7 mmHg in figure 17 to P2=46.1 mmHg in figure 18, resulting in a myogenic constrictor response to reduce flow. A decreased coronary flow with elevation of venous pressure is also consistent with the "tissue hypothesis" of coronary flow regulation. In accord with this theory, elevation of venous pressure would result in increased tissue pressure, vascular compression (increased arteriolar and venous resistance) and, hence, reduction in coronary flow. Preliminary experiments from our laboratory on autoregulating isolated hearts, indeed, show a reduction in coronary flow with increased right atrial pressure.

It could also be argued that in an unloaded isolated heart experiment (left ventricular pressure = 0 mmHg), the effect of an elevated left ventricular end diastolic pressure, LVEDP, is not taken into account. It is thought that increased LVEDP

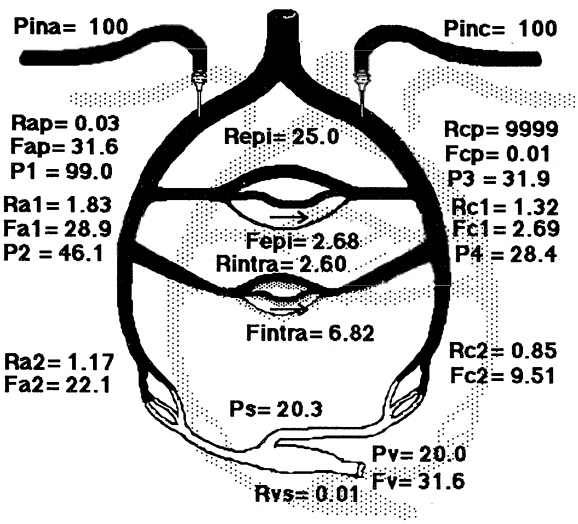

Fig. 18. Model simulation of left circumflex coronary occlusion , Rcp=9999 mmHg/(ml/min). In this simulation it was assumed that right atrial pressure was 20 mmHg, in contrast to the condition shown in figure 17.

reduces coronary flow (47, 48, 49, 50). However, in preliminary studies we separated the effects of right atrial pressure from LVEDP by placing a balloon in the left ventricle which allowed control of LVEDP independent of right atrial pressure. We found that increasing LVEDP to 30 mmHg had little effect on late-diastolic left coronary flow. On the other hand, when LVEDP was maintained constant and right atrial pressure was elevated, late-diastolic left coronary flow was significantly reduced.

In summary, model predictions although not totally substantiated, suggest that an increased venous pressure could reduce coronary and collateral flows. Each by itself could lead to a reduction in pump function of the heart during congestive heart failure; together they could introduce a positive feedback or vicious cycle that would continue to weaken the heart, resulting in a further elevation of right atrial pressure, hence, further reduction in coronary and collateral flows.

FINAL OBSERVATIONS

With the writing of this chapter we have come to the realization that the interactions between the coronary and collateral circulations can lead to rather complex hemodynamic consequences that are not readily apparent without the quantitative aspects of the computer model, particularly when the dynamics of chronic coronary occlusion and collateral growth are considered. Although this model, which was developed over several years, may not be totally accurate and may contain inconsistencies, it is a helpful tool in analyzing experimental results and exploring possible approaches to new questions and, in some instances, resolve apparent inconsistencies. It is our hope that we were able to demonstrate how questions posed by model solutions led to the design of experiments to verify predictions which, in turn led to modifications of the model and insights into mechanisms dictating the hemodynamic functions of the coronary and collateral circulations.

Acknowledgements: The authors are particularly indebted to Ms Gina Belsito for the art work and to Mark Jackson of the Medical Arts Department for his continued advice and help. And, without the competent technical support and counsel of Ms Sue Williams, this work would not have been possible.

Supported by the National Institutes of Health, NHLBI, and the American Heart Association, Texas Affiliate.

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13

EXERCISE INDUCED CORONARY COLLATERAL DEVELOPMENT: A COMPARISON TO OTHER MODELS OF MYOCARDIAL ANGIOGENESIS

Francis C. White, David M. Roth, M. Dan McKirnan, Susan M. Carroll and Colin M. Bloor
Department of Pathology , University of California, San Diego, School of Medicine,
La Jolla, California, U.S.A.

"Be not the first by whom the new are tried,
Nor yet the last to lay the old aside."
Alexander Pope

INTRODUCTION

Many investigators consider exercise to be a stimulus for coronary collateral development. According to Schaper (1) the primary factors affecting coronary collateral development include dilation secondary to hypoxia and increased tangential wall stress. Investigators still debate whether these factors occur during prolonged exercise stress. The effect of endurance exercise training, i.e. a decreased heart rate at a given work load, indicates more efficient cardiac function which implies less cardiac stress, thus reducing the stimulus for collateral development. However, chronic exercise training accentuates coronary collateral development when a critical coronary artery stenosis is present (2). To summarize the current knowledge of the interrelationship between exercise and coronary collateral development, we will analyze exercise's influence on angiogenesis in the heart, discuss different animal models of angiogenesis and indicate what similarities exist in these different models that merit further investigation.

Our discussion addresses the following topics: (1) exercise induced angiogenesis; (2) coronary collateral development in the pig; (3) angiogenesis and vascular remodeling in the bed at risk; (4) exercise and coronary collateral development in the ischemic pig heart; (5) angiogenesis in pig models of pressure overload hypertrophy; and (6) molecular biology approaches to vascular remodeling and collateral development. The first four topics review past and recent studies from our laboratory and other investigators addressing the general interrelationship between exercise and coronary

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

collateral development emphasizing the unique advantages of the pig model. The last two topics summarize recent studies from our laboratory and other investigators that indicate both differences and similarities during angiogenesis depending on the stimulus. Also these recent studies suggest what the focus of future studies should be.

Background

The development of coronary collateral vessels is advantageous for the myocardium when occlusion of a major coronary artery occurs or when a stenotic artery can no longer meet the blood flow demands of the myocardium. The quest for the mechanisms of this vessel growth has been ongoing in several laboratories for many years (1, 3, 4, 5, 6, 7, 8) and there is extensive literature on exercise induced collateral development (9, 10, 11, 12, 13). Early experiments in dogs, pigs, and rats established that blood vessels will grow when subjected to ischemia, although the mechanisms of this growth remain elusive. However, the recent technological achievements of molecular biology portend a bright future for elucidation of growth mechanisms (14). We have developed several models of myocardial angiogenesis in our laboratory and hypothesize that common mechanisms of angiogenesis occur in these divergent models. Endothelial and smooth muscle cells in the healthy heart rarely replicate. However, we found that replication of endothelial and smooth muscle cells and the development of new blood vessels occurs as a short but dramatic response to a variety of stimuli. Here we present our data and data of others that support our hypothesis.

EXERCISE INDUCED ANGIOGENESIS

For many years we have characterized the physiological and cardiac responses of pigs to acute and chronic exercise (15, 16). We have found, in these extensive experiments, that pigs adapt quickly to treadmill running. After 10 weeks of training at heart rates 80% of maximal (220-230 beats/min) for 50 minutes daily, the maximal oxygen consumption increases and average of 25% (8). In these studies we measured changes in cardiac dimensions and contractility and concluded that the heart adapts to chronic exercise primarily by increasing myocardial mass (+25%), end diastolic dimension and wall thickness. However, there is no increase in intrinsic contractility. Therefore, the effect of exercise training on the heart is one of increased stroke volume associated with a reduced peripheral resistance.

The goals of these studies were to determine the degree of blood vessel growth and to examine whether blood flow and minimal coronary resistance changes correlate with increases in arteriolar cross-sectional area. We also made morphometric measurements of blood vessels and compared them with blood flow measurements made using microspheres (17). Previously we determined minimal coronary resistance in exercising pigs by running the pigs to maximal heart rate and then infusing adenosine (1.25 mg/kg/min iv) (18). In that study, pigs exercising at maximal heart rates used approximately 88% of their maximal coronary reserve, and after infusion of adenosine, the pigs used 100% of their maximal coronary reserve. Using this protocol, we compared 8 pigs trained for 10 weeks to 8 untrained pigs, and determined whether exercise training could increase maximal perfusion capacity of the heart and whether the changes correlated with blood vessel growth. Table 1 shows the morphometric data and the calculated minimal coronary resistances. The morphometric data presented describes arterioles ranging 25-75 μm in internal diameter. The number of arterioles increased significantly (+56%) while minimal coronary resistance significantly decreased (-9%). This is the first study that directly measured coronary reserve to show

	LV/BW (g/Kg)	Capillary Density #/mm^2	Arteriolar Numerical Density** #/mm^2	Minimal Coronary Resistance (mmHg/ml/min/g)
Normal (n=8)	3.21 ± 0.08	2550 ± 110	2.42 ± 0.20	13.9 ± 0.6
Trained (n=8)	3.74 ± 0.09*	2653 ± 119	3.78 ± 0.19*	12.6 ± 0.5*
10 weeks	14% ↑	4% ↑ NS	56% ↑	9% ↓
Trained 10 weeks (n=8)	4.34 ± 0.14*	2181 ± 96*	3.39 ± 0.23*	13.0 ±0.6
Mild Hypertension	26% ↑	-14% ↓	40% ↑	6% ↓ NS
Trained 3 weeks (n=5)	3.25 ± 0.09	2840 ± 120*	2.65 ± 0.30	13.7 ± 0.5
	1% ↑	11% ↑	10% ↑ NS	1% ↓ NS

Table 1. Exercise induced angiogenesis. Vascular morphometry and minimal coronary resistance
Values are means ± SEM
Adapted from White et al (8) and Breisch et al (17) and unpublished data trained 10 weeks, mild hypertension
LV/BW = Left ventricular weight (g) / body weight (kg)
** $p<0.05$ compared to normal; NS = not significant; **Arterioles 25-75 μm in diameter*
Trained pigs were exercised daily at 70% of their maximal HR for 30 min to 1 hour
Minimal coronary resistances measured during near maximal heart rates

that exercise significantly improves the perfusion capacity of the heart. In another group of exercise trained animals that were mildly hypertensive, left ventricular (LV) weight increased (+26%), the number of arterioles increased (+40%), but the minimal coronary resistance remained unchanged, likely due to the level of hypertrophy. However, total blood flow to the left ventricle increased, indicating blood vessel growth was adequate to maintain coronary blood flow reserve. Tomanek et al (19) reported similar results in hypertensive dogs, i.e., LV mass increased (+46%), the number of arterioles increased and minimal coronary resistance decreased slightly. However, total blood flow to the heart increased proportionately to the increase in left ventricular weight. Thus, coronary angiogenesis occurs in left ventricular hypertrophy but coronary reserve changes depend on the degree of hypertrophy.

We also investigated capillary angiogenesis in these models (Table 1). Various investigators have used capillary density as an index of myocardial angiogenesis (20, 21, 22). To determine the flow reserve of the capillary bed we made measurements in the left anterior descendens (LAD) coronary bed using an isolated perfusion system (23). We perfused the coronary artery with a pressure dependent auto-regulating pump and injected 6.5 μm diameter latex beads into the artery until coronary reserve, measured during adenosine infusion, decreased. Coronary reserve did not decrease until at least 5,000 beads per mm^3 of tissue lodged in the capillaries. If all capillaries are parallel and about 70 μm long, we estimate that one can plug 20-25% of the capillary bed before capillary reserve decreases. The capillary reserve may be even higher if the capillaries are in a mixed series or with parallel elements. We measured minimal coronary resistance in pigs after 3 weeks of chronic exercise training (Table 1). These pigs

	# of Collaterals 20-60 μm ID	# of Collaterals 60-120 μm ID	# of Collaterals 120-180 μm ID	Total # (Mean)
Control (n=3)	115 ± 19	NO	NO	115
Ameroid 8 Weeks (n=3)	202 ± 25*	46 ± 9	8 ± 3	256
Ameroid and Trained (n=3)	188 ± 22*	55 ± 7	14 ± 2 +	257

Table 2. Coronary collateral growth.
Mean values ± SEM. ID = internal diameter; NO = none observed. Adapted from ref. 31.
*Ameroid animals had an ameroid placed on the LC artery for 5 weeks; trained animals also were exercise trained for 5 weeks. *P < 0.05 compared to control; + P < 0.05 comparing occluded trained to occluded.*

showed a significant increase in capillary density (+11%) but their minimal coronary resistance did not change. Thus, capillary density is not an index of perfusion capacity. However, capillaries are the critical precursor vessels in the development of arterioles. Finally in animals chronically exercised for 10 weeks and rested for 6 weeks we observed that the numerical density of arterioles returned to control levels. This indicates that like coronary collaterals (24, 25), arterioles also can regress. In conclusion, exercise induced angiogenesis has been shown to occur and has positive physiological consequences.

Coronary collateral development in the pig

The use of the pig model to study development of coronary collaterals has a long but intermittent history (7, 8, 26, 27). The pig has few innate coronary collaterals, thus abrupt occlusion of a pig coronary artery either results in death or a large infarct (28). Millard (27) found that by banding the LAD in piglets and occluding the artery gradually, tissue preservation occurs and collaterals develop. Knowing that the left circumflex (LC) coronary bed in the pig is only 25% of the left ventricle and that innate coronary collateral blood flow is low (0.05 ml/min/g) (29), we reasoned that coronary collaterals may grow fast enough to preserve this relatively small segment of the myocardium in an ameroid (gradual) occlusion model. Our initial studies showed that infarct size in this model averaged 7% of the bed at risk (8). The coronary collateral circulation developed to a level that provided normal coronary blood flow 2 weeks after occlusion. Also transient ischemia of the myocardium during exercise in this model persisted for at least 16 weeks after coronary artery occlusion (30). This exercise induced under-perfusion primarily occurs in the endocardium and mid myocardium with the epicardial region showing normal blood flow. In other studies we have measured coronary flows during 6 months of coronary artery occlusion. In those animals there was almost no change in exercise induced regional under perfusion. Thus we have a static model of persistent exercise induced ischemia.

These findings prompted an in depth examination of the anatomy of these coronary collaterals correlated to minimal coronary collateral resistance over a period ranging from 0 to 16 weeks after coronary artery ameroid closure. Tables 2 and 3 present data from coronary collateral morphometric studies and minimal coronary collateral resistance measurements (31) including measurements from animals subjected to exercise training after the onset of gradual coronary occlusion. In the first study we

counted coronary collaterals and measured their internal diameters and wall thicknesses in 3 control pigs and 3 pigs 8 weeks after ameroid placement (Table 2). We identified coronary collaterals using injection masses of 3 different colors, one for each coronary artery (32). In these studies we quantitated DNA synthesis by ^{3}H-thymidine autoradiographs (Figs. 1A and 2A). In control animals we identified an average of 115 coronary collaterals per animal. These vessels averaged 40 μm in internal diameter and were present around the periphery of the LC bed. They had thin walls compared to arterioles of the same size. Average wall thickness of the coronary collaterals was only 50% of wall thickness of normal arterioles. Most collaterals were in the endocardium

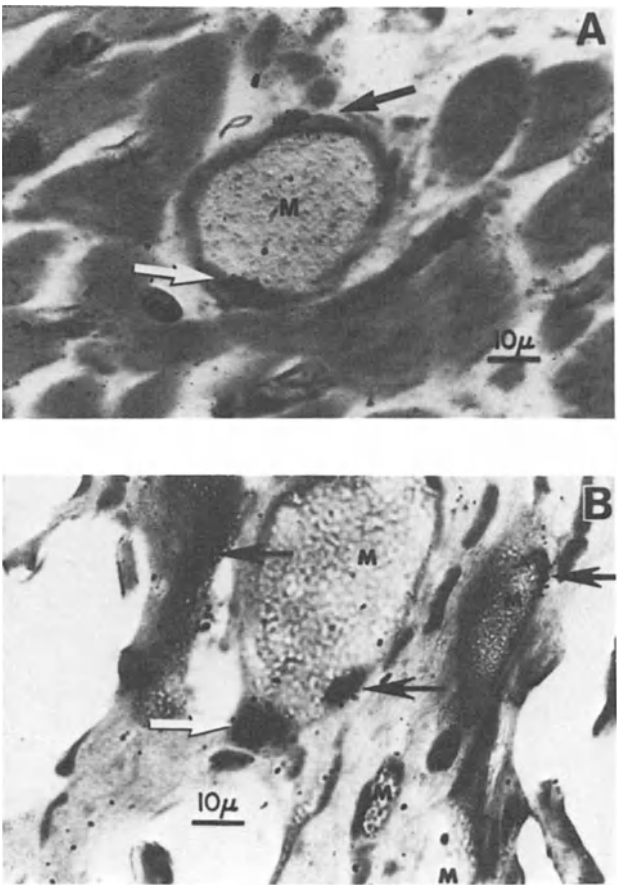


Fig. 1. Photomicrographs of coronary and collateral vessels showing ^{3}H-thymidine labeling during DNA synthesis, 3 weeks after ameroid placement on the LC coronary artery. We gave this animal ^{3}H-thymidine before euthanasia and injected the arteries with Microfil to identify collateral vessels and arterioles. Panel A shows a 50 μm vessel with a labeled endothelial cell nucleus (white arrow). The black arrow points to a labeled smooth muscle cell and an endothelial cell nucleus in proximity to each other. Panel B shows the LC bed at risk in the same pig. Numerous endothelial cell nuclei are labeled (black arrows). The white arrow points to a very enlarged labeled nucleus at the confluence of 3 small vessels. The entire bed at risk showed this marked DNA synthesis in blood vessel cell nuclei was observed in the entire area at risk.

and mid myocardium and very few were in the epicardium. At times, the collaterals were found grouped in clusters, particularly in the posterior papillary muscle. After 8 weeks of gradual ameroid occlusion, the number of coronary collaterals increased to an average of 256 vessels per animal. These vessels had larger diameters ranging to a maximum of 180 μm (Table 2), nevertheless they remained thin walled and lacked smooth muscle development. Transmural distribution of the collaterals in experimental animals was similar to that of the control animals. At later times (6 months) we saw little continued growth of these coronary collaterals either in number or size.

Figures 1A and 2A show ^{3}H-thymidine labeling of coronary collaterals in animals from these groups. In figure 1A, we show an autoradiograph of a 50 μm coronary collateral vessel 8 weeks after ameroid placement. The thin walled vessel shows DNA synthesis occurring in both an endothelial cell and a smooth muscle cell. Control cells have a mitotic index (number of labeled cells expressed as percentage of total cells measured) of 0.01%, as shown in figure 2A. In contrast, the index for endothelial and smooth muscle cell DNA synthesis increased dramatically to about 0.6% in the coronary occluded animals. Over time this index drops dramatically, and at 6 weeks it is similar to control levels. While our measurements focused on vessels larger than 20 μm in diameter, it was obvious that numerous smaller collaterals existed. In small sections (1 mm^2) of normal tissue we observed as many as 6 small collaterals (<20 μm diameter) in the periphery of the LC bed. After coronary artery occlusion we visualized as many as 25 of these vessels in a similar size region near the posterior papillary muscle.

These data strongly suggest that the innate collaterals play an important role as precursors to new collateral vessels (33). During the 2 days when the coronary artery goes from critical closure to complete closure, new blood vessels need to grow or existing vessels need to expand to meet the demands of the myocardium. Considering the limited time it is likely that expansion of pre-existing vessels is the predominant mechanism. However, we have identified new blood vessels sprouting as capillaries and developing into venules and arterioles. It is noteworthy that, when this increased collateral growth ceases, there are still numerous small collaterals present that contribute little to the flow reserve and do not grow further to meet the demands of a stressed myocardium. The questions as to why certain vessels grow and others do not grow are important domains for future analysis. In addition, an explanation of the

down regulation of collateral growth will be crucial to our overall understanding of angiogenesis in the heart.

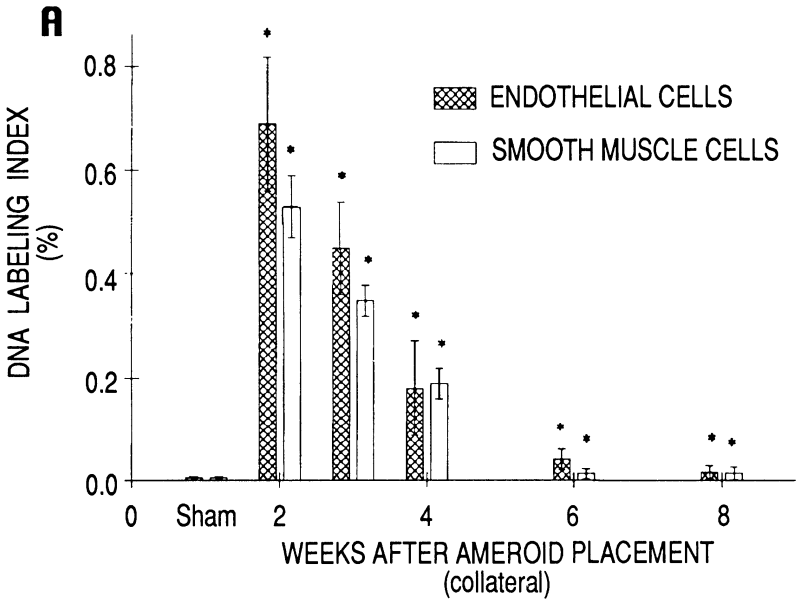
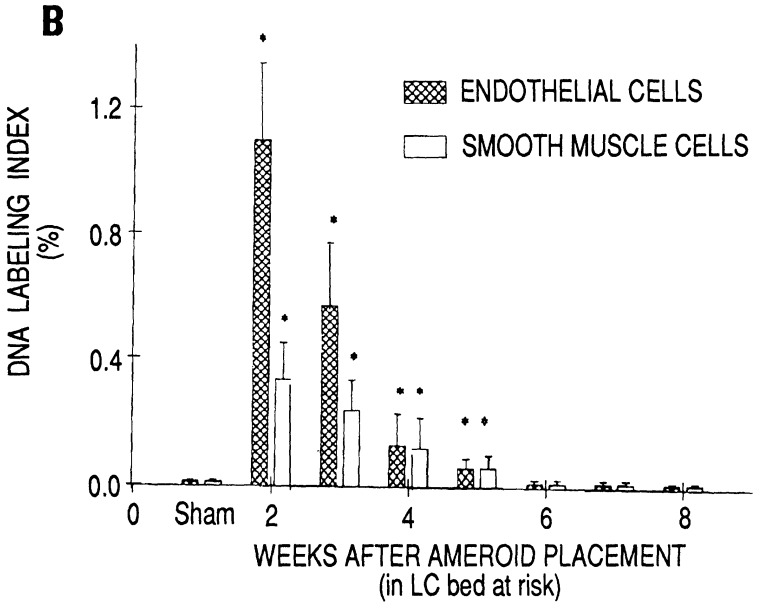


Fig. 2. A quantitative mitotic index of endothelial and smooth muscle cells as a percentage of the total cells observed. Panel A shows the labeling of coronary collateral vessels, 2,3,4,6, and 8 weeks after ameroid placement. Ameroid closure occurs at approximately 2 weeks. Labeling in a sham animal was about .01 %. There is a highly significant ($p < 0.05$) increase in the index at 2 weeks that rapidly diminished by 6 or 8 weeks. Panel B shows the labeling index for the bed at risk of these pigs, excluding the coronary collateral vessels shown in Panel A. The time course for the labeling is very similar to the index for the collateral vessels. The one striking difference is that the endothelial cell index for these vessels is much higher than for the collateral vessels at 2 weeks. This was due to the very high labeling index for capillaries in the bed. By 3 weeks the indices were similar due to diminished capillary endothelial cell DNA synthesis. Panel C shows the mitotic labeling for endothelial and smooth muscle cells 4, 8, 14, 30 and 60 days after pulmonary artery banding. The right ventricular weights had nearly doubled by 3 weeks. We maintained the right ventricular overload at nearly 100 mmHg systolic pressure through the 8 weeks, but there was no additional increase in right ventricular weight after one month. The labeling index and the time course for DNA synthesis are very similar to the ameroid model bed at risk. All values, means ± SE, are from 3 pigs at each time point except the sham animals.*


B
ENDOTHELIAL CELLS
SMOOTH MUSCLE CELLS
DNA LABELING INDEX (%)
1.2
0.8
0.4
0.0
0
Sham
2
4
6
8
WEEKS AFTER AMEROID PLACEMENT
(in LC bed at risk)

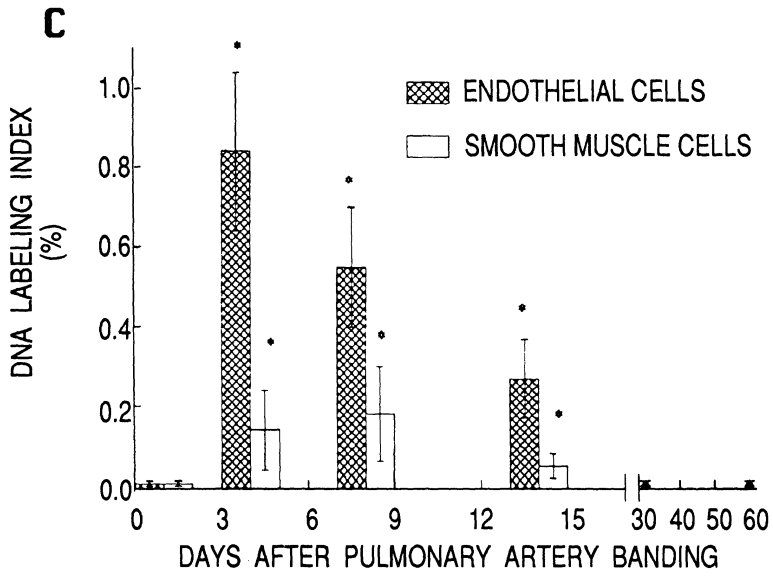

C
ENDOTHELIAL CELLS
SMOOTH MUSCLE CELLS
DNA LABELING INDEX (%)
1.0
0.8
0.6
0.4
0.2
0.0
0
3
6
9
12
15
30
40
50
60
DAYS AFTER PULMONARY ARTERY BANDING

Table 3 shows minimal coronary collateral resistance before and after exercise training in pigs with ameroid occlusion of the LC artery. In the normal coronary bed during brief occlusion of the LC artery, minimal coronary collateral resistance exceeds 2,000 mmHg/ml/min/g. This contrasts markedly with the normal levels of minimal coronary resistance, i.e., about 14 mmHg/ml/min/g (Table 1). By 5 weeks minimal coronary collateral resistance had decreased to about 52 mmHg/ml/min/g in the endocardium and 20 mmHg/ml/min/g in the epicardium. However, the minimal coronary collateral resistance was still significantly higher than control minimal coronary resistances of 14 mmHg/ml/min/g in normal animals (Table 1). Minimal coronary collateral resistance measurements at 10 weeks were not different from 5 week measurements.

In a separate series of experiments (33) we have shown that the initial level of innate coronary collateral flow correlates to the developed minimal coronary collateral resistance. This data corroborates the findings of Yamamoto and colleagues (34) in a dog model of repetitive occlusion. They suggested that the initial level of coronary collateral development is predictive of the final development of coronary collaterals. All of these data show that coronary collateral development in the pig heart is extraordinary, but it occurs over a limited period and is suppressed before complete restoration of collateral reserve occurs.

	5 Weeks		10 Weeks	
	MCCR ENDO	MCCR EPI	MCCR ENDO	MCCR EPI
Normal (n=8)	-	-	14.1 ± 0.06	13.0 ± 0.05
Untrained Occluded n=10	51.7 ± 11.2 +	20.0 ± 3.1 +	55.6 ± 8 +	18.5 ± 2.0 +
Trained Occluded n=9	46.0 ±6.3 +	19.1 ± 3.2 +	28.9 ± 6.4 *+	14.4 ± 2.3 *

Table 3. Exercise induced coronary collateral development. Minimal coronary collateral resistance (MCCR)
Values are means ± SEM. Adapted from Roth et al (30) and White et al (8).
**P < 0.05 compared to 5 weeks; + P < 0.05 compared to normal.*
Minimal coronary resistance measured during severe exercise (mmHg/ml/min/g)

Angiogenesis and remodeling in the bed at risk

We investigated the coronary bed distal to the site of ameroid occlusion to determine if blood vessel remodeling had occurred in the LC bed. To quantify infarct size in the bed at risk, we did routine histochemical surveys of formaldehyde perfused tissue, and noted a marked increase in small blood vessels in the jeopardized tissue compared to normal tissue. We tested the hypothesis that minimal coronary resistance would decrease in the jeopardized myocardium distal to the ameroid occlusion (23). In 8 control and 9 ameroid pigs occluded for 2 weeks, we used a pressure-controlled pump cannulated distally to the LC bed during maximal adenosine vasodilation to measure minimal coronary resistance. In figure 3 we show that at 100 mmHg perfusion pressure maximal flow in the jeopardized bed increased more than 3-fold while minimal coronary resistance decreased 3fold. To characterize the model we measured the number of arterioles and capillaries and measured minimal coronary resistance in 9 pigs 5 and 10 weeks after coronary occlusion (31). In table 4 we show data from the bed at risk of these pigs, 5 weeks after coronary occlusion. The numerical densities of arterioles and small muscular arteries, 25-75 μm in internal diameter, increased more than 100%. The calculated increase in their cross-sectional area agrees with the measured vasodilated flow increase of 3-fold. In figures 1B and 2B we demonstrate the marked increased in

	LV/BW g/Kg	Capillary Density #/mm^2	Arteriolar Numerical Density** #/mm^2
Normal n=8	3.21 ± 0.08	2550 ± 110	2.42 ± 0.20
Ameroid Occluded n=9	3.1 ± 0.15	2740 ± 160 (Bed at Risk)	5.15 ± 0.65* (Bed at Risk)
Ameroid Occluded Trained n=3 (unpublished data)	3.1 ± 0.34	2899 ± 170* (Bed at Risk)	4.84 ± 0.84* (Bed at Risk)

Tab.4. Ischemia and exercise induced blood vessel growth in the bed at risk
Values are means ± SEM.
Ameroid occluded group 10 weeks of ameroid placement. Ameroid trained group 5 weeks of ameroid placement followed by 5 weeks of exercise training.
Values for ameroid animals are taken from the collateral dependent bed at risk.
*$P < 0.05$ compared to normal animals. **Arterioles 25-75 μm in diameter.*

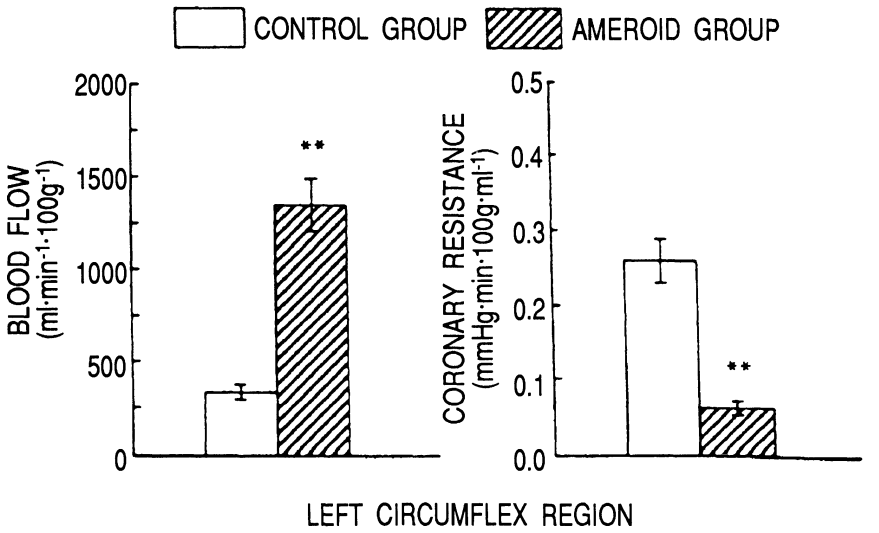


Fig. 3. Blood flow and calculated minimal coronary resistance are shown for 9 pigs 2 to 3 weeks after ameroid closure and 8 control pigs. We generated these data using a pressure-controlled pump cannulated to the LC artery during adenosine infusion. We measured blood flows both with a calibrated pump and radio-labeled microspheres. There is a remarkable increase in blood flow and an accompanying decrease in minimal coronary resistance in the bed at risk. These data strongly suggested an increase in the cross-sectional vessels in the bed (Roth et al (23)).

^{3}H-thymidine labeling in small blood vessels in the bed at risk and the similarity of the mitotic indices between the bed at risk (Fig. 2B) and the developing collateral circulation (Fig. 2A). This indicates that the up and down regulation of DNA synthesis in the bed at risk and the collateral circulation occurs at identical times. We also looked at animals 10 weeks after coronary occlusion. While the decreased minimal coronary collateral resistance was still present and an increased number of blood vessels was still there, the number of vessels had decreased from the 5 week levels and minimal coronary resistance had increased. These changes meet the criteria for blood vessel remodeling (35).

In assessing the status of the coronary capillary bed in this model we note an increased mitotic index in endothelial cells (Fig. 2B), including those of the capillaries. However, there was no increase in the number of capillaries after 5 weeks of exercise training (Table 4). We propose that there is progressive enlargement of capillaries into small arterioles and venules simultaneously with obliteration of capillaries that do not receive maintenance blood flow. Since the capillary bed's cross-sectional area is not

limiting and the stimulus for maintenance of the unused capillaries is not present, one would expect that capillaries are self limited by the level of blood flow perfusing them.

In figure 4 we show a photomicrograph of a cross-sectional view of the blood vessels in the LC bed in a control pig (top panel) and in a pig subjected to ameroid occlusion for 8 weeks (bottom panel). There is a dramatic increase in the number of small vessels after ameroid occlusion. Interestingly they seem to have replaced the

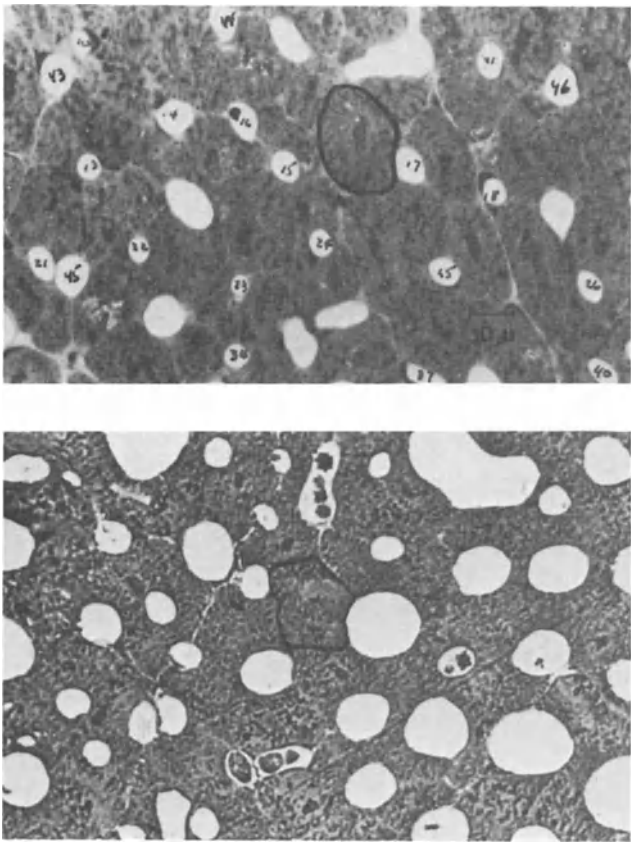


Fig. 4. A photomicrograph of the a normal LC coronary artery bed (top) and a bed 8 weeks after ameroid occlusion (bottom). These hearts were perfusion fixed at 100 mmHg. In the ameroid animals there is a dramatic increase in the number of small vessels, 8-40 μm in diameter. There was no increase in the number of capillaries in the ameroid animals, although the capillaries were larger. In the upper panel the capillaries are numbered for identification. A myocyte is circled in both panels and appears to be of similar size. The upper panel has a 10 μm bar. Both photographs were made at the same magnification.

capillaries so that overall there was no increase in the number of capillaries.

To further document the growth of blood vessels in the jeopardized myocardium, we injected a silicone elastomer into an ameroid occluded bed (8 weeks) and into the LC bed of a normal heart of the same weight (Fig. 5). In panel A we see marked development of blood vessels in the jeopardized bed compared to the normal LC bed. The weight of the ameroid cast was 55% greater than control indicating vessel growth. Also, the blood vessels clearly grew in length as well as in number. The open white arrows point out regions where there are large numbers of coronary collateral vessels. Görge et al (36) previously noted this "bloom" of new blood vessels using angiography studies.

In summary, there is a marked increase in blood vessels in the jeopardized myocardium and this increase in blood vessels coincides with the growth of coronary collaterals that supply the bed. This growth of small blood vessels correlates with a dramatic decrease in minimal coronary resistance. The results suggest a similar mechanism stimulating growth in both coronary collaterals and blood vessels in the

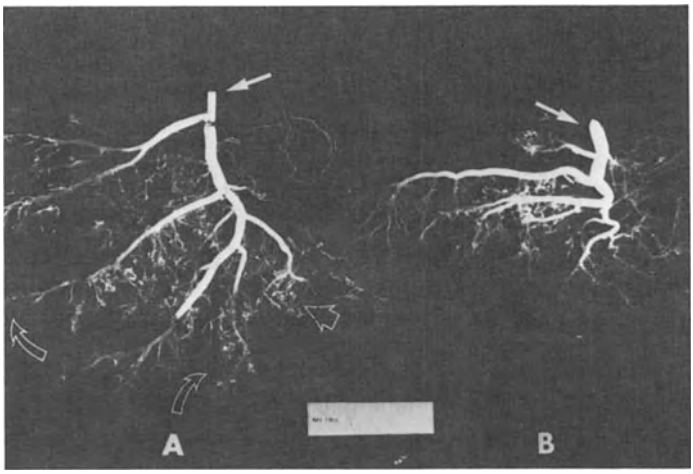


Fig. 5. Silicone elastomer casts of the LC coronary artery bed in a pig following 8 weeks of ameroid occlusion (A) or a normal pig (B). The pigs were of the same body weight and left ventricular weight. There is a remarkable growth to the bed at risk that appears to result in an increase in the length of the arterioles as well as increased numbers of small vessels. The white arrows indicate the point of ameroid closure. The open arrows point to regions where there are large numbers of coronary collateral vessels. These collateral vessels are not present in the control animal. The rule at the bottom of the photograph is 3 cm long.

jeopardized region. Furthermore, there is strong evidence for coronary remodeling in the bed at risk.

EXERCISE INDUCED ANGIOGENESIS IN THE ISCHEMIC PIG HEART

The majority of animal studies have shown that some improvement in coronary collateral blood flow occurs after exercise training in hearts with occluded or stenotic blood vessels (2, 10, 11, 30, 37, 38, 39). There are notable exceptions however (40, 41) and the human studies are not compelling. This may be because the methods of evaluating improvement in heart function by collateral growth are indirect and to some degree subjective (12, 13).

Several studies have evaluated collateral growth in normal animals beginning with the classical study of Eckstein (37). There is consensus that exercise training does not improve coronary collateral growth or blood flow in the normal heart (15, 37, 42). In one study from our laboratory (15), we exercise trained our animals for 10 months. We know of no other study that trained animals for that time. We used radiolabeled microspheres to measure collateral flow. The data showed no differences in collateral flow during coronary occlusion between a control group and an exercised trained group. These data suggest that ischemia is necessary for the development of new vessels or a perfusion pressure differential between adjacent beds must be present to stimulate the potential for collateral blood flow. The presence and level of blood flow may be important to stimulate angiogenic factors upregulated through exercise, ischemia or other potentiators of angiogenesis.

We have tested the potential of coronary collateral growth in pigs with a critical coronary stenosis of the LC, both with and without 5 months of exercise training after ameroid placement on the LC (2). The critical stenosis in these animals promptly closed, resulting in a completely occluded coronary artery and relatively large infarcts. Pigs without exercise training had infarct sizes more than 60% of the bed at risk, while exercise trained pigs had infarcts half that size. Coronary collateral flow in these studies was significantly higher in the trained group. In retrospect it was clear that these animals developed infarcts soon after the application of the critical stenosis and the exercise training was only effective in influencing infarct size and critical collateral development over a very short time. These studies emphasize the critical timing of collateral growth when there is a rapid stenosis of a coronary artery in a species with small naturally developed coronary collaterals. It is interesting to speculate how any

porcine myocardium survives with acute occlusion. In another study (28), we acutely occluded the LC artery in conscious pigs and found that in some pigs more than half of the myocardium survived and nearly normal resting flows were present in this surviving tissue, while in other animals 80% of the LC bed was infarcted. The difference between the two groups was due to different levels of innate collateral development. In subsequent studies where we acutely occluded the LC artery in uninstrumented pigs about 20% of the bed at risk survived. The rational for the survival of this tissue is that all the collateral flow available in the larger piece of tissue redistributes to a smaller piece of myocardium. Furthermore it is likely that even sparse, small innate vessels can rapidly dilate to increase blood flow to a level needed for tissue survival. These data suggest that coronary collaterals in the pig are capable of very rapid but limited dilation and proliferation.

Recently we described collateral blood flow and function (sonomicrometry wall thickening) in trained and untrained pigs following ameroid occlusion (30). This study is the first to correlate myocardial function and blood flow with coronary collateral development in exercised trained animals. Some details of this study are referenced in the chapter by Dr. Longhurst and Dr. Symons. The goal of the study was to exercise pigs with a collaterally dependent jeopardized bed. This bed would become ischemic during exercise and the level of ischemia could be monitored to maintain a constant level. From our previous study (43), where we measured blood flow and systolic wall thickening during graded exercise in ameroid occluded pigs, we knew that we could exhaust collateral blood reserves at heart rates of 220 - 240 beats per minute. Furthermore, the collateral dependent bed was incapable of responding to vasodilation using nifedipine or adenosine. This is in marked contrast to exercising ameroid occluded dogs, who respond to nifedipine (44). Therefore we could elicit a nearly maximal level of ischemia at heart rates of 75% of maximal (225 b/m). We exercised the animals 50 min/day for 5 weeks for a total exercise time of 930 minutes. Also we measured function and blood flow in a graded stress test before and after exercise training over 5 weeks (Table 5) (30). Blood flow in the collateral dependent region increased during severe exercise (+38%). This flow increase was greater in the endocardial region than in the epicardium. Wall thickening in the same animals increased (+29%), while resting function decreased (-25%). This decrease in resting function while resting flow is constant suggests myocardial stunning (45). Since infarct

		Rest	Moderate Exercise	Severe Exercise
Trained Group (n=10)				
Collateral Development	pre	42.4 ±4	26.5 ± 4	18.4 ± 3
Left Circumflex Bed	post	33.8 ±4 * ↓	29.0 ± 3	23.6 ± 4 * ↑
Sedentary Group (n=10)				
Collateral Development	pre	44.3 ±5	26.4 ± 4	19.0 ± 3
Left Circumflex Bed	post	39.5 ±5	29.9 ± 5	19.2 ± 4
Control (n=9)				
Left Anterior	pre	40.3 ±10	34.6 ± 6	30.9 ± 8
Descending	post	39.5 ± 5	39.3 ± 9	33.6 ± 11
Heart Rates	pre	87 ± 5	208 ± 2	263 ± 4
(Beats/Min)	post	85 ± 5	208 ± 3	264 ± 3

Table 5. Regional myocardial function during exercise
Values are means ± SEM **p<0.05 comparing post to pre*
Pre=beginning of training or sedentary period *post=end of 5 weeks of training or rest*

sizes in the trained animals were very similar it is unlikely that the loss of resting function is the result of infarct enlargement. Clearly if ischemia is the major stimulus, we maximized this potential. Our results showed that there remained a 40% decrease in function and flow reserve even after training. Additional support for these findings include the following observations: 1) measurements made when the animals were half way through their training showed no functional improvement; 2) functional improvements occurred in a graded manner when looked at on a week by week basis; 3) each animal showed the same changes, i.e., the function-flow curves of most animals had similar slopes (see chapter by Dr. Longhurst and Dr. Symons, Fig. 8 and (30)); and 4) when analyzing the function-flow curves, the most ischemic animals did not improve any more than the least ischemic animals arguing that ischemia is not the only stimulus for collateral development. One also can argue that if the exercise continued longer then there would be more improvement. However several arguments disagree with this conclusion. In an animal exercised for 5 months (2) we saw no more improvement in

blood flow than what we observed in the present study. Furthermore, all models of angiogenesis that we have studied suggest that the major growth of blood vessels occurs within a few weeks of the stimulus even when the stimulus continues. This is true of many stimuli including exercise, myocardial hypertrophy, triiodothyronine infusion (46) and ischemia.

Chronic exercise-induced myocardial stunning in our ameroid model of coronary occlusion was first suggested by Roth et al (47). We investigated systolic wall thickening in the collateral dependent LC region of animals acutely exercised. Figure 6 shows the response of wall function in two animals subjected to ameroid occlusion prior to a 30 minute exercise period. Wall thickening in % in the ischemic region remained depressed for up to one hour after the cessation of exercise. Peak heart rate achieved at 30 minutes of exercise was approximately 220 beats/min for each animal. Subendocardial blood flow in the LC and LAD regions had returned to resting values by

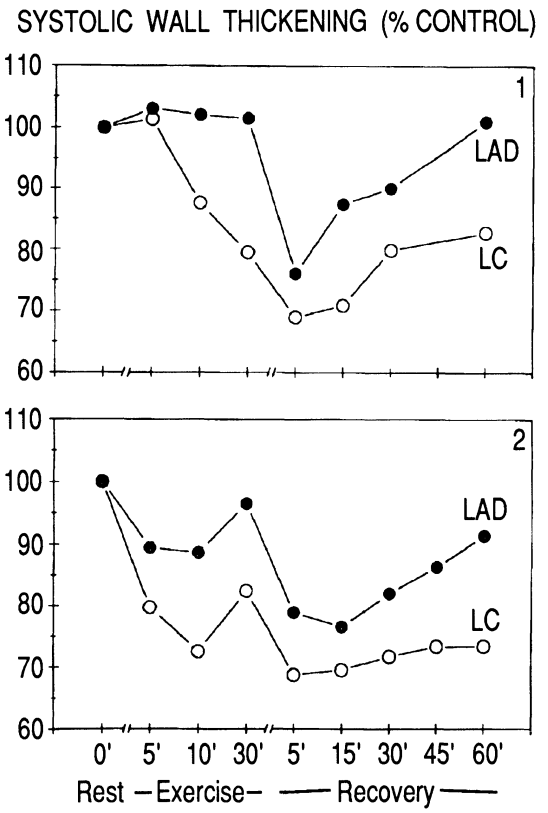


Fig. 6. Normalized systolic wall thickening during progressive exercise and recovery in two animals. These animals had coronary artery occlusion approximately 3 weeks previously. These data strongly suggest myocardial stunning.

30 and 45 minutes of recovery in these pigs. The decrease of LC function compared to LAD function suggests a very prolonged period of myocardial stunning following acute exercise. These observations are analogous to those made following treadmill exercise testing in patients with coronary artery disease (48). Wall motion abnormalities measured by 2-dimensional echocardiography persisted for more than 30 minutes post exercise. Exercise intensity and to a lesser extent exercise duration determined the severity of postischemic dysfunction in a dog model of transient myocardial ischemia (49). These studies suggest that exercise in conjunction with ischemia leads to myocardial stunning.

We do not fully understand the consequences of these changes. Is exercise useful as an adjunct to improvement of heart function by improving coronary collateral blood flow? The study of Fujita et al (50) is encouraging. Using repeated cineangiography they showed in patients receiving heparin and undergoing mild exercise that increased collateralization in ischemic regions occurred compared to patients receiving only exercise. Clearly patients will not or should not exercise at high enough intensity to provoke marked ischemia. Therefore it is likely that exercise will be used in conjunction with some administered angiogenic agent. The studies of Symons et al (51), where dipyridamole was infused for 90 minutes a day for 8 weeks in the pig ameroid model, showed improved collateral reserve. Again this argues that mechanical stimulation of the vessels brought on by the increased flow may stimulate collateral development or that dipyridamole is a mitogenic agent or both are responsible for the improvement. One might also argue that the pig or human heart can not develop sufficient collaterals to reverse completely the occlusion of a coronary artery. Recently (43) we showed that surgical stimulation of extra coronary collateral arteries in the pig may completely reverse the effects of LC occlusion. Also we have shown that heparin, at carefully controlled doses, can provoke a remarkable increase in coronary collateral reserve (52). Thus, we can induce collaterals to grow in the pig model to a level that will restore coronary collateral reserve to levels comparable to normal coronary reserve.

We conclude that exercise in itself is insufficient to provide marked increases in coronary collateral reserve and in some cases be may contraindicated. It is much more likely that exercise will be an adjunct to co-administration of angiogenic factors. In order for this to be a reality we must understand the molecular mechanisms of myocardial angiogenesis.

ANGIOGENESIS IN PIG MODELS OF PRESSURE OVERLOAD HYPERTROPHY

Studies by Breisch et al (17, 53, 54) and Tomanek et al (19) have shown that left ventricles, subjected to pressure overload that increases ventricular weight by more than 50%, have increased total coronary perfusion even though minimal coronary resistance increases. Furthermore this increase in total flow correlated with increased numbers and cross-sectional areas of small arterioles showing that angiogenesis can occur in the left ventricle subjected to pressure overload. Using a model of right ventricular pressure overload where right ventricular weight increases by more than 80%, we showed (55, 56) that minimal coronary resistance stays constant. Furthermore, we showed that there was an extraordinary increase in the dimensions of the arterioles. Figure 7 shows a silicone cast of the right coronary arterial bed of the right ventricle of two pigs. The artery on the left shows an increased girth of the same arterioles (arrows) as the one on the right The animals were of nearly identical body weight and left ventricular weight but the right ventricular weight of the animal on the left had increased by almost 100% following one month of pulmonary banding. In figure 8 we show the relative numbers of capillaries and small arterioles in pigs with moderate right ventricular hypertrophy

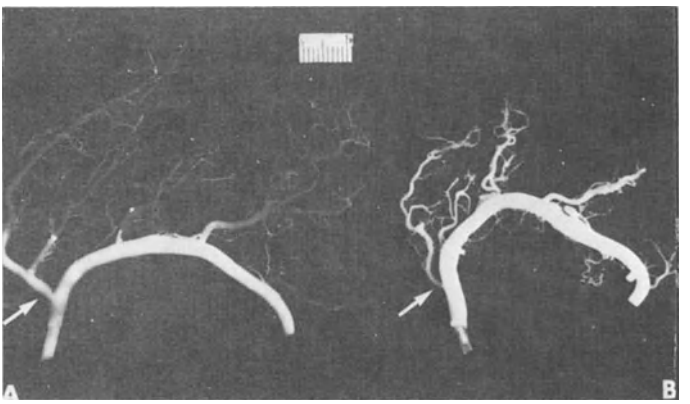


Fig. 7. A silicone cast of a portion of the right main coronary artery near its origin at the ostia. The left artery (A) is from an animal where we banded the pulmonary artery for one month and right ventricular systolic pressures increased from 35 to 90 mmHg. Right ventricular weight increased by more than 90%. The cast on the right was from a similar sized pig with a normal right ventricle. White arrows point to the same coronary branch in each artery. The small arteries increased both in length and in diameter. Interestingly the main coronary artery did not appear to increase in girth. The rule at the top of the photograph is 1 cm long.

(50%) and severe right ventricular hypertrophy (105%). The number of capillaries diminished significantly while the number of small arteries increased by almost 100%. Again this data shows that pressure overload can stimulate angiogenesis and that the maintenance in minimal coronary resistance occurs by the growth of small arterioles not by changes in capillary density.

MOLECULAR BIOLOGY APPROACHES TO VASCULAR REMODELING AND COLLATERAL DEVELOPMENT

Our previous studies demonstrated a tremendous increase in the number of endothelial and smooth muscle cells synthesizing DNA soon after placement of an ameroid constrictor. Presumably these cells divide in response to signals emitted by the increasingly ischemic myocardium. Preliminary experiments with cultured endothelial cells subjected to hypoxia indicate that hypoxia causes perturbations in the signal transduction pathway (57). To understand the mechanisms by which ischemia induces angiogenesis, we have initiated studies on gene expression and growth factor availability in the ischemic heart. Importantly, similarities in our observations regarding growth factors in the ischemic heart and in right ventricular hypertrophy (RVH) suggest that a single paradigm of angiogenesis may apply to both of these models.

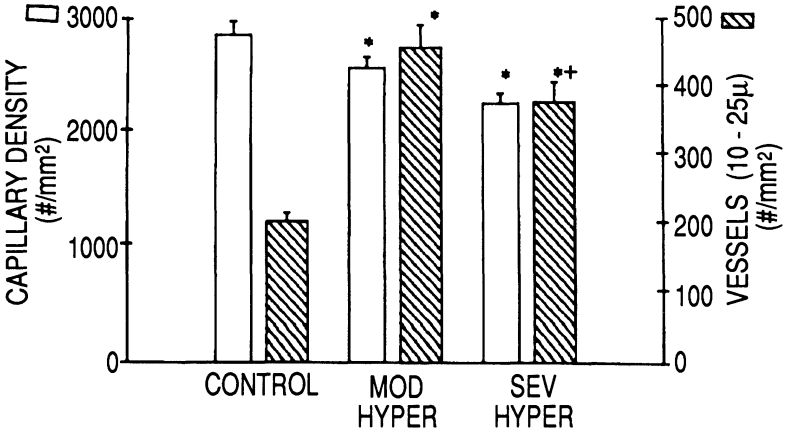


Fig. 8. Capillary density and small vessel density in the right ventricles of control pigs (N = 7), pigs with moderate hypertrophy (N = 8), and severe hypertrophy (N = 4). Capillary density decreased significantly in both hypertrophied groups while small vessel density increased by almost 100%. Minimal coronary resistance measured with radiolabeled microspheres and during adenosine infusion was maintained at control levels in the hypertrophied groups.

Protein kinase C (PKC) is an important element of the signal transduction pathway that allows cells to recognize and respond to extracellular signals. Increased levels of intracellular diacylglycerols activate this enzyme and its activation is associated with translocation of PKC to the membrane. We have investigated the effects of hypoxia on PKC in primary cultures of porcine aorta endothelial cells. Our results indicate that reduced PKC activity is present in both the cytosolic and particulate fractions isolated from endothelial cells incubated in 2% oxygen for 1 hour (57). Also we have observed a similar decrease in PKC activity in extracts made from tissue isolated from the ischemic bed. Interestingly, Western blots reacted with antibody to PKC revealed that, in preparations from the ischemic bed, the enzyme is translocated to the membrane, even though activity significantly decreases.

To investigate changes in gene expression in the chronically ischemic heart, we instrumented pigs with ameroid constrictors on the LC and harvested their hearts at 5, 7 and 13 days after surgery. The ameroid used in this series of experiments closes at approximately 5 days. By Northern blot analysis of total cellular RNA and

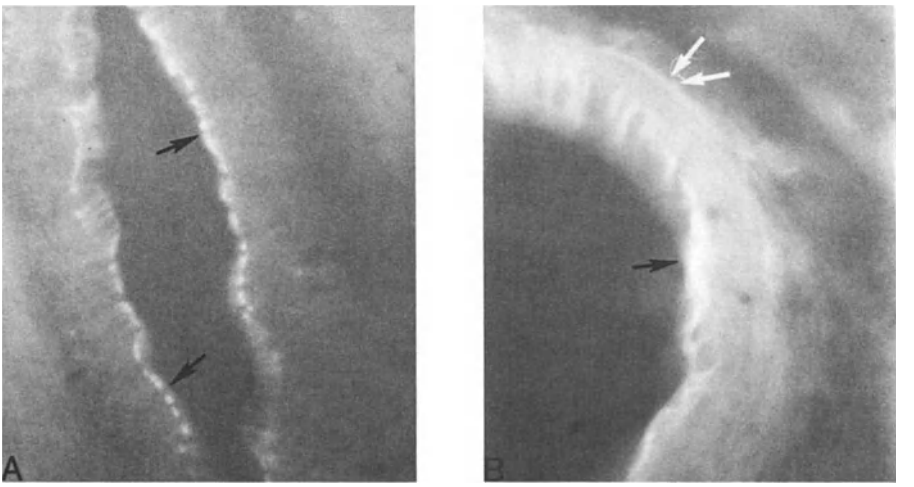


Fig. 9. Fluorescent histochemistry using a primary antibody to TGF-β1 at 4 (Panel A) and 8 (Panel B) days after pulmonary banding. The vessels are arterioles 200 μm in diameter located in the hypertrophied right ventricle. In the 4 day animal endothelial cells (black arrows) are clearly labeled while at 8 days the fluorescent staining includes the smooth muscle cells (white arrow). This fluorescence did not occur in this tissue when the primary antibody was omitted.

hybridization to ^{32}P labeled probes, we observed that expression of the early response genes, c-fos and jun B, was upregulated in the ischemic region compared to controls. In addition, we observed an increase in the message for transforming growth factor-β1 (TGF-β1). This agrees with published results concerning the upregulation of TGF-β1 in rats with myocardial infarction (58). Interestingly we have also observed a transient increase in the message for TGF-β1 in the early phases of right ventricular hypertrophy.

Using fluorescent histochemistry to detect protein instead of RNA, we observed TGF-β1 in the ischemic bed 8 days after ameroid closure. In addition, as shown in figure 9, TGF-β1 was present in the hypertrophied right ventricle 8 days after banding of the pulmonary artery (White, unpublished). Also we have observed by immunochemical techniques, similar temporal regulation of basic fibroblast growth factor (bFGF) in myocardium of both the ischemic and RVH models. As shown in figure 10, bFGF associates with the capillaries and arterioles in the RVH after 4 days of pulmonary constriction. At 8 days, bFGF was still present, but we detected no signal 14 days after banding. Likewise, we observed bFGF associated with blood vessels in tissue sections from pigs 2 or 3 days after ameroid closure, but we did not detect it at 14 days. These results suggest that angiogenesis occurs by similar mechanisms in both

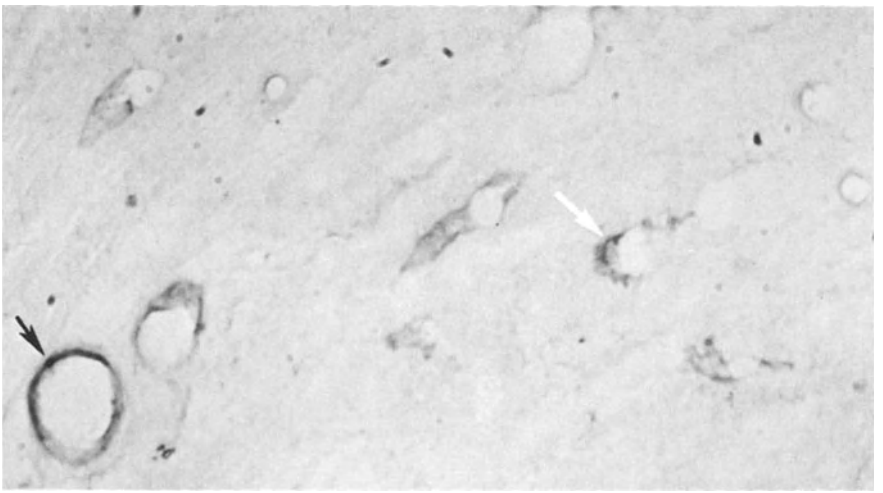


Fig. 10. Using immunohistochemistry methods we detected bFGF in blood vessels 4 days after pulmonary artery banding. The secondary antibody was to alkaline phosphatase and the substrate appeared as a dark purple precipitate in capillaries (white arrow) and small arterioles (black arrow).

the ischemic and pressure over-loaded heart and raises the interesting possibility that both conditions produce similar initiating signals.

DISCUSSION

Several stimuli can promote the growth of blood vessels in the mature heart. These include ischemia, pressure overload, exercise, bradycardiac pacing, and triiodothyronine infusion. It is unlikely that the stimuli are the same in all of these models. For instance it is improbable that exercise in the normal heart induces ischemia. What is more likely is that the stimuli for angiogenesis produce a similar scenario regarding upregulation of polypeptide growth factors and proteolytic enzymes. Our studies suggest that the first response to a stimulus is the growth of endothelial cells primarily in capillaries and to a lesser extent in small arterioles and venules. Remodeling of capillary size vessels to small arterioles and venules quickly follows this early response. The growth of existing arterioles >75 μm is minimal. Since the restriction to maximal blood flow probably is in the small arteriolar network 10-40 μm in diameter, the increase in size and number of these blood vessels is critical to changes in minimal coronary flow resistance. When new blood vessels are developing or mature blood vessels are growing in the case of myocardial hypertrophy, they are responding to an increase in muscle mass or a remodeling of myocytes and collagen. In the case of ischemia this angiogenesis is associated with the degradation of myocytes and the accompanying inflammation. Since a relative underperfusion or "ischemia" can occur in developing hypertrophy, this suggests that focal necroses or inflammation play critical roles in angiogenesis in these models.

The number and location of the preexisting collateral vessels influence the rate and expansion of the coronary collateral bed following ischemia. In the pig heart we found that the number of collateral vessels doubled from the time of ameroid closure until there was no longer any evidence of growth (usually a month later). Clearly the innate vessels and their potential to grow and remodel are critical for the preservation of ischemic tissue. The growth of *de novo* new vessels, while occurring, is less important and can only enhance the potential for collateral flow. While it is plausible that in the exercise induced hypertrophied model the stimulus for blood vessel growth ceases with the normalization of muscle mass, wall stress, and blood flow, it is not clear why collaterals would not continue to grow to meet their potential demand. We see numerous small collateral vessels in pig hearts that still show a substantial coronary

collateral blood flow reserve deficit. Furthermore, even when the myocardium is subjected to intense daily bouts of exercise the increase in blood vessel growth is small and only a 20-30% increase in function and flow occurs. There obviously has to be a dominant down regulation of blood vessel growth in these models such that either the stimulus for growth is no longer present or down regulation is achieved by the presence of inhibitors of angiogenesis.

Our own approach to this problem is to identify the growth factors and investigate the transducing signal pathway for these growth factors during both the initiation and inhibition of angiogenesis. Schaper and colleagues suggested a similar approach (14, 35). Our most compelling finding is the simultaneous growth of endothelial cells in all the models. Most recently we identified the presence of bFGF associated with new blood vessels both in the ischemic model and in the hypertrophied models. bFGF is a known in vivo and in vitro mitogen of endothelial cell growth (59). Its sources in the heart are not clear, although it is present in the endothelial cells as well as in platelets, macrophages and myocytes (60). It is unlikely that only a single growth factor is responsible for the growth of these blood vessels. We continue to look for additional peptides that may be present in the myocardium during the stimulation phase. It is also becoming increasingly clear that proteolytic enzymes associate with this growth phase. Just as important as the cause of the growth in these models is its inhibition (61, 62). We and others find that TGF-β1 associates with ischemia and additionally we have found that it also associates with blood vessel growth during hypertrophy. Several investigators have shown that TGF-β1 is both stimulatory and inhibitory of endothelial cell growth (63, 64). We propose that it acts both to promote and to inhibit the growth of blood vessels in the heart. It is also likely that the complex interplay of the various isoforms of TGF may provide answers for this complex problem. Wünsch et al (65) also showed the localized presence of TGF-β1 in the chronic ischemic model.

CONCLUSIONS

Myocardial angiogenesis in the porcine model occurs in various conditions, e.g., exercise conditioning, ischemia and hypertrophy. During exercise training changes occur both in the capillary and arteriolar beds. These changes result in increased coronary blood flow reserve in the exercise trained heart. However, a net increase in

tissue perfusion only occurs if the exercise level is insufficient to induce moderate or severe hypertrophy. The stimulus for angiogenesis in this setting remains elusive at this time since an inflammatory component, that has a strongly identified role in other settings, is not present. Thus further studies of angiogenesis in this model are of definite future interest.

Angiogenesis plays a critical role in the setting of ischemia, in that tissue preservation depends on its extent. In this environment an inflammatory component plays a critical role. Capillary sprouting and remodeling occur when the early changes of inflammation and repair continue after the onset of coronary occlusion. Angiogenesis in this model results in a remarkable increase of coronary reserve evidenced by the nearly threefold increase in maximal coronary blood flow during adenosine dilated perfusion. The changes appear to primarily involve the arteriolar bed with little net change occurring in the capillary bed, however, the initial budding and remodeling arise in the capillaries. It is of particular interest that when exercise conditioning is superimposed on the setting of ischemia there is additional structural development of the collateral bed. How exercise interacts with ischemia to further promote angiogenesis is unclear. Since the exercise level needed to elicit this increased vascular development is severe; it is possible that the severity of inflammation also increases.

Angiogenesis also occurs in a porcine model of hypertrophy. These changes are more pronounced in right ventricular hypertrophy compared to left ventricular hypertrophy, predominantly occur in the arteriolar bed and increase proportionately to the degree of hypertrophy. Inflammation probably plays a role in this setting too, since functional studies provide evidence that a relative underperfusion or ischemia is present in early stages of developing hypertrophy in our models.

Molecular biology approaches now provide us the opportunity to dissect some of the puzzles arising from previous studies. Since angiogenesis occurs in various settings that, at first, do not appear to share similar stimuli to initiate vascular growth, these new methods permit more detailed descriptions of the mechanisms involved in angiogenesis in these diverse settings. Our hypothesis is that components of inflammation play a major role in the initiation of events leading to vascular development and remodeling. The porcine model provides an additional advantage, i.e., these changes occur during a brief interval and then cease. This provides a model in which studies can focus on the mechanisms involved in angiogenesis and on

interventions that may influence angiogenesis. Thus the porcine model will be extremely useful in future studies of vascular development.

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COLLATERAL CIRCULATION OF THE BRAIN

Konstantin-A. Hossmann
Department of Experimental Neurology, Max-Planck-Institute for Neurological Research
Cologne, F. R. Germany

INTRODUCTION

The brain is an electrical organ with a high energy utilization rate. In the awake state energy consumption of cerebral cortex amounts to approximately 15 mmol~P/g/min, i.e. as much or more than that of liver or heart muscle. The energy demands of the brain are almost exclusively covered by oxidative metabolism of glucose which yields 38 mol~P per mol glucose. If the oxygen supply to the brain is reduced, glucose is metabolized anaerobically which reduces the energy yield by 95% to 2 mol~P/mol glucose.

Since the energy reserves of the brain are limited, functional activity depends crucially on the continuous supply of oxygen and glucose from the blood. The vascular system of the brain is organized in a way that assures adequate blood flow under a wide range of physiological and even pathological conditions. Systemic disturbances of cardio-respiratory function are compensated by powerful autoregulatory mechanisms which respond to the decrease of blood pressure or of the arterial oxygen tension by lowering the cerebrovascular resistance (1, 2). Local constrictions or occlusions of supplying arteries are compensated by a network of preformed anastomotic connections which provide collateral channels for the blood supply by unobstructed vessels (3, 4, 5, 6). In contrast to heart and kidney (7, 8, 9) formation of new collateral vessels under the conditions of progressing ischemia is not a prominent response (10, 11), probably because the brain is not able to survive for any extended period once the autoregulatory capacity of the cerebrovascular system has been exhausted. The following report will, therefore, focus mainly on the morphological, hemodynamical and pharmacological aspects of the preformed anastomotic systems. However, in view of the potential therapeutical implications, the angiogenic properties of the brain will also be addressed. Finally, a brief account will be given of the therapeutical approaches that have been discussed for establishing collateral flow to the brain although the benefits of such procedures are still controversial.

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

PREFORMED ANASTOMOSES OF THE CEREBRAL VASCULATURE

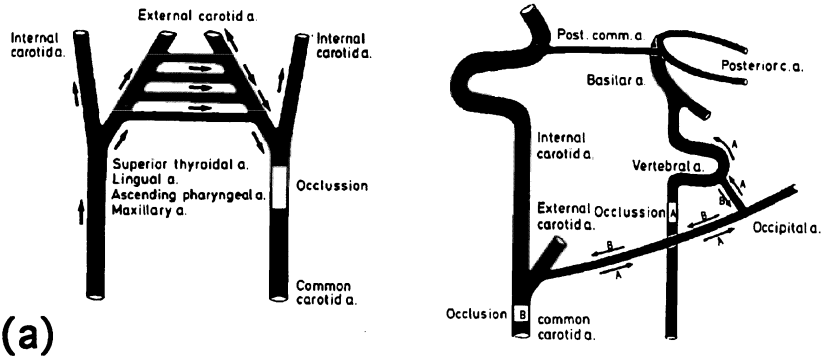
Morphological aspects

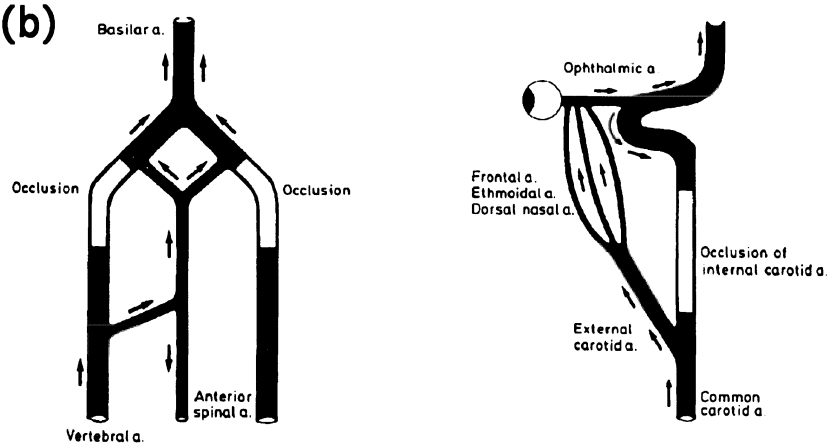
The brain receives its blood supply from four major arteries, the two internal carotid and the two vertebral arteries. The left vertebral artery originates from the left subclavian artery, and the other three arteries from the brachiocephalic or innominate artery. The vertebral arteries merge into the basilar artery which is connected to the internal carotid arteries by the circle of Willis at the base of the brain. From this circle originate the right and left anterior, middle and posterior cerebral arteries which supply the respective brain territories of the two hemispheres. The cerebellum and the brain stem are supplied by branches from the basilar artery and, therefore, may receive blood from either the vertebral arteries or the circle of Willis.

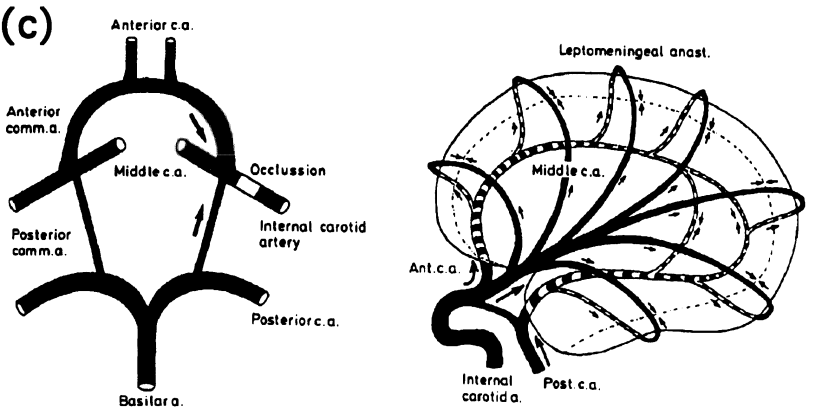
The brain is protected against focal interruptions of its blood supply by a number of extra- and intracranial anastomoses which connect the supplying systems at various levels of the vascular tree (3, 6) (Fig. 1). The most important ones are located a) between the two external carotid arteries; b) between the extracarotid artery and the vertebral artery; c) between the vertebral arteries and the anterior spinal artery; d) between the external carotid artery and the intracranial circulation through anastomoses with the ophthalmic artery; e) through the intracranial system of the circle of Willis at the base of the brain; f) across the leptomeningeal anastomoses of Heubner; g) through the arterial ring anastomoses of Schmidt; and h) through the capillary anastomoses of Pfeifer.

The extracranial anastomoses and the circle of Willis are low resistance collateral channels which assure unimpaired blood supply to the brain in case of the obstruction of one or more of the main neck arteries. The intracerebral collateral circulation is more critical because the parenchymal arteries are essentially end-arteries (12, 13). The collateral blood supply depends almost entirely on Heubner's arterial network of pial

Fig. 1. Schematic representation of the preformed anastomotic channels of the cerebral vasculature.
a) Extracranial anastomoses between the external carotid arteries (left) and between the external carotid and the vertebral arteries (right).
b) Anastomoses between the vertebral and the anterior spinal artery (left) and between the external carotid artery and the intracranial circulation via the opththalmic artery (right).
c) Intracranial anastomoses of the internal carotid and the basilar arteries by the circle of Willis (left) and between the cortical branches of the anterior, the middle and the posterior cerebral arteries via the leptomeningeal anastomoses of Heubner (modified from Zülch (6)).


(a)
External carotid a.
Internal carotid a.
Internal carotid a.
Superior thyroidal a.
Lingual a.
Ascending pharyngeal a.
Maxillary a.
Occlussion
Common carotid a.
Post. comm. a.
Posterior c. a.
Basilar a.
Internal carotid a.
Vertebral a.
External carotid a.
Occlussion
Occipital a.
Occlusion
common carotid a.


(b)
Basilar a.
Occlusion
Occlusion
Anterior spinal a.
Vertebral a.
Ophthalmic a.
Frontal a.
Ethmoidal a.
Dorsal nasal a.
Occlusion of internal carotid a.
External carotid a.
Common carotid a.


(c)
Anterior c.a.
Anterior comm.a.
Middle c.a.
Occlussion
Internal carotid artery
Posterior comm.a.
Posterior c.a.
Basilar a.
Leptomeningeal anast.
Middle c.a.
Ant.c.a.
Internal carotid a.
Post.c.a.

(leptomeningeal) anastomoses on the surface of the brain (14, 15). These anastomose form a macro-network of small arteries which connect the distal branches of the anterior, middle and posterior cerebral arteries and which provide the major collateral blood supply after occlusion of one of these vessels. There is no overlap flow by interdigitated vessels between the territories of the three arteries (16).

The micro-network of the aterial ring anastomoses described by Schmidt (17) are also located on the surface of the brain but are able to provide collateral flow only between adjacent branches of the same supplying artery. They are of functional importance during chronic obliterations of small surface arteries, as in the case of thromboangiitis obliterans, but do not compensate for the occlusion of a major brain vessel (18).

The capillary anastomoses of Pfeifer (19, 20) are transverse intracerebral microchannels which are of considerable importance for the nutritional adjustment of blood flow at the microcirculatory level. They may compensate for the flow reduction after microembolisation but they are not able to provide collateral flow to a major intracerebral artery. It is also important to note that the medial penetrating branches of the middle cerebral artery supplying the basal ganglia and the brain stem are not connected to other vascular territories and behave functionally like end-arteries (21).

Hemodynamic aspects

General pattern of collateral circulation: The hemodynamic efficiency of the preformed anastomotic systems after acute interruption of the blood supply varies greatly depending on the level of the vascular occlusion. The rich anastomotic connections between the carotid and vertebral arteries provide a powerful collateral system which is able to compensate for the occlusion of up to three of the four arteries (22). If the vascular occlusions are carried out sequentially with an interval of a few weeks between each ligation, additional low resistance collaterals develop between the peripheral branches of the thyrocervical and the costocervical trunks which enable the healthy primate to survive even after ligation of all four neck vessels (23). Only under conditions of advanced artherosclerosis or if the vascular obstructions are combined with a lowering of systemic arterial pressure, the extracranial anastomotic systems may not be able to provide adequate collateral blood supply. As a result cerebral blood perfusion pressure (measured at the level of the circle of Willis) declines and cerebral blood flow is compromised first in those areas which are most distant to the

arterial inflow. Since these regions represent the borderlines between the supplying territories of the main cerebral arteries, the resulting lesions have been termed "borderzone" or "water-shed infarcts" (24, 25) (Fig. 2). The hemodynamic situation of this type of circulation failure has also been referred to as the "law of the most distant field" (Gesetz der letzten Wiese) in analogy to the pattern of irrigation failure in agriculture (26). The localization of such infarcts on the surface of the brain is along the bounderies between the territories of the anterior, middle and posterior cerebral arteries, and in the basal ganglia in the terminal area of the strio-lenticulate arteries (24) (Fig. 2).

In contrast to the extracranial occlusion of neck vessels, the intracerebral obstruction of a major brain artery does not change the global cerebral blood perfusion pressure but it increases the local cerebral vascular resistance. Blood is supplied to the territory of the occluded vessel from the collateral vessels located in the periphery of the vascular bed. This reverses the flow direction from the peripheral towards the central parts of the vascular tree, leading to critical impairment of flow first in the core of the supplying territory. The corresponding infarct, therefore, develops in the center of the affected vessel and expands concentrically into the more peripheral parts (Fig. 2).

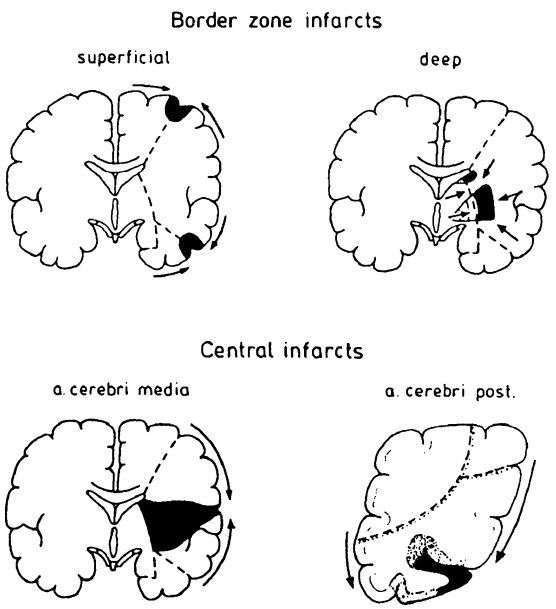


Fig. 2. Schematic representation of the localization of infarcts after stenosis or occlusion of the main supplying arteries of the brain. According to the "law of the most distant field" extracranial occlusion results in ischemic infarcts in the periphery (border zone infarcts), and intracranial occlusion in the center of the corresponding vascular territory (central infarcts). The size of the infarct is a function of the collateral blood supply and varies with the local perfusion pressure and the resistance of collateral vessels (modified from Zülch, (6)).

Resistance of collateral vessels: According to the law of Hagen and Poiseuille the functional efficiency of the collateral circulation depends on the perfusion pressure and the collateral vascular resistance which, in turn, is a function of the length and diameter of collateral vessels, and the apparent viscosity of the blood. As described above, the intracerebral collateral circulation is mainly supported by the network of Heubner's pial (leptomeningeal) anastomoses. The vascular resistance of these collaterals can be assessed by measuring cortical blood flow in combination with pressure recordings in a pial artery, the carotid artery and the cerebrovenous system (27). The strategy of this measurement is as follows. According to Gillilan (28), two different types of brain vessels have to be distinguished: the superficial or conducting vessels and the nutrient or penetrating vessels. The conducting vessels comprise the extracerebral segment of the vascular bed and include the carotid and basilar arteries, the anterior, middle and posterior cerebral arteries and the network of Heubner's pial anastomoses on the surface

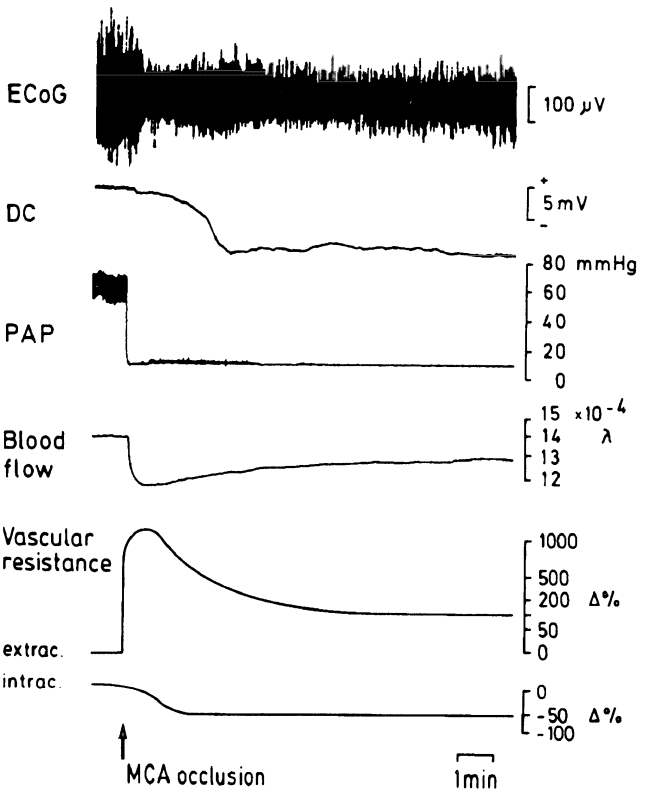


Fig. 3. Acute transorbital occlusion of the middle cerebral artery in cat: polygraphic recording of the electrocorticogram (ECoG), the cortical steady potential (DC), the pial arterial pressure (PAP), cortical blood flow (measured by heat conductance), and the calculated extra- and intracerebral vascular resistance. Note the abrupt increase of extracerebral vascular resistance, followed by a more gradual decline of both extra- and intracerebral resistance. The "activation" of collateral circulation results in the partial reversal of the initial decline of flow which however does not prevent the suppression of EEG and the negative shift of the cortical steady potential (modified from Shima et al (27)).

of the brain. The penetrating vessels and the capillary network supplied by them comprise the intracerebral segment of the brain circulation. By recording the blood pressure difference between the carotid and a pial artery on the one hand, and between a pial artery and the cerebrovenous outflow on the other, segmental resistances of the

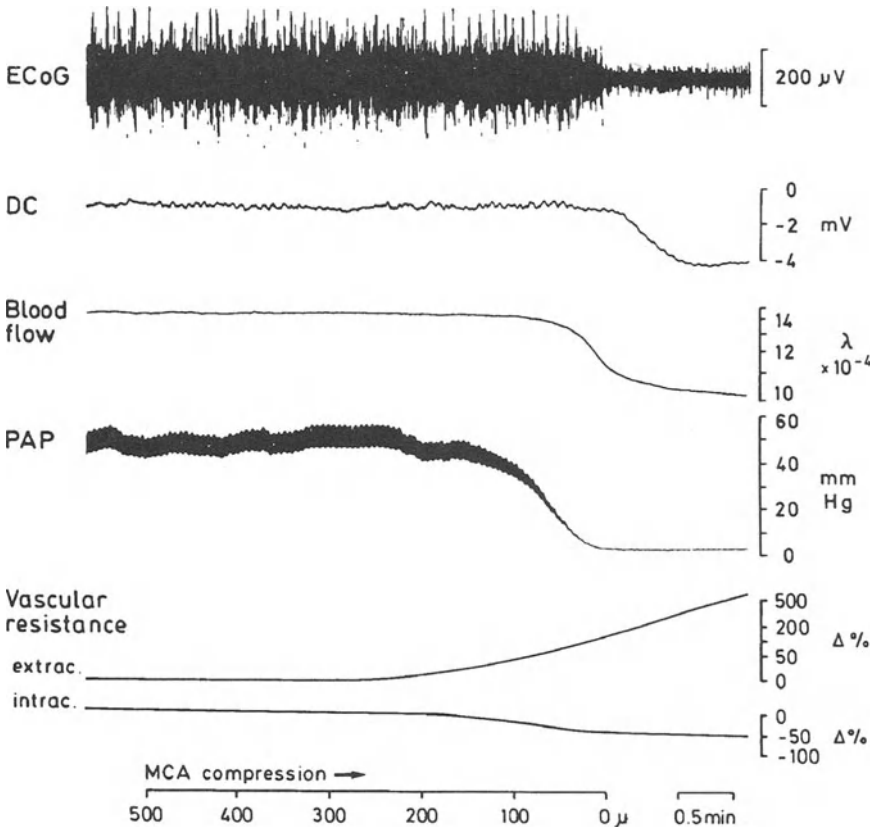


Fig. 4. Gradual compression of the middle cerebral artery in cat: polygraphic recording of the electrocorticogram (ECoG), the cortical steady potential (DC), cortical blood flow (measured by heat conductance), the pial arterial pressure (PAP) and the calculated extra- and intracerebral vascular resistance. The diameter of vascular lumen is given below. Compression of vascular lumen to less than 200 µm (corresponding to 25-30% of original diameter) results in an increase of extracellular vascular resistance and a decline of pial arterial pressure which however is compensated by an autoregulatory dilation of the intracerebral vessels. With further compression to less than 50 µm, blood flow begins to decline and electrophysiological signals deteriorate (modified from Date et al (33)).

extracerebral conducting and the intracerebral nutrient vessels can be calculated according to Ohm's law (27, 29). Collateral vascular resistance after occlusion of a major supplying brain artery is the difference between the pre- and post-occlusion resistance of the extracerebral conducting vessels.

In animal experiments pial arterial pressure can be recorded non-occlusively with micropipettes connected to a servo-nulling micropressure recording device (30). A typical recording of pial arterial pressure, cortical blood flow and the extra- and intracerebral segmental resistances after acute middle cerebral artery occlusion in the cat is shown in figure 3. Before occlusion pial arterial pressure amounts to about 50% of the systemic arterial pressure. Since the pressure of the cerebrovenous outflow can be neglected, the pressure differences - and in consequence the resistances - across the extra- and intracerebral segments are similar. Immediately after vascular occlusion extracerebral resistance sharply rises and blood flow drops to near zero. However, within a few minutes, both the extracerebral and the intracerebral resistances decline, and blood flow rises to a steady although reduced level which remains remarkably constant over the following hours. The delayed onset of collateral flow confirms earlier observations by Denny-Brown and Meyer (5) and indicates that the collateral system requires some time for "activation".

The importance of the speed of vascular occlusion for the activation of the collateral system has been documented in gerbils by comparing the mortality after abrupt carotid occlusion with that after a stepwise reduction of the vessel lumen (31). Stepwise occlusion decreased the mortality by more than 50% but this effect was abolished by the infusion of angiotensin II inhibitors. It, therefore, has been suggested that activation of the collaterals is mediated - at least in part - by the endogenous renin-angiotensin system (32).

When the middle cerebral artery is gradually compressed, the vascular lumen has to be reduced to less than 25-30% of its original diameter before pial arterial pressure begins to decline (33) (Fig. 4). This is in line with computer simulations (34, 35) and the neurological observation that a considerable degree of a local vascular obstruction is tolerated without changes of blood flow (36). Further compression of the middle cerebral artery causes a progressing decline of pial arterial pressure indicating that the preformed collateral system is not able to fully compensate for the increased resistance of the stenotic vessel. This, however, does not immediately lead to a decline of cerebral blood flow because the increased resistance of extracerebral conducting vessels is

compensated by the autoregulatory dilation of the intracerebral vascular bed. Only when pial arterial pressure decreases to below 30 - 40 mmHg or about 60% of the normal value, cerebral blood flow begins to fall and electrophysiological function begins to deteriorate (33).

The relationship between the efficiency of collateral circulation and ischemic brain injury can be quantified by stepwise compression of the middle cerebral artery to allot some time for the evolution of the ischemia-induced functional disturbances (33) (Fig. 5). According to these experiments EEG begins to decline when pial arterial pressure decreases below 30-40 mmHg, and the cortical steady potential begins to shift towards negativity at a pial arterial pressure below 20-25 mmHg (Fig. 6). There is also a relationship between pial arterial pressure and the size of the ischemic infarct developing after middle cerebral artery occlusion (37). One hour after the vascular occlusion the cross sectional area of ATP depletion (measured by ATP-induced

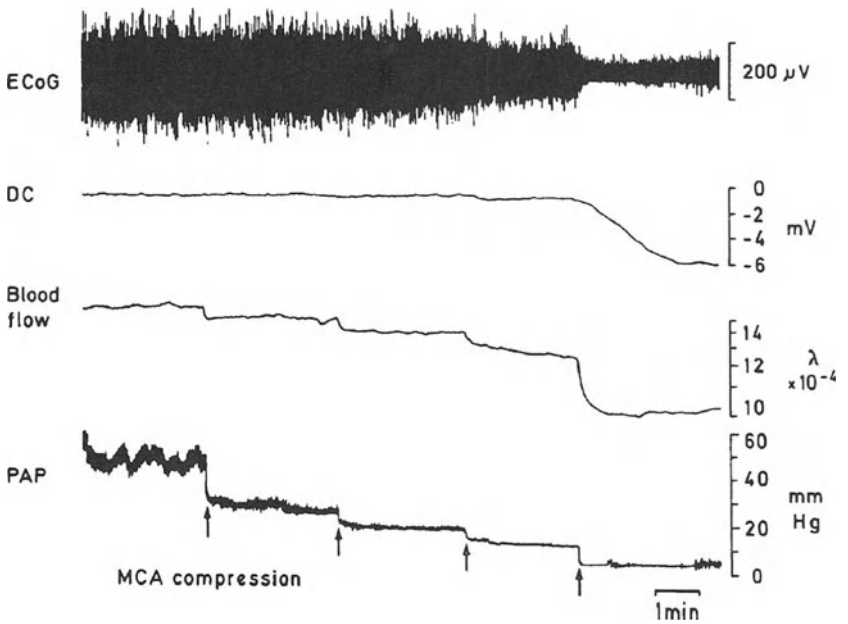


Fig. 5. Stepwise compression of the middle cerebral artery of cat for the determination of the threshold relationship between pial arterial pressure and the changes of electrocorticogram (ECoG), the cortical steady potential (DC) and cortical blood flow (measured by heat conductance). For evaluation of data see figure 6 (reproduced with permission from Date et al (33)).

bioluminescence on coronal sections passing through the territory of the middle cerebral artery) affects about 20% of the hemisphere at a pial arterial pressure of 20 mmHg but it increases to 60% when pial arterial pressure further declines to 5-10 mmHg. This explains that relatively small decrements of blood pressure substantially deteriorate the survival chances of the ischemic tissue.

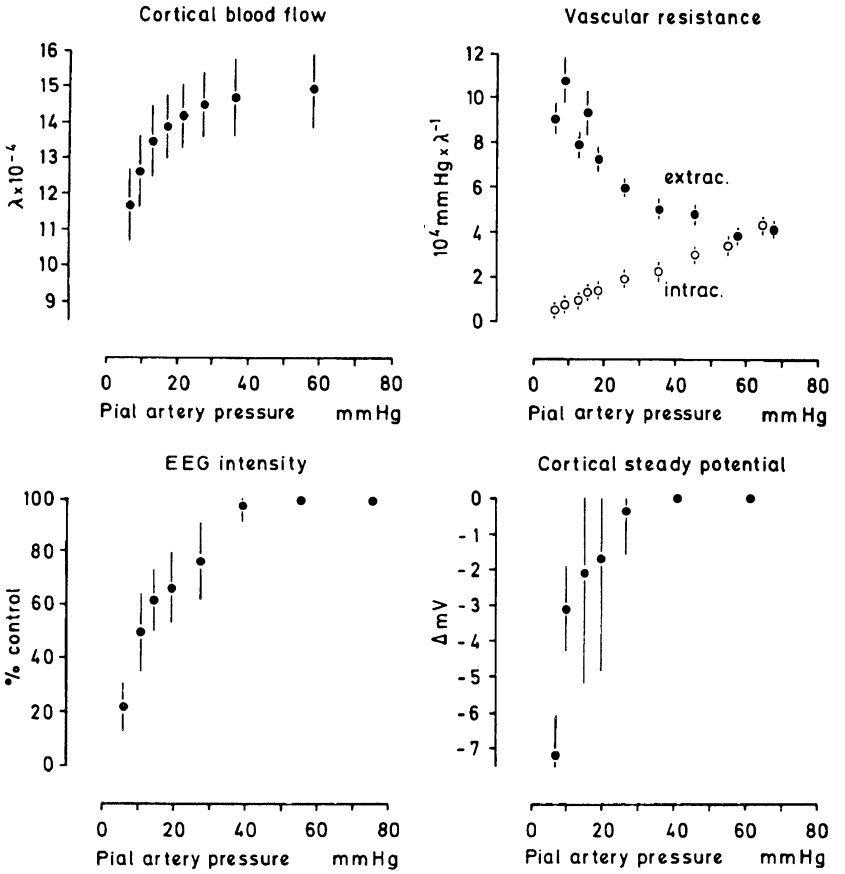


Fig. 6. Relationship between pial arterial pressure and the changes of collateral blood flow, extra- and intracerebral vascular resistance, EEG intensity (measured by Fourier analysis) and cortical steady potential during vascular compression of the middle cerebral artery in cat. Pial arterial pressure decreases with increasing resistance of the extracerebral conducting vessels but cerebral blood flow remains constant down to a pressure of about 35 mmHg due to the autoregulatory dilation of the intracerebral vessels. With further lowering of pial arterial pressure blood flow sharply declines and the EEG and cortical steady potential deteriorate (modified from Date et al (33)).

Interestingly, the threshold of the beginning flow impairment is not equivalent with the maximal dilation of the intracerebral vessels, as evidenced by the continuing decline of intracerebral vascular resistance when pial arterial pressure is further reduced (Fig. 6). An explanation for this phenomenon is the dual control of cerebrovascular resistance which changes as a function of blood pressure and the ionic composition of perivascular fluid (1, 2). The autoregulatory response, therefore, is enhanced by hydrogen ions and other mediators of vasodilation which are released from the brain parenchyma as soon as the tissue suffers ischemic injury.

Reactivity of collateral vessels: The relationship between collateral blood flow and blood pressure has been investigated by several laboratories (38, 39, 40, 41) but there is only one study in which the response of the collateral vessels has been assessed by quantitative measurement of the collateral vascular resistance (27). In this study blood pressure was raised by balloon occlusion of the abdominal aorta to avoid pharmacological side effects, and cerebral blood flow and pial arterial pressure were measured in the territory of the middle cerebral artery before and after middle cerebral artery occlusion (Fig. 7). Before occlusion, an increment of systemic arterial pressure by about 35 mmHg caused an increase of pial arterial pressure by 25 mmHg but no alterations of blood flow, indicating preserved cerebral autoregulation. Calculation of segmental vascular resistance revealed that autoregulation of blood flow is achieved mainly by an increase of intracerebral resistance which rises more than twice as much as that of the extracerebral conducting vessels (38, 39, 40, 41). After middle cerebral artery occlusion the same increment of systemic arterial pressure led to an increase of pial arterial pressure by only 4 mmHg because the high resistance of the collateral system absorbs most of the pressure increment. Despite this modest increase in collateral blood perfusion pressure, blood flow slightly rose because autoregulation of the ischemic blood vessels is reduced (Fig. 7). Interestingly, vascular resistance of the extracerebral conducting vessels which includes that of the collateral vessels, increased more than before middle cerebral artery occlusion, indicating that the collaterals respond to the pressure increment by an autoregulatory vasoconstriction. This observation is of considerable pathophysiological significance because suppression of the autoregulatory response of the collaterals would lead to a much more pronounced increase of pial arterial pressure and, in consequence, to better collateral supply to the ischemic territory.

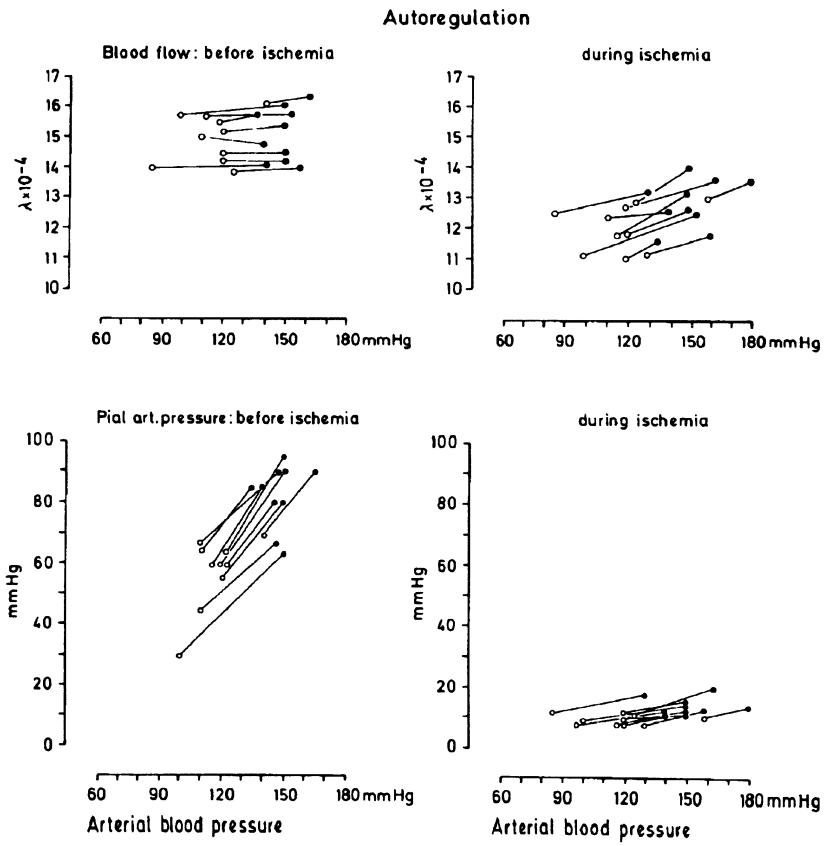


Fig. 7. Autoregulation of cortical blood flow before and after acute middle cerebral artery occlusion of cat. Changes in blood pressure were induced non-pharmacologically by inflation of an intra-aortic balloon. Before ischemia the systemic blood pressure rise increases pial arterial pressure, indicating that the autoregulatory stabilization of blood flow is mainly mediated by vasoconstriction of the intracerebral vascular bed. After middle cerebral artery occlusion the increment of pial arterial pressure is greatly reduced because of the high resistance and the autoregulatory vasoconstriction of the collateral vessels; blood flow nevertheless slightly increases because intracerebral vessels do not respond to the pressure pulse (modified from Shima et al. (27)).

In contrast to the alterations in blood pressure, changes in arterial carbon dioxide have little if any effect on the cerebral collateral circulation. In the healthy brain hypercapnia leads to a sharp rise of blood flow which is associated with a marked fall of pial arterial pressure (Fig. 8). Calculation of segmental vascular resistance revealed that the resistance of the intracerebral nutrient vessels declines more than that of the extracerebral conducting arteries. After middle cerebral artery occlusion neither pial

artery pressure nor blood flow changes. This demonstrates that in the ischemic brain neither the collateral system nor the intracerebral vascular bed respond to changes of arterial carbon dioxide tension. This observation is in line with studies from various laboratories who were unable to demonstrate an effect of hypo- or hypercapnia on either blood flow or infarct size (42, 43, 44).

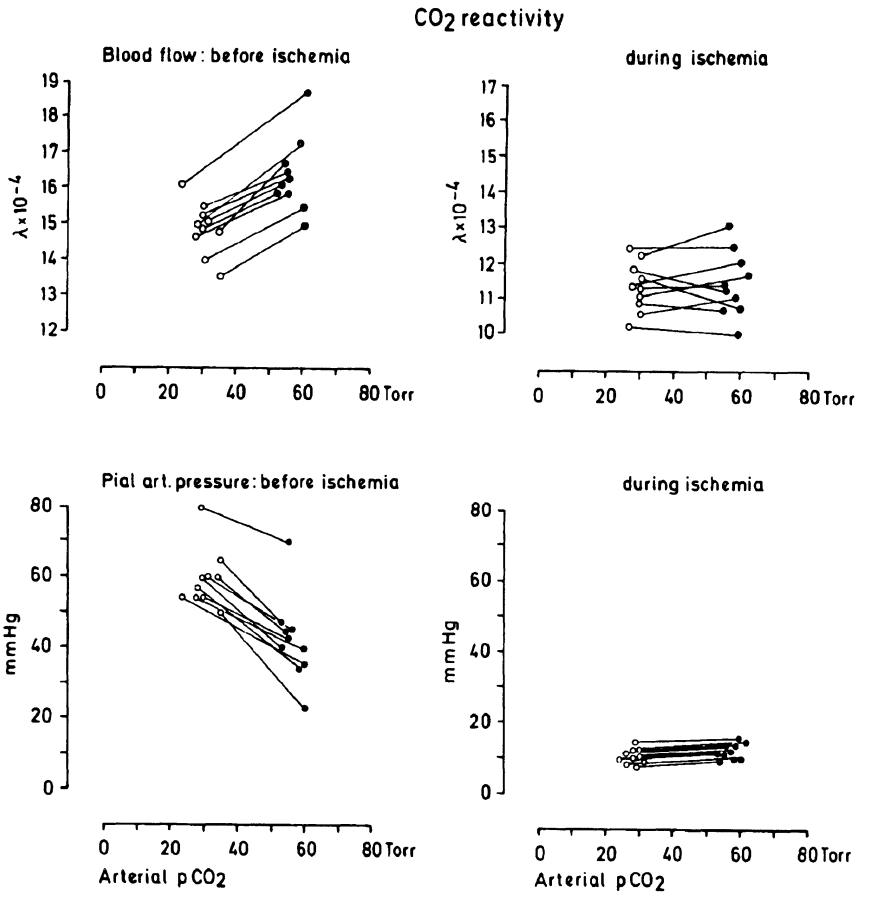


Fig. 8. CO_2 reactivity of cortical blood flow before and after acute middle cerebral artery occlusion in cat. Changes in arterial CO_2 tension were induced by adding 6% CO_2 to the inhalation air. Before vascular occlusion hypercapnia induces an increase of blood flow despite the sharp decrease of pial arterial pressure, indicating that the flow response is mainly mediated by dilation of the intracerebral vessels. During middle cerebral artery occlusion neither blood flow nor pial arterial pressure react to changes of arterial CO_2 due to complete abolition of CO_2 reactivity of both extracerebral collateral and intracerebral ischemic vessels (reproduced with permission from Shima et al (27)).

Steal phenomena: The anastomotic connection of two vascular territories may divert blood from one region to the other, depending on the magnitude and the direction of the blood pressure gradients across the collateral vessels (45, 46). The associated change of regional blood flow is called "steal" if it results in a decrease of flow and "inverse steal" if it results in an improvement of flow. Inverse steal has also been referred to as the Robin Hood-Syndrom (47) in analogy to the legendary hero who took from the rich and gave to the poor.

Steals may be symptomatic or asymptomatic, depending on the magnitude of the flow changes. It has been suggested that the term "steal syndrom" or "true steal" should be used only in the presence of neurological symptoms whereas in the absence of such symptoms the terms "steal phenomenon", "steal effect" or "false steal" would be more appropriate (48, 49).

Steals are not limited to particular vascular territories and may affect the extra- and intracerebral circulation. Examples of extracerebral steals are the subclavian, the occipital-vertebral, the external carotid and the ophthalmic steal-syndroms (for review see 45, 46). These syndroms reduce the blood supply to the brain by shunting the flow from the brain to the extracerebral ischemic tissue, e.g., the proximal occlusion of the left subclavian artery may reverse the flow in the left vertebral artery in order to support

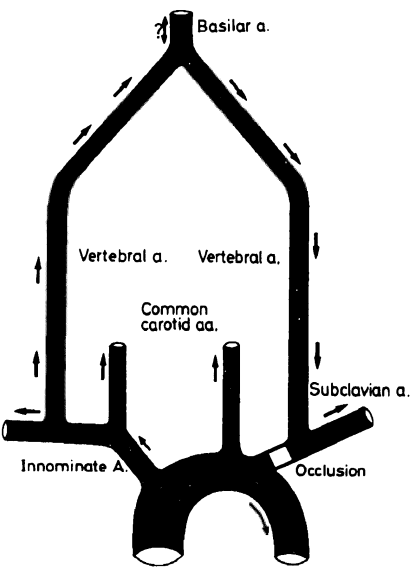


Fig. 9. Schematic representation of the subclavian steal syndrom. Occlusion of the subclavian artery close to its origin at the aortic arch leads to the reversal of flow from the basilar via the vertebral artery to the ischemic brachial artery. As a consequence, blood flow of brain regions supplied by the vertebro-basilar system may decline (reproduced with permission from Zülch (6)).

collateral blood flow to the ischemic arm (Fig. 9).

The occurrence of intracerebral steal during the evolution of infarct has been vividly disputed in the past and still remains controversial. Evidence in favour of intracerebral or interhemispheric steal was provided in patients and animal experiments following ventilation with carbon dioxide or anesthesia with halothane (50, 51, 52). These procedures led to the reduction of blood flow and in animal experiments to the reduction of the back pressure in the stump of the occluded middle cererbral artery. The effect was explained by vasodilation in non-ischemic brain regions, causing a decrease of blood pressure in the pial arterial network and, hence, a reduction of the collateral blood supply to the ischemic territory.

Conversely, an inverse steal resulting in the amelioration of blood flow and an increase in the stump pressure of the middle cerebral artery was noted during hyperventilation (53), barbiturate treatment (54), or occlusion of the external carotid artery (55). The improvement was related to vasoconstriction in the intact brain or - indirectly - to the decrease of intracranial pressure causing an improvement of blood perfusion pressure.

Demonstrations of steal and inverse steal are, however, not equivocal. Other authors were unable to detect alterations of flow during either hypo- or hypercapnia (42, 43). Kogure et al (56) and Yamamoto et al (57) even described an improvement of flow and a reduction of the size of cortical ischemia during ventilation with carbon dioxide. Micropressure recordings in the pial anastomotic vessels after occlusion of the middle cerebal artery did not reveal any changes during hypo- or hypercapnia although pressure responded promptly to alterations of systemic blood pressure (27). Steal — if it exists at all — seems to depend on the particular experimental situation.

NEWLY FORMED ANASTOMOSES OF THE CEREBRAL VASCULATURE

Morphological aspects

Collateral blood flow after acute occlusion of a major brain artery depends obviously on the preformed caliber of the anastomotic vessels. Only with ongoing ischemia or under the condition of a gradual constriction of the supplying artery, some adaptive processes may occur which lead to morphological alterations of the collateral vessels.

There is general consent that intracranial collaterals of the cerebrovascular system are essentially enlargements of preformed anastomoses with few if any newly formed vessels. These enlargements have been identified at all levels of the cerebrovascular system. Occlusions of both carotid arteries in rabbits or monkeys lead to an increase of the lumen and an increase of the tortuosity of the basilar artery (58, 59, 60). The increased tortuosity has been attributed to the lengthening of the basilar artery or — indirectly — to that of the vertebral arteries.

Following occlusion of one carotid artery in rats, the diameter of the ipsilateral communicating posterior artery which is part of the circle of Willis and which connects the basilar with the internal carotid artery, enlarged to almost twice the size of the opposite side after 6 weeks (61) (Fig. 10). This enlargement was highly protective against the additional occlusion of the contralateral carotid artery, as evidenced by the much lesser decrease in blood flow when the second occlusion was carried out after 6 weeks.

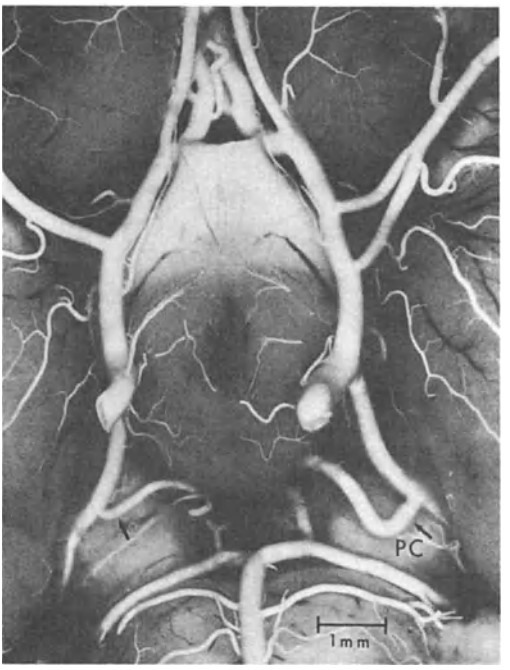


Fig. 10. Permanent occlusion of the left carotid artery in Fisher 344 rat results in marked increase of the diameter of the ipsilateral communicating posterior artery (PC). The circle of Willis is visualized by latex filling 6 weeks after vascular occlusion (reproduced with permission from Coyle and Panzenbeck (61)).

Occlusion of the middle cerebral artery leads to the enlargement and increased tortuosity of Heubner's pial anastomoses of the ipsilateral hemisphere (10, 62) (Fig. 11). Coyle and Heistad (63) reported an increase in the mean diameter of these anastomoses by more than 50% within three weeks following middle cerebral artery occlusion in normotensive rats. Coyle (10) determined the tortuosity factor by dividing the length of the anastomoses by their straight line distance and concluded that the vascular enlargement was accompanied by an elongation of the anastomoses by 24 - 29%. In spontaneously hypertensive rats the diameter of the preformed anastomoses and their increase after middle cerebral artery occlusion was much smaller (63) which explains that in this rat strain stroke incidence is higher and the size of infarcts larger than in normotensive rats (64, 65, 66).

The mechanisms for the enlargement of the collateral vessels are unknown. Obviously, this effect must be due to active growth because the increase in vascular

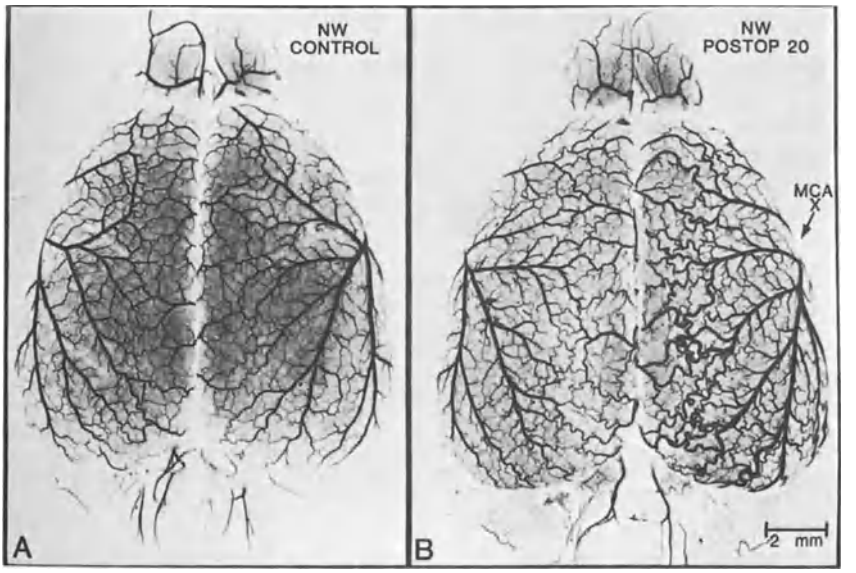


Fig. 11. Microangiograms of the pial circulation of a control rat (left) and a rat after 20 days following permanent occlusion of the right middle cerebral artery. Note the marked increase of the diameter and tortuosity of Heubner's leptomeningeal anastomoses on the surface of the right hemisphere of the MCA-occluded animal (right). Microangiography was performed by intraarterial infusion of latex (reproduced with permission from Coyle, (10)).

diameter is associated with an increase in length and an increase in the number of smooth muscle cells per unit of length (60). Ischemia is known to induce the expression of growth factors but it is difficult to conceive how the conducting vessel senses the accumulation of these factors in the distal parts of the peripheral bed. More likely candidates for the vascular response are the local change of hemodynamic drag or shear stress at the vascular wall which are detected by the endothelium and communicated to the smooth muscle cells by either endothelial growth factors or vasoactive substances (60).

In contrast to vascular enlargements, neogenesis of collateral vessels is probably of little if any importance for the development of collateral circulation in the brain. Turnbull (67) investigated the microvasculature of the rabbit brain for up to two months after middle cerebral artery occlusion but was not able to detect new collateral vessels between infarct and surrounding tissue. The number of Heubner's pial anastomoses was counted by Coyle and Heistad (63) in normotensive and hypertensive rats following middle cerebral artery occlusion but there was no difference between the affected and the contralateral hemisphere.

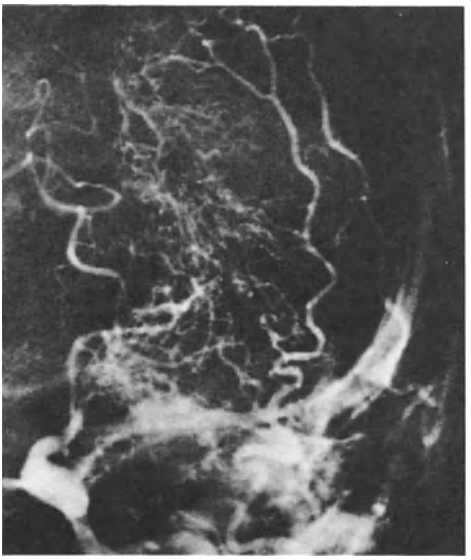


Fig. 12. Moyamoya pattern of dilated collateral vessels in a patient with occlusive cerebro-vascular disease. The name of this pattern refers to the Japanese word of tobacco smoke and has been coined in analogy to the smoke-like angiographic appearance of contrast-filled microvessels (reproduced with permission from Zülch, (6)).

Under clinical conditions the possibility of vascular neogenesis has been discussed in respect to the Moyamoya pattern of angioma-like vessels (Fig. 12). The name of this pattern (the Japanese word for tobacco smoke) has been coined by Suzuki and Takaku (68) because of the smoke-like angiographic appearance of numerous contrast-filled microvessels. The Moyamoya disease has been related to the formation of arterial collaterals in response to a slowly progressing vascular stenosis (68) but others suggest that it is a vascular malformation similar to the "rete mirabile" of some species such as cats or goats (69). More recently, Kono et al (70) concluded from morphometric studies that leptomeningeal vessels of Moyamoya disease are not newly formed but are dilations of pre-existing vessels which would be in line with the animal experimental findings that vascular neogenesis is not an important factor for the development of the cerebral collateral circulation.

Hemodynamic aspects

Enlargement of collateral vessels is obviously a highly protective mechanism against cerebral ischemia because vascular resistance decreases with the fourth power of the luminal diameter. Using a mathematical model of the cerebral circulation, Hudetz et al (34) predicted that infarcts after middle cerebral artery occlusion comprise 50% of the vascular territory when the mean diameter of the pial anastomoses is 80 μm, but can be completely prevented when the diameter increases to 180 μm. Coyle and Panzenbeck (61) produced an enlargement of the posterior communicating artery of the circle of Willis by occluding the ipsilateral common carotid artery, and observed that this enlargement greatly reduced the decrease of hemispheric blood flow when the opposite carotid artery was additionally ligated. This finding is in line with the classical observation of Symon and Russel (23) that both carotid and vertebral arteries of the primate can be safely ligated if the ligatures are carried out sequentially to allot some time for the development of low resistance collateral channels.

The functional efficiency of enlarged pial collaterals was tested by Coyle and Heistad (63) in young normotensive Wistar Kyoto rats submitted to permanent occlusion of the middle cerebral artery. In this species acute middle cerebral artery occlusion does not produce infarcts because the residual collateral blood flow remains above the threshold of morphological injury. One month after the vascular occlusion cerebral blood flow measured with microspheres returned to normal. Additional induction of seizures with bicuculline led to an equal increase of flow in the territories

of the occluded and the contralateral non-occluded middle cerebral arteries, indicating that not only resting flow but also the cerebral vascular reserve for induced flow changes returned to normal (Fig. 13).

The functional importance of collateral enlargements for the protection of the brain under clinical conditions, however, remains unclear. Bozzao et al (71) demonstrated that the size of an infarct measured 3 months after vascular occlusion is determined by the quality of the collateral circulation during the initial 6 h, i.e. long before collateral enlargments develop. Anatomical enlargements of collateral vessels may, therefore, protect against secondary ischemia but do not seem to improve the outcome of the primary ischemic impact.

THERAPEUTICAL IMPLICATIONS

The misrelationship between the rapid onset of ischemic injury after vascular occlusion and the much slower enlargement of the collateral vessels poses a major therapeutical problem for establishing an adequate collateral circulation for the protection of the brain tissue. In view of the high vascular resistance of the intracranial collateral vessels one should expect that pharmacological dilation with so-called vasoactive substances improves collateral flow. In fact, measurements of segmental vascular resistance after acute occlusion of the middle cerebral artery in cats revealed that various vasodilating agents such as papaverin, prostacyclin or nimodipine decrease the resistance of the collateral vessels (72). However, blood flow in the ischemic territory did not change or only marginally improved which is in line with numerous

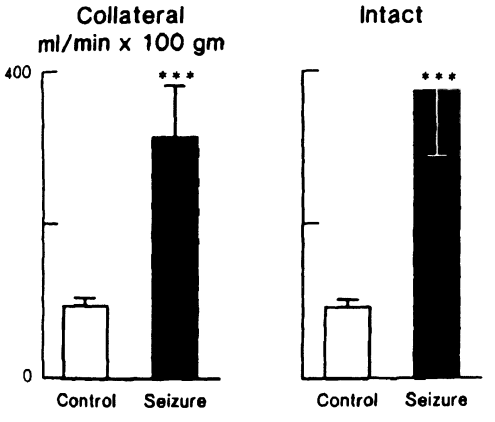


Fig. 13. Functional efficiency of the collateral system of Wistar Kyoto rat 1 month following permanent occlusion of the middle cerebral artery. Blood flow was measured in the territory of the occluded artery (collateral) and in the homotopic region of the opposite hemisphere (intact) before (control) and during bicucculine-induced seizures. The functional efficiency of collaterals is documented by the similar flow rate at rest and during seizures in both hemispheres (values are means ± SD, n = 12; reproduced with permission from Coyle and Heistad (63)).

clinical and experimental studies which also failed to improve blood flow by the use of vasodilating agents (73, 74, 75). The reason is the consistent fall of systemic blood pressure which annihilates the decline of collateral vascular resistance. Pharmacological improvement of collateral circulation, therefore, requires selective dilation of the collateral vessels without significant effect on the peripheral vasculature. Such drugs, however, are not available at the present. Several authors have, therefore, attempted to lower collateral vascular resistance by surgical or pharmacological interruption of the sympathetic pial innervation (76, 77, 78, 79). Although parasympathetic denervation seems to increase infarct size (80), reduction of the sympathetic tone does not produce the opposite effect (79).

Manipulations of blood pressure or arterial carbon dioxide are also of little benefit, the former because of the high collateral resistance and the latter because of the unresponsiveness of the vasculature during ischemia (see above). Rheological improvements of blood flow by hemodilution failed to improve ischemic injury (81, 82, 83) probably because the decrease of blood viscosity is counteracted by the lowering of the oxygen carrier capacity. Conversely, the increase of blood viscosity above normal by hemoconcentration (84) or hyperglycemia (85) clearly increases infarct size and, therefore, should be reversed.

The poor therapeutical outcome of pharmacological interventions have led to the development of surgical procedures for anastomosing the temporal artery with a pial branch of the middle cerebral artery (extra-intracranial bypass) (86, 87, 88). Despite numerous positive reports, the benefit of this intervention for the treatment or the prevention of impeding ischemia could not be substantiated by controlled studies (89), and is therefore disputed. An alternative way for surgical improvement of collateral flow is the implantation of muscle grafts (90) or of the omentum (91, 92) on the surface of the brain but the clinical usefulness is also disputed (93).

A potentially useful approach could be the pharmacological induction of neo-angiogenesis in the surrounding of a vascular territory with reduced blood supply. In the developing brain angiogenesis matches the metabolic needs of the tissue and is closely coupled to the functional and metabolic maturation (94, 95, 96, 97). In the adult brain angiogenesis ceases because nerve cells do not proliferate but the brain maintains its angiogenetic potential, as evidenced by the vascularisation of brain tumors (98, 99, 100) or intracerebral grafts (101, 102, 103). The absence of neo-angiogenesis under conditions of gradually increasing brain ischemia differs from that in the heart or

other peripheral organs and may be due to the fact that the brain is not able to reduce its work load and that for this reason the transition from adequate to non-adequate perfusion is much sharper. Therefore, a reduction of blood flow cannot be compensated by a reduction of oxygen consumption, and the brain may suffer irreversible injury before any ischemia-induced angiogenic mediators become operative. Another particularity of the brain is the shut-down of protein synthesis at a much higher threshold of blood flow than that required for the maintenance of energy metabolism (104, 105, 106). The expression of angiogenic factors may, therefore, be suppressed at an early stage of brain ischemia, preventing the spontaneous adaptation of the vascular system to the reduced blood supply.

However, the possibility of infarct prevention by therapeutical induction of neo-angiogenesis should not be dismissed. As discussed in other chapters of this book, considerable progress has been made in the past few years concerning the understanding of the molecular processes of angiogenesis in various organs including the brain. It is conceivable that the exploration of this knowledge for the therapeutical induction of neo-angiogenesis in brains with reduced hemodynamic capacity may provide a new way for preventing critical reduction of blood flow. It is possible that this could enlarge the otherwise unsatisfactory repertoire of the therapeutical procedures for the prevention of stroke, and future research should concentrate on this challenging perspective.

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15
LIMB COLLATERALS

Werner Schoop
Freiburg, F. R. Germany

Vascular reactions to stenoses and occlusions can be studied particularly well in limb vessels especially with regard to the conditions and factors which are relevant for the collateral arteries.

Development of anatomical conditions

In practice, a collateral circulation can only arise in regions of the circulation which have interconnected arteries, since the requisite precapillary anastomoses are only present there. In the limbs of humans, all arterial vascular regions are linked together by such anastomoses.

The anastomoses with the widest lumen are to be found in the region of the large joints, however the numerous connections in the skeletal musculature are of greater functional significance. Of the other vascular regions which are usually involved in collateral circulation, those of the skin should be mentioned. Many investigators (1, 2) have also found enlarged vasa vasorum and -nervorum functioning as collaterals in limbs with arterial occlusion. These are likely to correspond to the collateral networks which can often be imaged angiographically in patients with thrombangiitis obliterans in the course of obliterated arteries of the lower leg (especially the posterior tibial artery). Periosteal and interosseous vascular systems may also be involved in the collateral circulation. The foramina nutritiva are enlarged as a result of the morphological dilatation of the vessel (3). In principle, the arterial collateral circulation arises from preformed vessels. New vessels are only formed in the occluding thrombus (4, 5). Initially, these develop independently of the rest of the arterial system, but can occasionally link together the vessel lumina which are still open proximal and distal to the obliteration. Investigations showed that the arteries either divide into capillaries within the thrombus or lead to branches running outside the occluded vessel (4), hence they can only be accorded minor significance.

In the event of arterial obliteration, certain preformed collaterals are available for the various regions of the arteries supplying the limbs with blood. Depending on the

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extent of the occlusion and on individual variations in the vascular architecture certain differences may occur. When important exits or abouchements of the preformed collateral vessels are within the obliteration or are likewise occluded, new bypasses arise which are usually long and have a narrower lumen.

The dependence on the location and extent of an arterial occlusion of the bypasses has been repeatedly described synoptically (6, 7, 8, 9). The most important collateral systems of the limbs will be described and illustrated below (see Figs. 1-3).

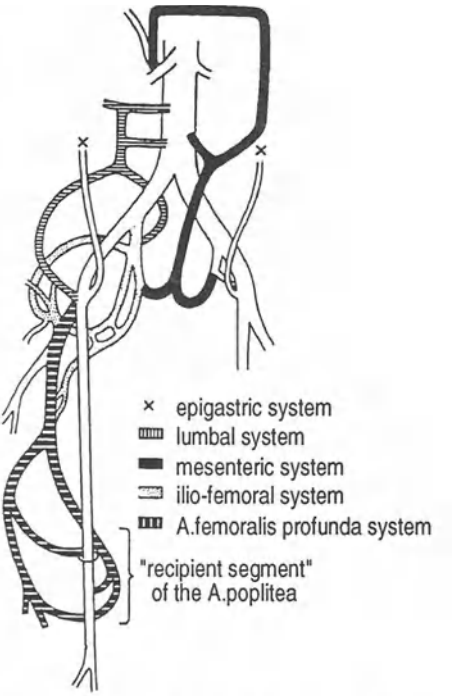


Fig. 1. In the presence of an occlusion in the aortic-iliacal system different collateral circulation pathways can develop (9).

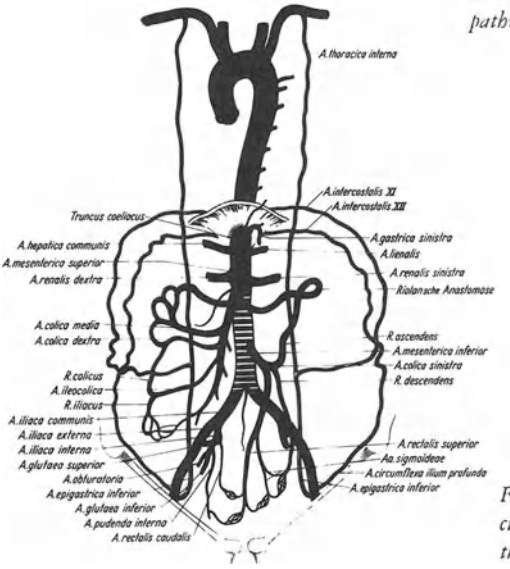


Fig. 2. Different systems of collateral circulation in the presence of a total occlusion of the abdominal aorta (23).

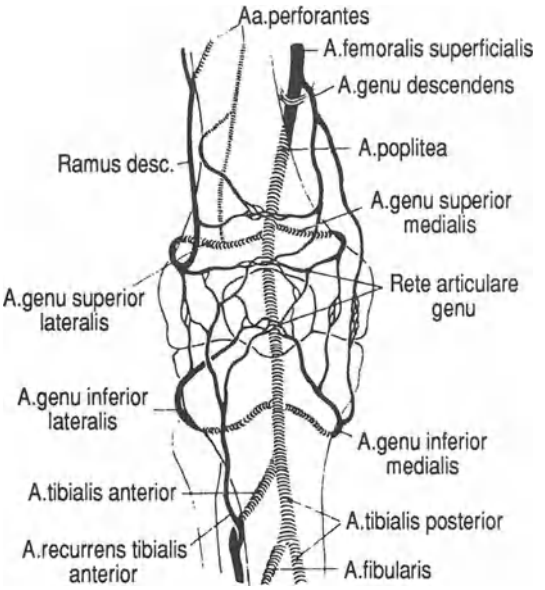


Fig. 3. Preexisting collateral arteries in the knee region (23).

Acute occlusion of a limb artery

The consequences of an acute limb arterial occlusion primarily depend largely on the effectiveness of the preformed collaterals. Only when certain sections of the large transport arteries are occluded does such a major restriction of the blood supply arise, owing to poor collaterals, that there is threat of necrosis (10). The acute occlusion of arterial branches or individual arteries of the lower leg is compensated so well by collaterals that there are no unequivocal symptoms of a blood flow disorder.

After the acute occlusion of a limb artery, characteristic changes and reactions occur which have been investigated in detail in animal experiments. Clinical observations indicate that analogous effects occur in humans.

In accordance with the new pressure conditions in acute arterial occlusion, a unidirectional (bulk) blood flow is established in the preformed collaterals. This increased blood flow abolishes the initial ischemia of the area within a few minutes or up to a few hours, and is evidently due to an increase in the functional dilation of arteries participating in the collateral circulation. The organic collateral enlargement which then begins can be demonstrated angiographically as early as five to six days later (2, 11, 12). Usually, several bypass collaterals are available. In most cases,

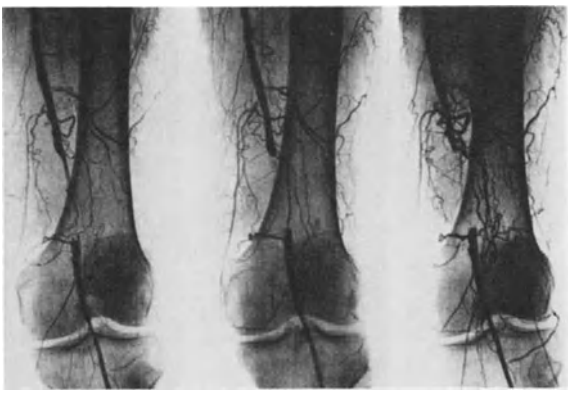
collaterals which bridge over the obstacle via the shortest route are dilated the most (13). Large-lumen arteries can develop from vessels which have not initially been visible or have hardly been visible angiographically; on the other hand, the dilation of long collaterals may finally regress (14) (Fig. 4a-c). The apparent preference for short collaterals proves to be especially effective and appropriate, since they have a lower flow resistance in the dilated state and since they can best compensate for the loss of the occluded vessel.

After processes of intense growth in the first weeks, the rate of organic collateral dilation (=remodelling) decreases in subsequent months. Growth of collaterals appears to have been completed only after years in many cases (see below).

Stenotic lesions

The increase in lumen size of the collateral arteries and the development of an arterial collateral circulation begins already at the stage of arterial stenosis and before final obliteration. Such a situation is recognizable in the angiogram by the dilation and in particular by the characteristic meandering of potential collateral arteries. Such characteristic remodeling, however, only ius found in high-degree stenoses (Fig. 5), which already exhibit unequivocal hemodynamic consequences (systolic pressure gradient, pathological exercise oscillogram).

Collateral remodelling and growth evidently developsonly when the stenosis gives rise to a post-stenotic lowering of pressure. Such a decrease possibly occurs only during and after exercise and in the presence of a significant narrowing. The angiograms



a b c

Fig. 4a-c.
a) 2 weeks of occlusion due to obliterating atherosclerotic alterations in a 66 year old male.
b) 4 weeks later.
c) 8 weeks later (from (14)).

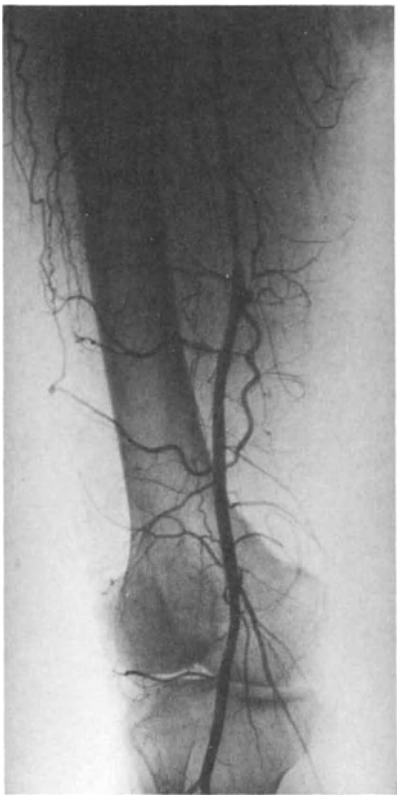


Fig. 5. Stenosis of the femoral artery with collateral circulation.

published by some authors (15, 16) to demonstrate an incipient dilation of arterial collaterals even in the presence of a flat intimal plaque, do not stand up to critical examination. Such slight dilations and meandering of branches (mainly of the femoral artery) are frequently found in elderly people, even if the lumen of the femoral artery has smooth contours in the angiogram.

The occurrence of a more pronounced decrease of post-stenotic pressure gives rise also to a pressure gradient along the preformed collaterals (primary collateral circulation), so that a unidirectional flow must occur resulting in collateral circulation. Initially, such a collateral circulation is likely to occur only temporarily, namely during the phase of peripheral vasodilation which markedly reduces the post-stenotic pressure. Our own experimental animal investigations show that not only the degree of stenosis, but also the peripheral flow resistance, is relevant for the stimulation of an arterial collateral circulation (14).

After creating an AV fistula distal to a high-degree arterial stenosis that has already been present for a long time, a pronounced collateral circulation develops within a few weeks (17). Since development of the arterial collateral circulation begins in the stage of stenosis in chronic stenotic lesions, the complete obliteration usually does not give rise to the severe symptoms of acute arterial occlusion. Indeed, the final obliteration may remain largely asymptomatic.

Collateral arteries in AV fistulae

An arterial collateral circulation also develops around large AV fistulae. Such a short circuit connection evidently has the same effect as a high-degree stenosis or an occlusion: it induces a collateral circulation from preformed arterial links which generally have much larger lumina than those following an occlusion. Occlusions and AV fistulae share the common feature that they create a large pressure gradient between proximal and distal arterial branches.

Depending on the size of the fistula, the blood transported in the collateral circulation either flows exclusively into the periphery or partly into the fistula (18). In animal experiments (19), the collateral circulation develops even if the limb is amputated distal to the fistula. After closure of the AV fistula, the arterial collateral circulation regresses in the same way as after elimination of an arterial occlusion.

Modifying factors

Factors which may be important for development of the collateral arterial circulation are

1. the underlying anatomical conditions
2. the chronological conditions
3. the age of the patient
4. therapeutic measures.

The underlying anatomical conditions: The anatomical conditions present have the greatest significance for development of an arterial collateral circulation. With individual variations, location and extent of the arterial stenosis determine the primary collateral circulation. These primary conditions are only of limited significance for further development, since the capacity for organic dilation is determined by internal principles.

For example, acute embolic occlusion of the popliteal artery usually leads to a severe underperfusion of the lower leg. If this is overcome without tissue loss, a well-compensated occlusion often results after years which prevents major impairments. Such a situation can be explained as follows: the primary collateral circulation in this region is relatively inefficient, since it consists of arteries with narrow lumina. Since these collateral arteries are short and since they dilate to a relatively large extent by lumen growth, the compensation attained in this way is often very good because the region to

be supplied with blood (the lower leg) is relatively small. On the other hand, an iliac occlusion usually does not lead to an equally large exercise tolerance of the leg.

In chronic occlusion of lower leg arteries, corkscrew-like collaterals with narrow lumina are frequently found in the course of occluded artery segments, especially in younger patients. These primarily involve the posterior tibial artery. Such forms are regarded as typical for the presence of a thrombangiitis ohliterans (Buerger's disease).

Occlusion of proximal vessel branches or distal collateral junctions lengthen the collateral circulation, especially in its narrowest parts. The change in flow conditions connected with this (lower blood flow velocity) only allows a limited organic dilation. A collateral circulation which has already formed can partially regress again when the occlusion is lengthened. Regression of collateral dilation after occlusion of collaterals has been demonstrated in animal experiments (17).

The chronological conditions: No definitive data can be given on the rate and duration of collateral remodelling since several factors are responsible for this phenomenon (20). In this regard, the anatomical conditions present are to be emphasized. The more efficient the primary collateral circulation, the more rapidly is the adaptive growth completed. From clinical experience and from measurements of blood flow in angiological patients, it can be concluded that these processes can persist for years.

After an acute occlusion, the collateral arteries evidently receive the strongest growth impulses in the first weeks (21). Clinical experience and comparative measurements of blood flow (22) indicate that the collaterals dilate more slowly in the following months and even years, provided that new obliterative lesions do not develop.

In view of the known correlations between the rate of blood flow and vascular lumen size which have been documented in numerous studies (14, 23), the morphological transformation can be expected to have been completed when an equilibrium between these two factors has been restored.

Today, the speed of arterial blood flow can be measured easily and noninvasively by means of the ultrasound Doppler method. At a site of particular clinical interest, i.e., at the initial part of the deep femoral artery, it can be determined whether in this important collateral artery the adaptation is already complete when the femoral artery is occluded. The flow rate is raised in femoral occlusion at this point, and in patients with occlusions that had been present for many years it is often of the same magnitude as that in a person with healthy arteries (own findings). In femoral artery occlusions, a higher

flow rate in the initial part of the deep femoral artery may indicate the presence of stenosis at the origin of the deep femoral artery exit stenosis, or an adaptation which is not yet complete or which has been impeded by atheromatous lesions.

The age of the patient: It is natural to assume that growth and remodelling of the collateral arteries decreases with increasing age of the patient (20). In fact, our own observations show that the collateral arteries can dilate substantially even in the 7th decade of life (Fig. 4a-c). However, this capability decreases substantially beyond the 70th year of life.

Therapeutic measures: Attempts have been and are being made to promote the development of collaterals with multifactorial therapeutic measures. Animal experiments have shown that there are factors which stimulate additional lumen growth of collateral arteries. The greatest dilation was observed when an AV-fistula was created distal to an arterial occlusion (14). This observation underscores the validity of the well-founded assumption (14, 23) that an increase in blood flow velocity is to be regarded as a crucial factor for the morphological remodelling of collaterals. Experimental observations which have shown an acceleration of the development of collaterals after an acute arterial occlusion by elimination of sympathetic innervation (24) and by muscular work (25) are also consistent with this hypothesis.

In terms of these criteria, lumbar sympathectomy has been proposed in patients with occlusion of the leg arteries and was frequently carried out in former times. Control angiograms taken some months after the operation not uncommonly showed collateral arteries with a much wider lumen than before. However, it has not been unequivocally proven until now that sympathectomy leads to an additional increase in the lumen of collateral arteries in humans. The results published by some authors (26, 27) in support of an improved development of collaterals after "periarterial sympathectomy" or after resection of the obliterated segment of the artery could not be confirmed (28, 29).

It has been considered likely that muscle training increases the efficiency of the collateral circulation, since such training in patients improves their ability to walk. However, it has so far not been definitively demonstrated that there is an increase in the lumen of collateral arteries due to training in angiological patients. There is also no evidence that vasoactive agents can increase the lumen of collateral arteries.

Functional characteristics of the collateral arteries in occlusion of limb arteries

In its narrowest segments, the collateral circulation consists of numerous small and very tiny arterial vessels the width of which can change to a relatively large extent depending on the contractile tonus of their wall musculature. Theoretically, it should thus be possible for the collateral flow resistance to increase and decrease substantially.

Many collateral arteries are likely to be subjected to a nervous tone, especially when they largely consist of cutaneous vessels, as in the case of in occlusion of lower leg and digital arteries. Changes in the lumen diameter are also to be expected via the sympathetic innervation.

A major constriction in the lumen of narrow-luminal collateral arteries which constitute the main flow resistance has substantial consequences for the blood supply to the dependent periphery. When a circulatory centralization arises owing to acute volume deficiency (hemorrhage, after angiography), severe ischemic states comparable to those in acute arterial occlusion can be experienced even in patients with adequately compensated occlusions. These conditions can usually be abolished readily by volume substitution.

Other states of increased peripheral sympathetic innervation are also likely to be accompanied by constriction of collateral arteries. Vasoactive drugs may also be expected to have such an effect.

Functional dilation of the peripheral arterial collateral vessels is interesting especially in terms of therapeutic criteria. The contractile tonus of the arteries participating in the collateral circulation initially decreases definitively after an acute occlusion; this is triggered by the increased blood flow velocity. This effect is likely to be most pronounced in the arterio-arterial anastomoses, since these vessels which had almost no blood flow before show the relatively greatest increase in flow velocity after occlusion. With the increasing organic dilation (remodelling) of these vessels, a tonus is likely to develop again. However, a functional dilation appears to be present even after lumen growth. This is indicated by the major and rapid decrease in caliber of collateral arteries in the angiogram when the occlusion is eliminated by thrombolysis, i.e. by nonsurgical therapy.

Despite the reduction in tonus in the collateral arteries that span an occlusion of long standing an additional dilatation appears to be possible. This is indicated by

animal experiments (30). One to two minutes after induction of post-stenotic vasodilation by muscular work or drugs, the collateral flow resistance decreased.

Apart from such indirect effects, vasoactive agents can evidently also dilate the collateral arteries by direct action (31). Similar findings were obtained in humans (32, 33), however the strength of this evidence is less good due to methodological reasons.

A decrease of the collateral flow resistance is not necessarily based on the dilation of collateral arteries through which blood is flowing. It may also be due to an increase in the lumen of previously "resting" connections which are now perfused.

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16

NEUROHUMORAL AND PHARMACOLOGIC REGULATION OF COLLATERAL PERFUSION

David G. Harrison
Cardiovascular Division, Emory University School of Medicine and Atlanta Veterans Administration Hospital, Atlanta, Georgia, USA

INTRODUCTION

In most species including humans, gradual coronary occlusion induces the development of coronary collaterals (1, 2). In some species these develop from preexisting vessels, and in others they develop anew (3). An aspect of coronary collateral development is the formation of a mature medial layer (4). This newly developed vascular smooth muscle provides a substrate for collateral vasomotion, and the regulation of collateral blood flow by neurohumoral influences. Furthermore, collateral perfusion must traverse not only collateral vessels but also vessels proximal to the collaterals per se, and also arterioles downstream from the collaterals. This series of resistances results in a unique situation in which regulation of collateral perfusion is subject to control mechanisms not present in normally perfused myocardium. In this chapter, several aspects of regulation of perfusion to collateral dependent myocardium will be considered, including unique vasomotor properties of mature collaterals, regulation of collateral perfusion by vessels proximal to the collaterals, and finally new findings regarding alterations of the vasomotor characteristics of recipient arterioles distal to collaterals.

COLLATERAL VASOMOTOR CHARACTERISTICS

While numerous papers have been published examining the effect of various neurohumoral agents or drugs on collateral perfusion, most have not used optimal conditions to study the effect of these substances. As discussed above, coronary collaterals exist within a series of resistances, and it is essential that experimental preparations are used which permit discrimination between changes in true collateral tone vs changes in the vasomotor tone of either upstream or downstream (arteriolar) resistances. Additionally, collateral blood flow can be influenced by changes in heart rate (5), left ventricular diastolic pressures (6, 7), and contraction (8). Many studies which have attempted to examine the effect of interventions of collateral resistance have

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not adequately controlled these variables, and thus the observed changes in collateral perfusion cannot be solely attributed to collateral constriction or dilatation. Studies of collateral perfusion in intact animals are particularly susceptible to this criticism. In the following paragraphs, studies will be reviewed which have attempted to control these many variables.

Alpha-adrenergic regulation of collateral vasomotion

Several groups have been interested in the potential role of α-adrenergic stimulation on collateral tone. The basis for this interest is that if collateral vessels contained functioning α-adrenergic receptors, either circulating or neurally released vasomotor catecholamines could result in collateral constriction, and potentially limit perfusion to collateral dependent myocardium. Several groups have examined the effect of α-adrenergic agonists on collateral resistance. Schaper reported no change in peripheral coronary pressure during administration of methoxamine in isolated hearts in which collaterals had been stimulated by chronic ameroid occlusion (9). In our laboratory, we sought to examine the presence of functioning α-adrenergic receptors on mature collaterals (10). Collaterals were stimulated to develop in dogs by placement of an ameroid constrictor on the circumflex coronary artery. Six months later, the animals were studied. An isolated blood perfused heart preparation was used to minimize extravascular compressive forces. The pressure gradient across the collateral circulation was determined by subtracting peripheral coronary pressure distal to the site of ameroid

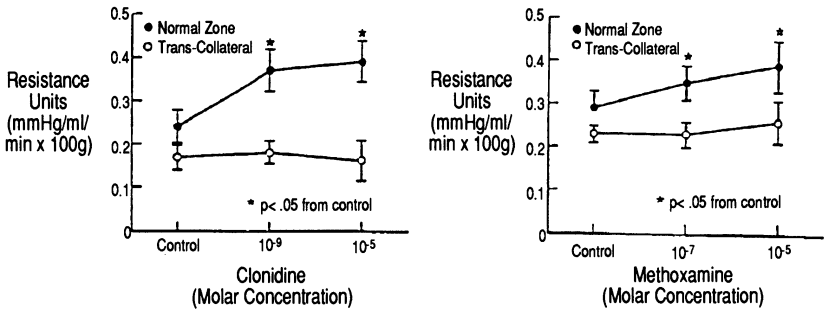

Fig. 1. Effect of Clonidine (left), and Methoxamine (right) on transcollateral and normal zone resistance. Studies were performed in isolated, blood perfused dog hearts with well developed collaterals stimulated by ameroid constrictor placement. Both alpha agonists increased resistance to perfusion in the normally perfused myocardium, but did not alter trans-collateral resistance. (From Harrison et al (10), with permission of the American Heart Association).

occlusion from aortic pressure, which was maintained at 80 mmHg. The studies were performed during maximal vasodilatation with adenosine, to maximize the constrictor effect of α-adrenergic agonists. Collateral blood flow was measured with radioactive microspheres, and transcollateral resistance was estimated by the ratio of transcollateral pressure divided by collateral flow. Neither the selective α_1-adrenergic agonist methoxamine, nor the selective α_2 agonist, clonidine altered transcollateral resistance (Fig. 1), although both caused constriction of resistance vessels within the normally perfused regions of myocardium. In parallel experiments, segments of collateral vessels were studied in organ chambers. These vessels constricted to numerous agonists, including KCl, angiotensin II, vasopressin, and $PGF_{2\alpha}$. In contrast,
neither of the alpha adrenergic agonists phenylephrine nor clonidine produced constriction of collateral segments (Fig. 2). These in-vitro experiments rather convincingly demonstrated that mature collateral vessels in the dog do not contain functioning α-adrenergic receptors. These results are in accord with more recent studies by others, using other experimental approaches. Hautamaa et al measured

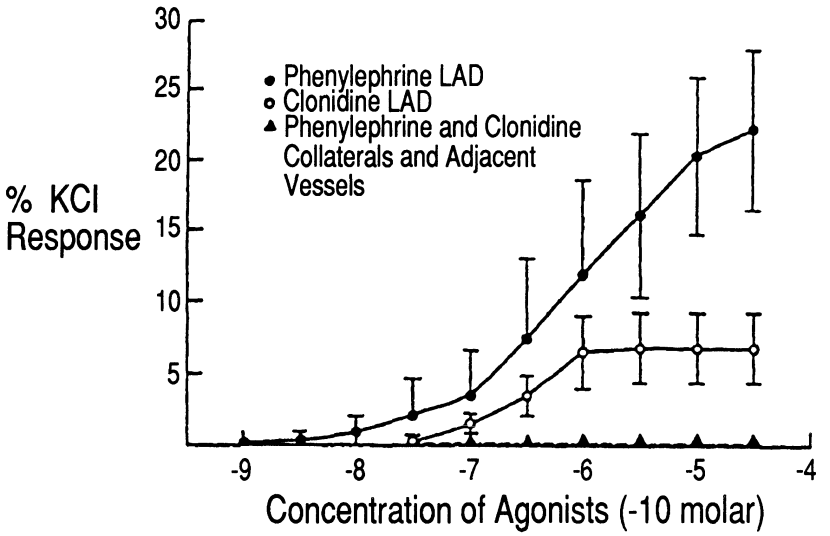

Fig. 2. Response of rings of isolated collateral vessels, the left anterior descending coronary artery, and coronary vessels immediately adjacent to collaterals to phenylephrine and clonidine. Vessel segments were studied in isolated organ chambers. Both alpha agonists caused contractions of the left anterior descending segments, while neither contracted the collateral or adjacent vessels. (From Harrison et al (10) with permission of the American Heart Association).

transcollateral resistance in open-chest dogs with mature collaterals, stimulated by gradual ameroid coronary occlusion (11). One study has suggested that mature collateral vessels may contain α_2-adrenergic receptors. Maruoka and co-workers studied open chest anesthetized dogs with collateral vessels developed by 2-3 months of ameroid constriction. Collateral resistance was estimated by measuring collateral perfusion with radioactive microspheres. Increases in collateral resistance were observed in response to BHT 920. This response was prevented by pretreatment of the α_2-adrenergic antagonist rawaulscine (12).

More recently, Bache and co-workers (13) examined the effect of nerve stimulation, the α_1 adrenergic agonist phenylephrine, an α_2 adrenergic agonist BHT 933, and ergonovine, which activates both serotonergic and α-adrenergic receptors on the collateral circulation. Both retrograde collateral flow and microvascular collateral perfusion (by the microsphere technique during retrograde flow diversion) were measured in dogs 4-16 weeks after collateral growth had been stimulated by embolization of the left anterior descending coronary artery. The responses measured by radioactive microspheres were felt representative of changes in intramural microvascular collaterals, and the responses measured by retrograde flow assumed to represent changes in collaterals between larger vessels. The experiments were performed in the presence of β-adrenergic blockade. Interestingly, neither phenylephrine nor nerve stimulation

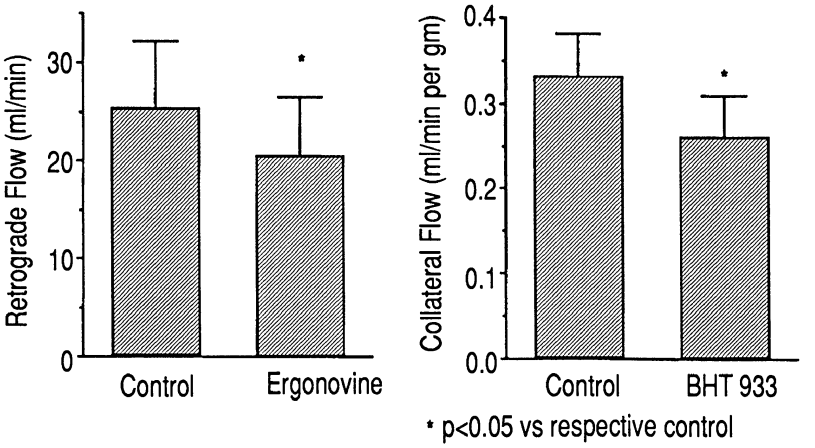

Fig. 3. Effect of ergonovine on retrograde measurements of collateral flow (left panel), and of BHT-933 on collateral perfusion (measured during retrograde diversion of epicardial collateral flow, right panel). From Bache et al, 1991 (13), with permission of the American Physiological Society.

altered either parameter of collateral perfusion. Ergonovine, however, decreased retrograde flow, and BHT-933 decreased microsphere measured collateral flow (Fig. 3). These data clearly indicate that ergonovine could constrict either collaterals themselves, or possibly the vessels proximal to the collaterals. The response to BHT 933 may indicate constrictions of intramural microvascular collaterals, or perhaps enhanced constrictions of the recipient arterioles downstream from the collaterals. This latter explanation is possible because of the alteration in endothelial function of the microcirculation perfused by collaterals (see below).

In the above studies, neither norepinephrine nor nerve stimulation altered collateral resistance or flow, even in the presence of β-adrenergic blockade. Norepinephrine is a potent agonist of both α_1 and α_2-adrenergic receptors, and sympathetic nerve stimulation would be expected to activate both subtypes of α-adrenergic receptors in a physiologcally relevant manner. Given these considerations, it seems that the effect of the BHT compounds may be encountered under normal physiological circumstances. Further, it is reasonable to conclude that mature collaterals in the dog do not contain α-adrenergic receptors responsive to most α-adrenergic agonists, and certainly not the neuronally released substance.

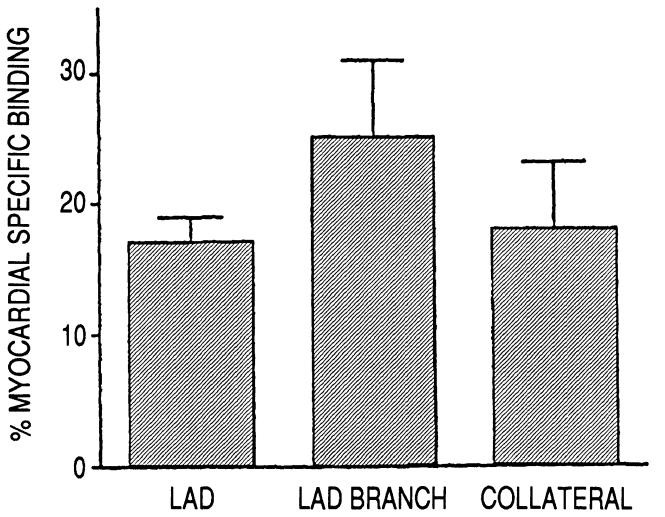

Fig. 4. Specific binding of labelled iodocyanopindalol to sections of canine left anterior descending coronary arteries, branches of the left anterior descending, and mature collateral vessels stimulated to grow by ameroid constrictor placement. From Feldman et al (14), with permission from the American Physiological Society.

Beta-adrenergic stimuli

In contrast to α-adrenergic receptors, mature collateral vessels possess a population of β-adrenergic receptors very similar to that in other native coronary arteries. Feldman et al used a cover slip autoradiographic technique to detect β-adrenergic receptors on the vascular smooth muscle of histologic segments of mature canine coronary collaterals (4 months after ameroid constrictor placement), adjacent similar sized vessels, and the left anterior descending coronary arteries (14). A similar population of β-adrenergic receptors was present in all vessels (Fig. 4). Binding was inhibited to a similar extent by both β_1- and β_2-adrenergic antagonist, suggesting a mixed population of β_1-adrenergic and β_2-adrenergic receptors. In parallel experiments, ring segments of collaterals were studied in isolated organ chambers. After constriction with $PGF_{2\alpha}$ these vessels demonstrated relaxations to both the β_1-adrenergic agonist prenalteral and the β_2-adrenergic agonist albuteral (Fig. 5). The contour of these dose-response curves were again compatible with a mixed population of β-adrenergic receptors. These responses were identical to those observed in studies of ring segments of the left anterior descending coronary artery.

The implication of these studies of isolated collateral segments would seem to be that the predominant functioning adrenergic receptor in collaterals are of the β-classification, and thus sympathohumoral adrenergic stimulation of collateral vessels would most likely lead to β-adrenergic mediated vasodilatation. This seems teleologically advantageous, because during periods of stress, the net effect of the increased adrenergic stimulation would seem to be collateral dilatation, and thus

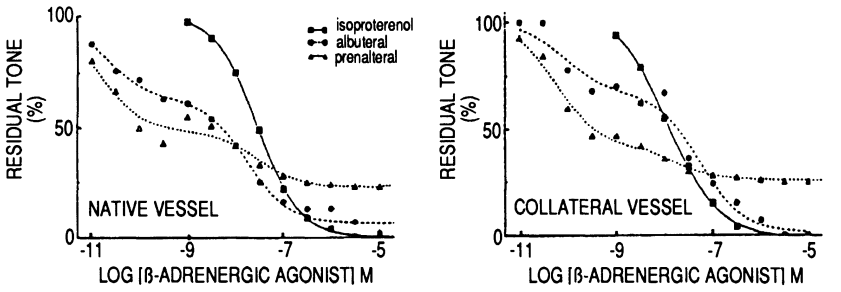

Fig. 5. Relaxations of collateral (panel a) and non-collateral (panel b) vessels to the β_1-adrenergic agonist prenalteral and the β_2-adrenergic agonist albuteral. Rings of vessel segments were studied in organ chambers. Both agonists produced dose dependent relaxations of both innate and collateral segments. From Feldman et al (14), with permission from the American Physiological Society.

improved perfusion to collateral-dependent myocardium.

Vasomotor responses to vasopressin

In preliminary studies of collateral vessels in organ chambers, the constrictions to vasopressin were noted to be quite marked, often exceeding those to depolarizing concentrations of KCl. In view of these in-vitro findings, subsequent experiments were performed to examine the effect of vasopressin on collateral perfusion in intact animals (15). Dogs were stimulated to grow collaterals by placement of an ameroid constrictor on the circumflex coronary artery for 4 months. Subsequently, the animals were studied in an open chest anesthetized state. The proximal left anterior descending coronary artery was cannulated and perfused from a pressurized reservoir, and a cannula placed in the circumflex coronary artery to allow measurement of peripheral coronary pressure beyond the collaterals so that some estimate of collateral resistance could be obtained. A snare was placed around the right coronary artery so that during subsequent measurements of collateral perfusion, the right coronary artery could be occluded, allowing the perfused left anterior descending to be the major source of collateral perfusion. Regional myocardial perfusion was measured by direct injection of radioactive microspheres into the left anterior descending perfusion line under baseline conditions, and during the infusion of increasing concentrations of vasopressin. The concentrations of vasopressin accomplished during the infusion were subsequently determined by radioimmunoassay, and ranged from approximately 10 to 1000 μU/ml. While these levels are clearly elevated beyond that encountered under physiological conditions, concentrations in these ranges (>150 to 1000 μU/ml) have been observed during severe physical stress, dehydration, hemorrhage, and cardiopulmonary bypass. The effect of these concentrations on collateral perfusion were dramatic, and are summarized in figure 6. Overall, these concentrations had minimal effect on the total resistance of perfusion to the non-collateral dependent region of myocardium. In contrast, collateral perfusion was markedly altered by vasopressin. The change in transcollateral resistance at the highest concentration of vasopressin was a 200% increase. In some instances, perfusion to the collateral dependent region decreased from normal values of >100 to <30 ml/min per 100 gms of myocardium.

More recently, it has been demonstrated that exogenously administered vasopressin can inhibit the increase in collateral perfusion that accompanies exercise in dogs with well developed collaterals stimulated by ameroid occlusion (16).

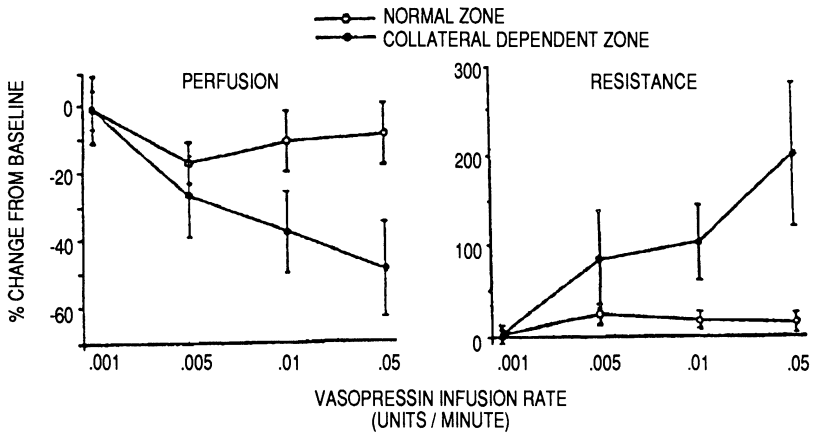

Fig. 6. Left panel; effect of vasopressin on perfusion to normal zone and collateral-dependent regions of myocardium. Right panel; effect of vasopressin on resistance to perfusion in normally perfused myocardium and on trans-collateral resistance. From Peters et al (15), with permission from the American Heart Association.

Whether or not human collaterals can exhibit such a response to vasopressin remains a subject of conjecture. The important conclusion that can be drawn from these experiments is that there is the potential for the newly developed vascular smooth muscle of collateral vessels to develop a functioning receptor population which may allow the collaterals to possess unique vasomotor characteristics. These theoretically may predispose to collateral constriction and reduce perfusion to collateral dependent myocardium and induce myocardial ischemia. In this regard, there are several clinical reports of unfortunate and catastrophic events during vasopressin infusion in individuals with coronary artery disease and in some instances "chronic coronary occlusion" (17, 18, 19, 20, 21). It is interesting to speculate that vasopressin induced collateral constriction may have contributed to these events.

Collateral opening

Another aspect of collateral vasomotion worthy of consideration is the phenomenon of collateral opening that occurs acutely after coronary occlusion. For several years it has been recognized that after abrupt coronary occlusion, collateral perfusion does not immediately reach a maximal value, but gradually increases over a period of several minutes to one hour. It has been suggested that this is merely a "popping open" of vessels previously closed because a pressure gradient across these vessels has developed due to the decrease in pressure in the recipient vascular bed. This

explanation is problematic, because the mean distending pressure across the collateral vessel is actually substantially decreased by occlusion of the recipient vasculature, and thus the tendency would be for these vessels to passively collapse rather than dilate if this were only a mechanical phenomenon. In fact, this line of reasoning has prompted the concern that the increase in collateral perfusion that occurs during the first several minutes after coronary occlusion is not due to collateral dilatation or opening, but perhaps related to other factors such as changes in extravascular compression of the collaterals. Recently, Lamping et al have examined this issue by directly visualizing collateral vessels on the surface of the canine left ventricle (22). These studies were performed using stroboscopic illumination of the left ventricle and collaterals were identified as bridging vessels extending from branches of the circumflex to branches of the left anterior descending coronary artery. Upon occlusion, collaterals greater than 100 μm in diameter did not change in size, but those less than 100 μm in diameter dilated substantially, by as much as 20% of their original diameter in 1 minute and to 40% by 15 minutes. These preliminary experiments are the first direct observation of collateral opening or dilatation in response to coronary occlusion, and support the concept that this is a process of active vasomotion. In a series of subsequent studies, Lamping et al have examined mediators of this process (23). One explanation for collateral dilatation following coronary occlusion was that after the development of a pressure gradient across the collaterals, a small amount of flow begins through each collateral vessel, and that the endothelium of these vessels senses this flow and releases the endothelium-derived relaxing factor, either nitric oxide or a related nitrosyl compound (ED(NO)), in response to this increase in shear, leading to a shear induced dilatation. This explanation is unlikely however, because collateral opening was not prevented by pretreatment with an antagonist of the formation of the ED(N0), nitro-L-arginine. In subsequent studies, Lamping et al have shown that inhibition of ATP sensitive potassium channels with glibenclamide completely blocks dilatation of collaterals following coronary occlusion (24). These findings suggest that opening of ATP sensitive potassium channels in the collateral vascular smooth muscle leads to local hyperpolarization, and subsequent dilatation of the collaterals. The precise signal for opening of these channels is not clear. One explanation is that the increase in shear induced by the initial flow induces the release of an endothelium-derived factor which hyperpolarizes the underlying vascular smooth muscle via opening the ATP sensitive potassium channel (25), the so called endothelium-derived hyperpolarizing factor (26).

A second explanation is that chemical signals released from the recipient ischemic myocardium directly open the ATP sensitive potassium channel of the collateral vascular smooth muscle. Finally, chemical signals released within the vascular smooth muscle (such as the reduction of ATP levels) may modulate this response. Nevertheless, these preliminary experiments clearly demonstrate that collateral opening is a process of active vasomotion which is likely mediated by an effect of coronary occlusion on the collateral vascular smooth muscle membrane potential. This phenomenon may have important clinical implications. It is interesting to speculate that pharmacologic agents that open ATP sensitive potassium channels may maximally open collateral vessels *before* coronary occlusion. Such a pharmacologic manipulation may be beneficial in the setting of coronary angioplasty, where maximizing collateral perfusion may be helpful in minimizing the ischemic events that occur during balloon inflation.

EFFECTS OF COLLATERAL PERFUSION ON THE VASOMOTOR CHARACTERISTICS OF RECIPIENT VESSELS

Recently, it has become evident that perfusion through mature collaterals over a period of time alters the reactivity of the downstream resistance vessels. During studies of the reactivity of collaterals to vasopressin, we were able to estimate the response of the arterioles within the normally perfused and within the collateral-dependent regions of myocardium (15). The low concentrations of vasopressin used in this study had minimal effect on the normally perfused myocardium. In contrast, vasopressin produced substantial increases in arteriolar resistance in the collateral-dependent myocardium. Because vasopressin's vasoconstrictor effect can be modulated by the release of the endothelium-derived relaxing factor (27), we reasoned that one explanation for this observation may be that the endothelium within the collateral-dependent region may be dysfunctional. In subsequent studies, arterioles from the collateral-dependent regions of myocardium were studied in-vitro, using a microvessel imaging apparatus (28). Endothelium-dependent vascular relaxations to ADP and acetylcholine were strikingly abnormal in these vessels, while responses to the calcium ionophore A23187 were unchanged compared to control vessels (Fig. 7). These findings suggest that chronic perfusion through collaterals produces an impairment of membrane signalling within the endothelium, such that neurohumoral agonist which require interaction with a receptor and subsequent second messenger activation have a diminished effect. The calcium ionophore A23187, which does not require

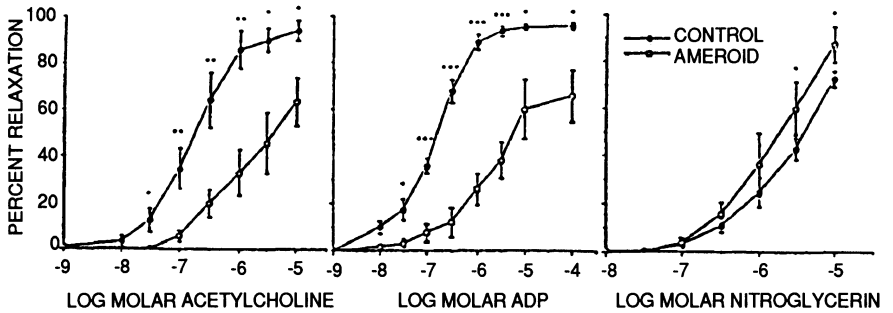

Fig. 7. Relaxation of arterioles from normally perfused myocardium and collateral-dependent myocardium to acetylcholine, ADP, and nitroglycerin. Vessels were studied in a microvascular imaging apparatus after pre-constriction with the thromboxane analogue U44619. From Sellke et al (28), with permission from the American Heart Association.

receptor/second messenger interactions, produced a normal response in these vessels, indicating that the NO synthase enzyme was not impaired and that vascular smooth muscle responsiveness was not altered. Interestingly, responses to nitroglycerin were actually increased in these vessels. Supersensitivity to exogenous nitrovasodilators has been reported in conditions where the production of the EDRF has been inhibited or the endothelium denuded (29, 30).

This observation has been confirmed in pigs 4 months after ameroid constrictor placement to stimulate collateral development (31). More recently, it has been shown that endothelium-dependent vascular relaxations of collaterals themselves are similar to native coronary arteries (32). Thus, endothelial dysfunction in the coronary circulation of hearts with collaterals seems limited to the arterioles downstream from collaterals themselves.

Maseri and co-workers have shown in humans, that intracoronary ergonovine causes the angiographic disappearance of collaterals and recipient vessels downstream from collaterals (33). This is associated with clinical evidence of ischemia. The mechanism for this phenomenon remains unclear, but it is interesting to speculate that endothelial dysfunction may predispose to an enhanced effect of ergonovine on the coronary microvessel's vascular smooth muscle, leading to microvascular spasm.

The mechanisms underlying this alteration in endothelial cell function remain obscure. It is conceivable that transient ischemia during early collateral development may result in endothelial cell damage which persists after collaterals are fully developed (34). This seems unlikely because the endothelium of these vessels does not

possess the regenerated appearance of previously injured endothelial cells. A second possibility relates to the characteristics of flow and perfusion pressure distal in arterioles distal to collaterals. The perfusion pressure distal to collaterals is usually reduced compared to aortic pressure, in most animal models by as much as 50%. Further, the pulsatile nature of perfusion is likely markedly altered. Chronic changes in perfusion pressure clearly change endothelial regulation of vascular smooth muscle. It is likely that the expression of NO synthase and perhaps other cellular components mediating endothelium-dependent vascular relaxation are influenced by the pulsatile nature of flow. Finally, it is interesting to speculate that substances such as growth factors and cytokines released from the growing collaterals may diffuse downstream and influence vascular reactivity of the recipient arterioles. It is likely that a combination of these factors alter endothelial modulation of arterioles within the collateral dependent myocardium.

MODULATION OF COLLATERAL PERFUSION BY RESISTANCES PROXIMAL TO THE ORIGIN OF COLLATERALS

The precise size of vessels which give rise to coronary collaterals remains a matter of debate. We have estimated that the pressure at the origin of the coronary collaterals to be within a few mmHg of aortic pressure suggesting that they arise predominantly from conduit coronary arteries (35). Others, using different experimental approaches, have suggested that many collaterals arise from the smaller coronary vessels (36, 37, 38). This is obviously a critical issue, because coronary microvessels less than 300 μm in diameter undergo very active vasomotion, and approximately 50% of the total coronary resistance exists in vessels between 100 and 300 μm in diameter (39). If a large component of the collateral circulation originated from vessels substantially smaller than 200-300 μm diameter, these vessels could contribute to the regulation of collateral perfusion. Constriction of these vessels would decrease collateral perfusion pressure, and dilatation would increase perfusion pressure, effectively increasing collateral blood flow. At present, little is known about this potential source of collateral regulation, although most would conclude that under conditions of basal perfusion, its contribution is small.

In contrast, during conditions of vasodilatation, the small resistance proximal to the origin of the collaterals may become very significant. Because of the high flow state, a pressure gradient may develop over this proximal resistor, effectively decreasing collateral perfusion pressure and reducing collateral flow. Under these conditions, a

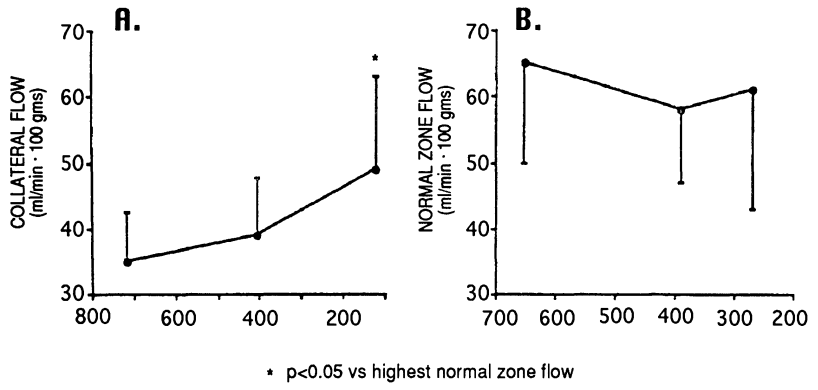

Fig. 8. Relationship between normal zone perfusion and collateral perfusion during vasodilatation with adenosine (left panel) and diltiazem (right panel). Studies were performed in isolated, blood perfused canine hearts. Native collateral perfusion was measured with radioactive microspheres during circumflex coronary artery occlusion. From Harrison et al (40), with permission from the American Heart Association.

reciprocal relationship can be shown to exist between perfusion to the normal myocardium and the collateral-dependent myocardium. Such a mechanism for collateral steal has been modeled and suggested to vary depending on the vasodilator employed (40). Interestingly, potent small vessel dilators such as adenosine and dipyridamole are more likely to decrease collateral perfusion than vasodilators of more proximal vessels like nitroglycerin and more homogeneous vasodilators like the calcium channel antagonists (41) (Fig. 8).

CONCLUSION

In this review, current knowledge about the effect of vasoactive agents on the collateral circulation has been summarized. Virtually all of these data are derived from studies of experimental animal models, and care must be taken to extrapolate these findings to the case of human with mature collaterals. Nevertheless, certain concepts are likely quite applicable to clinical situation. The development of mature collaterals in response to gradual coronary occlusion prevents myocardial infarction and preserves ventricular function, and must be viewed as an extremely fortunate and beneficial process. It is clear however, that the need to utilize collaterals alters neurohumoral regulation of myocardial perfusion, not only because of unique vasomotor properties of

the collaterals themselves, but also because of changes in other aspects of the vasculature.

Acknowledgments: Dr. Harrison is an Established Investigator of the American Heart Association. Supported by NIH grants HL32717 and HL 390016, Ischemic SCOR HL 2011046, and a Merit Review Grant from the Veterans Administration

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17

VENOUS LEVEL COLLATERALS IN THE CORONARY SYSTEM

Michael V. Cohen and James M. Downey***
*Departments of Medicine * and Physiology**, University of South Alabama, Mobile, Alabama, U.S.A.*

This chapter will recount a series of experiments that we have recently performed to evaluate the function of the venous level coronary collateral vessels. In reviewing those studies the hemodynamics of venous level collateral connections in the heart will be explored and their potential nutritional contribution will be evaluated. We will then attempt to discuss those studies in light of work done by others in the field.

Coronary venous collateral channels have largely been ignored in favor of the more approachable and often studied arterial collateral vasculature. The residual nutrition delivered to ischemic myocardium by collateral connections is a major determinant of the extent of cell death following a coronary occlusion (1, 2). To that end a great deal of study has been devoted to measuring collateral flow and identifying interventions which might augment it. While the tracer microsphere method has emerged as the method of choice for measuring collateral flow in the coronary system, one shortcoming of this technique is its inability to detect any blood entering the ischemic zone from post-capillary anastomoses such as possible venous level collateral vessels. Measurement of regional coronary venous blood flow by a catheter positioned at selected sites in the coronary venous circulation (3, 4) has become a clinically popular technique for measuring residual flow to ischemic myocardium. Unfortunately, venous-level collaterals corrupt such measurements whenever the venous flow is contaminated by venous blood from other arterial perfusion beds. Despite a relative lack of interest shown in venous-level coronary collaterals by physiologists and other scientists, clinicians have seized on the coronary venous system as an alternate pathway to deliver oxygenated blood to beleaguered myocardium in the perfusion territory of stenotic or occluded arteries. Thus, retroperfusion of the coronary sinus and great cardiac vein with arterial blood has been proposed to provide supplemental perfusion to ischemic myocardium which would limit infarct size (5, 6), preserve myocardium during surgical procedures necessitating cardiac arrest (7), and promote revascularization of ischemic myocardium when other surgical approaches are not feasible (8). In this chapter we will

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

attempt to estimate how much retroperfused blood actually has nutritional access to the tissue and how much is simply shunted through anastomotic channels to the right atrium. Hence, coronary venous collaterals must now be viewed differently. The coronary veins are not merely conduits for drainage of deoxygenated, metabolite-laden blood but also must be regarded as having an important potential role in the salvage of ischemic myocardium. This review will summarize what is currently known about the coronary venous circulation. To begin, we would like to summarize a series of studies that we have performed which illustrate the presence and function of venous level collateral connections.

Hemodynamic and dye studies reveal venous level collaterals

Experiments demonstrate that low-resistance anastomotic channels do exist between adjacent venous segments in the heart (9). The distal left anterior descending coronary artery (LAD) and great cardiac vein (GCV) in open-chest dogs were cannulated, and the perfusion tubing connected to a femoral artery and a jugular vein respectively. A continuous infusion of indocyanine green was administered proximally into the arterial perfusion line while distal arterial and venous blood samples were simultaneously withdrawn.

In only 1 of the 11 dogs examined was the concentration of indocyanine green in the coronary venous drainage appreciably less than that in the simultaneously collected coronary arterial blood. Clearly the vein in this one animal did not exclusively drain the tissue supplied by the cannulated LAD. In the remaining 10 dogs, the venous absorbance averaged 92.1 ± 4.1% (pNS) of that in the coronary artery indicating that *during normal hemodynamic conditions the GCV drainage was derived almost entirely from the LAD territory.*

In the 10 animals whose GCV blood had little contamination with blood from sources other than the LAD, the venous effects of separate and combined LAD and GCV occlusions were remarkably consistent, and are exemplified by the typical response shown in figure 1. Under baseline conditions mean arterial and venous flows were equal in this heart. GCV occlusion abruptly decreased venous efflux to zero and pressure proximal to the obstruction increased to 31.7 mmHg. LAD flow decreased only slightly, however. Additional occlusion of the LAD lowered proximal GCV pressure to 13.3 mmHg while distal LAD pressure (peripheral coronary pressure) was 13.8 mmHg. Release of the arterial occlusion resulted in a striking reactive hyperemia with peak flow

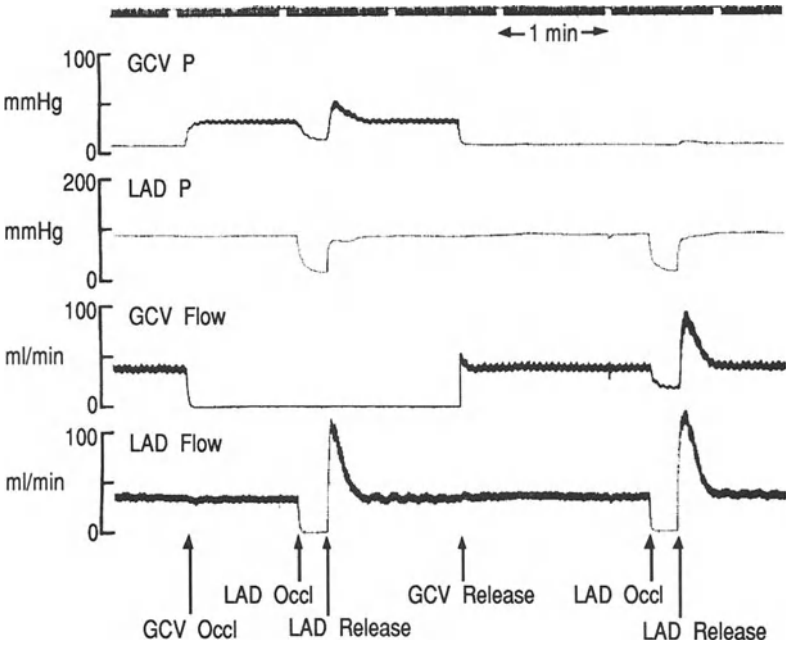

Fig. 1. Changes of great cardiac vein (GCV) and left anterior descending (LAD) pressures (P) and flows during combined GCV and LAD occlusions and then isolated LAD occlusion (occl) in one representative animal. Under baseline conditions GCV and LAD flows are identical. GCV occlusion produces a significant increase in GCV pressure, but following concomitant LAD occlusion GCV and LAD pressures decline to almost identical values. During isolated LAD occlusion GCV flow falls to approximately one-half of the control level. Immediately following release of the occlusion, there is a 3-fold arterial reactive hyperemia, but peak LAD flow exceeds peak GCV flow by 23%. Therefore, the hemodynamic responses of the coronary veins are similar to, but do not exactly mimic, those of the coronary arteries. Reprinted from Cohen et al *(9) with permission from the American Physiological Society.*

nearly triple baseline flow. Concomitant with the increase in arterial flow, venous pressure in the occluded GCV rose to 49 mmHg. Finally release of the venous occlusion caused a transient venous reactive hyperemia.

The response during occlusion of just the LAD is also depicted in figure 1. LAD occlusion again decreased peripheral arterial pressure to 13.8 mmHg but GCV flow was only halved. Release of the occlusion caused a transient three-fold increase in arterial flow over the baseline level and this was mimicked by a sharp increase in venous flow. The peak reactive hyperemia in the arterial circuit, however, always exceeded peak venous flow.

During combined LAD and GCV occlusions, proximal GCV pressure for the entire group of 10 dogs ranged from 6.9 to 18.2 mmHg, while the range in the distal LAD was 10.0 to 22.5 mmHg. These two pressures were typically within 2 mmHg of each other. The average pressure in the arterial segment was 13.6 ± 1.3 mmHg and in the venous segment was 12.4 ± 1.2 mmHg (pNS).

During LAD occlusion when LAD flow fell to zero, GCV flow in the group of 10 dogs decreased by only 56.5 ± 3.7%. The increase in venous flow during arterial reactive hyperemia consistently fell short of the peak arterial level. Whereas peak LAD flow averaged 102.2 ± 6.1 ml/min, peak venous flow was only 60.9 ± 8.2 ml/min or 58.6 ± 6.7% of peak LAD flow. Even when the venous flows were scaled to account for the differences in baseline arterial and venous flows before arterial occlusion in 4 dogs, peak GCV flow averaged only 74.8 ± 2.7% of the LAD response. *The dissociation between arterial and venous flows can best be explained on the basis of collateral channels at a venous level.*

The resistance model

In order to quantitate the amount of collateralization and its position in the venous bed, we used a modeling approach. The coronary circulation can be represented

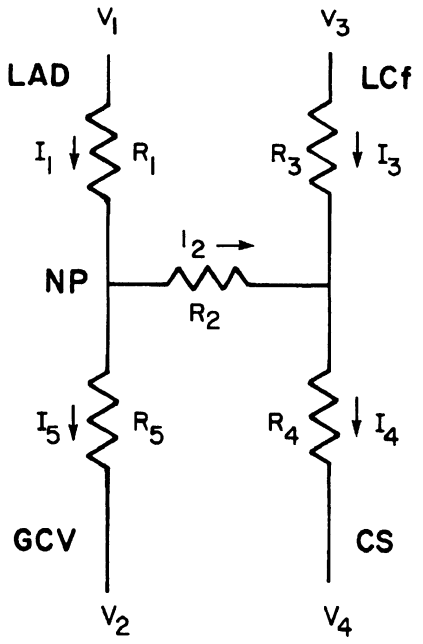

Fig. 2. Electrical analog model of the coronary vasculature. Abbreviations: CS = coronary sinus; I_1, R_1, V_1 = flow, resistance, perfusion pressure of left anterior descending coronary artery (LAD); I_2, R_2 = flow, resistance of venous collaterals; I_3 R_3, V_3 = flow, resistance, perfusion pressure of left circumflex artery (LCf); I_4, R_4, V_4 = flow, resistance, pressure of CS; I_5, R_5, V_5 = flow, resistance, pressure of great cardiac vein (GCV); NP = node pressure. Reprinted from Cohen et al *(9) with permission from the American Physiological Society.*

by the simple electrical model depicted in figure 2 where V_1, V_3, V_4, V_5 represent the LAD, left circumflex (LCf), coronary sinus, and GCV pressures, respectively. R_1 and R_3 are the net resistances across the LAD and LCf arterial beds, while R_5 and R_4 are resistances of the GCV and the coronary sinus, respectively. R_2 is the resistance of venous collaterals connecting the LAD and LCf venous beds. Flow across each of the 5 resistors represents flow in that vascular segment and is represented by I_1 through I_5. The node pressure (NP) is the pressure at the junction of the venous collaterals with the main venous channels. Using Ohm's law, algebraic expressions can be written to define I_1, I_3, V_1-V_2, V_3-V_4, and V_1-V_3. Solution of the simultaneous equations yields the following expressions:

$$I_1 = \frac{(V_1-V_2 + R_5I_2)}{(R_1+R_5)}$$

$$I_2 = \frac{(R_1+R_5)(R_3+R_4)(V_1-V_3) - R_1(R_3+R_4)(V_1-V_2) + R_3(R_1+R_5)(V_3-V_4)}{R_1R_5(R_3+R_4) + R_2(R_1+R_5)(R_3+R_4) + R_3R_4(R_1+R_5)}$$

$$I_5 = I_1 - I_2$$

$$NP = V_1 - I_1R_1.$$

To facilitate solution of these equations certain assumptions had to be made. As noted above, simultaneous occlusion of the LAD and GCV produced near-equalization of pressures in the obstructed arterial and venous segments. Under these conditions it was assumed that the peripheral coronary artery pressure was equivalent to NP. It was also assumed that occlusion of both the LAD and GCV would abolish venous collateral flow making NP at the LAD and LCf ends of the venous collateral equal. Hence the ratio of R_3/R_4 would be $(V_3 - PCP)/(PCP - V_4)$. Additional assumptions that LAD occlusion does not affect the R_3/R_4 ratio, that this ratio and the baseline R_1/R_5 ratio are equivalent, and that $V_4 = V_2$ permitted determination of the R_1/R_5 ratio.

Because V_1, V_2, I_1, and I_5 under baseline conditions are known and since V_1-$V_2 = I_1R_1 + I_5 R_5$, R_1 and R_5 could be calculated. Flow and resistances were normalized for the mass of the LAD perfusion territory. Because normalized resistances were assumed to be equal in the LAD and LCf regions, we set $R_1 = R_3$ and $R_5 = R_4$ for baseline conditions. To simulate GCV occlusion R_5 was set to 10,000 resistance units (RU). One RU is equal to 1 mmHg.min/ml of blood. With the GCV clamped, NP was assumed to equal the pressure in the stagnant venous bed. A value for R_2, an unknown, was selected and then varied until the calculated I_1, I_5 and NP approximated the measured arterial and venous flows and distal venous pressure during GCV

occlusion, respectively. R_2 was then assumed to remain constant for the other experimental conditions. R_1 was set to 10,000 RU when the LAD was occluded.

Using the assumptions noted, calculated baseline arterial resistance (R_1) averaged 1.77 ± 0.17 mmHg·min·ml^{-1}. In the 7 dogs in which the mass of LAD and LCf regions was determined, the LAD perfusion territory averaged $33.8 \pm 0.9\%$ of the total left ventricular mass. When R_1 was normalized for the mass of LAD myocardium, the average resistance was 0.058 ± 0.009 mmHg·min·ml^{-1}·g^{-1}. Venous resistance (R_5) averaged $10.0 \pm 1.5\%$ of arterial resistance, while venous collateral resistance (R_2) was $9.9 \pm 3.0\%$ of R_1. Thus, the model revealed that the venous collateral had a very low resistance (only 10% of the vascular resistance for the entire segment, $R_1 + R_5$). That it was post-capillary was revealed by the fact that it was located distal to 90% of the segment's resistance.

To determine the ability of the model to describe the hemodynamic behavior of the coronary arterial and venous circuits, the arterial (I_1) and venous (I_5) flows predicted from real pressure data and the derived vascular resistances were compared to actual

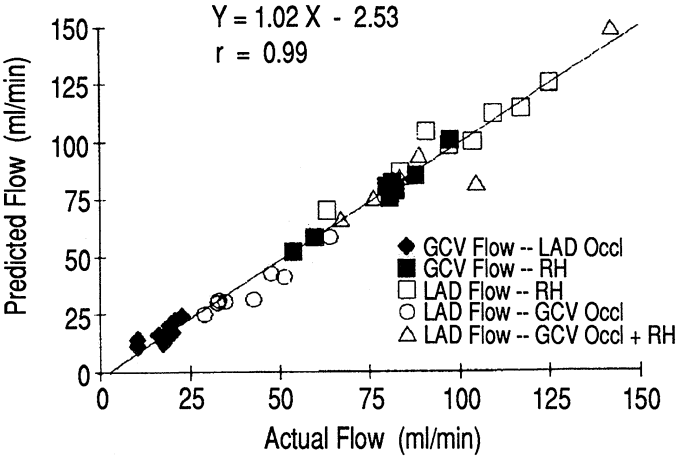

Fig. 3. Graph of predicted arterial and venous flows calculated by computer based on an electrical analog model (see Fig. 2) plotted against actual flows during either left anterior descending (LAD) occlusion (occl), LAD reactive hyperemia (RH), great cardiac vein (GCV) occlusion, or LAD reactive hyperemia and simultaneous GCV occlusion. Solid symbols represent GCV flows and open symbols represent LAD flows. For purposes of this comparison the measured venous flows in 4 animals were adjusted upwards by the same percentage necessary to make baseline arterial and venous flows equivalent, a necessary prerequisite of the model. The linear regression line drawn through the data points is almost superimposed on the line of identity. Reprinted from Cohen et al *(9) with permission from the American Physiological Society.*

flow measurements during GCV occlusion, LAD occlusion, LAD reactive hyperemia, and combined GCV occlusion and LAD reactive hyperemia. Additionally, calculated node pressure was compared to coronary venous pressure during the three perturbations (GCV occlusion, combined GCV and LAD occlusion, GCV occlusion and LAD reactive hyperemia) in which the venous circuit was obstructed. The flow and pressure correlations are depicted in figures 3 and 4. *The calculated linear regression equations for flow (predicted flow = 1.02 * actual flow - 2.53, r=0.99) and pressure (node pressure = 1.00 * GCV flow + 0.17, r=0.97) demonstrate near superimposition on the line of identity indicating that the model did accurately predict the hemodynamics.*

Retrograde flow collection prevents uptake of microspheres but not a diffusible tracer

The above study demonstrated that low resistance venous level collateral vessels course between adjacent veins in the dog heart. It must now be asked whether these vessels affect the hemodynamics of the coronary system. Because they occur deep in the microcirculation, they might be capable of some nutritional support to an ischemic

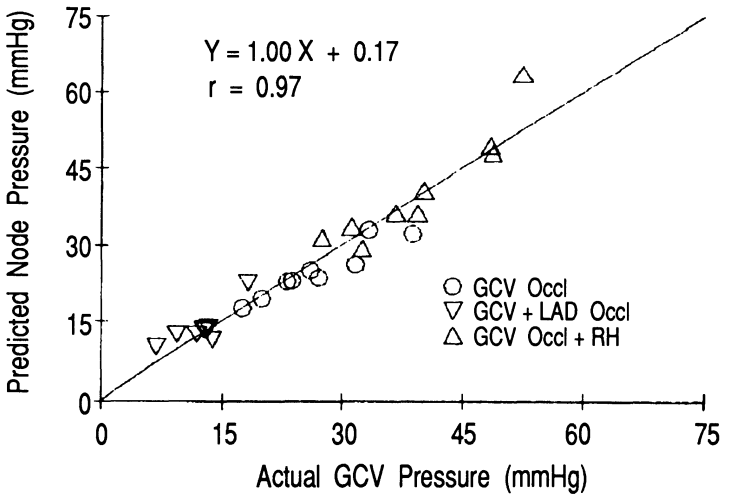

Fig. 4. Graph of predicted node pressure calculated by computer based on electrical analog model (see Fig. 2) plotted against actual great cardiac vein (GCV) pressure. All data were collected during GCV occlusion (occl). In addition to the latter some pressures were recorded during simultaneous left anterior descending (LAD) occlusion, while others were recorded during reactive hyperemia (RH) after release of the LAD occlusion. The linear regression line drawn through the data points is nearly identical to the line of identity. Reprinted from Cohen et al *(9) with permission from the American Physiological Society.*

segment. The key to testing for such a role would be to greatly reduce the pre-capillary collateral blood flow by collecting retrograde coronary blood flow. In this condition a dog's coronary arterial branch is occluded proximally and the distal segment is vented. When the distal segment is vented, arterialized blood flows retrogradely and issues from the segment. The blood flowing retrogradely represents blood flowing through the pre-capillary collateral blood vessels, most of which occur at the large artery level. Normally that blood is destined for the microcirculation, but when the obstructed artery is opened to atmospheric pressure the blood takes the path of least resistance by bypassing the high resistance of the capillaries and instead flowing backwards through the artery and cannula.

It was first determined whether either radiolabeled 15 μm microspheres (a particulate blood flow tracer) or simultaneously injected ^{86}Rb (a diffusible blood flow tracer) could be deposited in the ischemic zone during retrograde flow collection (10). Open-chest anesthetized dogs were used for the experiment. The isotopes were mixed in a 5 cc syringe and injected as a bolus into the left atrium while the LAD branch was occluded and retrograde flow collected. Blood flow was calculated by the method of Rudolph and Heymann (11). At the end of the study fluorescein dye was injected into the left atrium to mark the well perfused regions. As soon as the dye was seen to stain the heart, the heart was removed and sectioned. Care was taken to cut samples from the geometric center of the ischemic zone such that no fluorescent tissue was included in the samples. The samples were then weighed and the activity of each label was measured in a gamma counter.

Radioactive microspheres revealed a flow of 1.36 ± 0.19 ml/min/gm to the well perfused circumflex zone but only 0.002 ± 0.001 ml/min/gm to the ischemic LAD region. At the same time an appreciably greater amount of ^{86}Rb was found in the ischemic zone. Flow to the normal region measured by rubidium was 0.93 ± 0.12 ml/min/gm and that to the ischemic LAD region was 0.04 ± 0.01. *Thus 20 times more of the diffusible ^{86}Rb gained access to the ischemic tissue than the particulate microspheres during retrograde flow diversion.* Analysis showed that on the average only 117 radioactive microspheres were seen in each ischemic sample, whereas as many as 2,047 would have been expected based on the ^{86}Rb measurements. Clearly an occult or hidden component of blood flow was reaching the ischemic zone which was not indicated by the microsphere measurements. This is exactly the pattern that would be expected if venous-level collaterals were providing nutritional support to the ischemic

region since venous blood would be devoid of microspheres completely extracted by the myocardium in the first pass and therefore never reaching the coronary veins but rich in recircu-lating ^{86}Rb.

Xenon wash-out also reveals venous level collaterals

In additional studies (10) the occult component of blood flow to ischemic tissue was estimated by a wash-out method which is more sensitive to low flows than a wash-in method using either microspheres or ^{86}Rb. Again open-chest dogs were used. The cannulated arterial branch served less than 10% of the left ventricle and thus coronary occlusion had a negligible effect on overall cardiac performance. Figure 5A shows a characteristic ^{133}Xe washout curve from normally perfused myocardium. When the tissue is loaded with ^{133}Xe and then perfused with non-radioactive blood the flow/unit volume of tissue is reflected in the logarithmic slope of the washout curve. The semi-

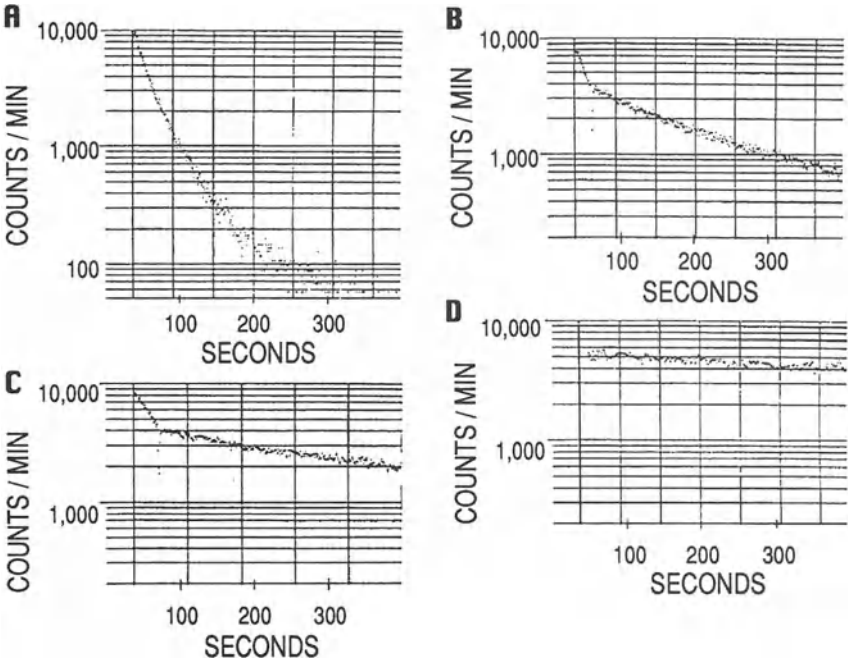

Fig. 5. ^{133}Xe washout curves from a representative dog. A: during normal perfusion; B: during occlusion of the perfusion line; C: during collection of retrograde flow; and D: during collection of retrograde flow after embolizing the segment with 15 μm non-radioactive microspheres. Reprinted from Yoshida et al *(10) with permission from the American Physiological Society.*

log plot reveals a fast phase for the first 60 seconds followed by a slower phase. For all animals studied the fast phase yielded an average flow rate of 0.79 ± 0.07 ml/min/g and accounted for more than 90% of the total injected isotope. The slow phase accounted for the remaining 10% and yielded a flow rate of 0.39 ± 0.04 ml/min/g. The fast phase was assumed to represent perfusion of ventricular muscle while the slow phase was assumed to represent washout from connective tissue (12).

Figure 5B shows the washout following coronary occlusion after 50 seconds in the same heart. Note that occlusion of the artery during either the fast or the slow washout phase produced an identical residual washout rate. Although two distinct compartments could be resolved in resting hearts, only one monoexponential washout rate was evident following occlusion. This rate averaged 0.26 ±0.04 ml/min/g and was assumed to represent total collateral flow.

Figure 5C shows the effect of proximally occluding the perfusion line and opening it distally to the atmosphere to collect retrograde flow. Collection of retrograde flow should shunt most of the pre-capillary collateral flow away from the exchange vessels. Note that ^{133}Xe continued to be washed out at a rate of 0.11 ± 0.02 ml/min/gm, despite the successful diversion of pre-capillary flow as retrograde flow. Although there was some ^{133}Xe activity in the blood flowing retrogradely, figure 6 reveals that most of the ^{133}Xe was leaving via the coronary sinus. Furthermore, because washout continued monoexponentially during retrograde flow collection this residual flow must have had uniform access to all of the tissue in the ischemic zone.

To determine whether perhaps a small fraction of the flow from the pre-capillary collaterals found it favorable to flow in the antegrade direction, the pressure against retrograde flow was elevated. No effect on washout rate was seen until back pressure exceeded 15 mmHg. The results suggested that the flow which washed out the ^{133}Xe was not simply an antegradely-flowing portion of the pre-capillary-derived collateral flow since a high back pressure was required before any of the retrogradely-flowing blood could be diverted to the antegrade direction (10). The fact that no detectable antegrade diversion occurred until a driving pressure exceded 15 mmHg suggested that a vascular waterfall in the antegrade vasculature probably opposed such flow (13).

Figure 5D shows that complete embolization of the microcirculation with 15 μm microspheres introduced into the LAD still did not abolish the ^{133}Xe washout although flow was slowed to 0.03 ml/min/g. All dogs were fibrillated at the end of the study in the middle of a washout curve and in every case no subsequent washout could

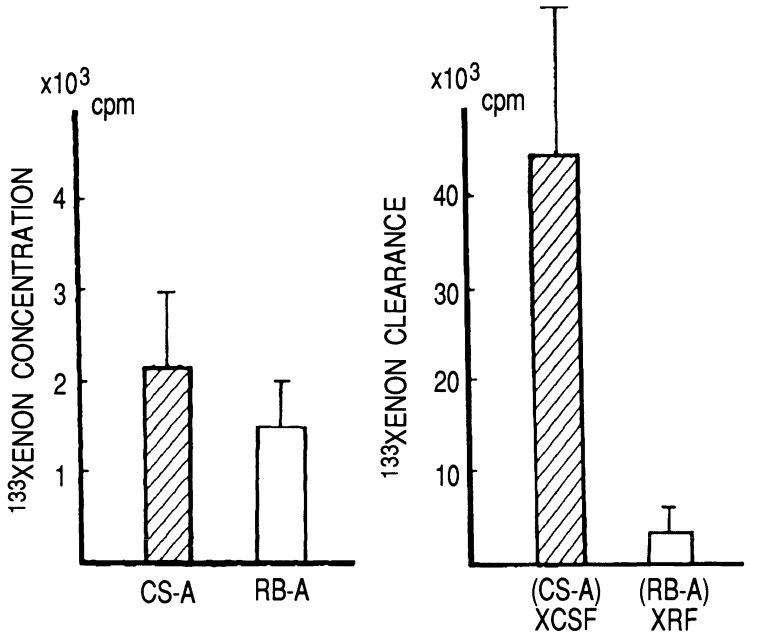

Fig. 6. ^{133}Xe concentration (left) and clearance (right) in the coronary sinus (CS), and in the blood flowing retrogradely (RB) from the LAD cannula. Clearance was calculated as concentration times flow. Note that although the concentration of isotope is similar in both locations, much more isotope is being removed via the veins than in the blood flowing retrogradely from the artery. Reprinted from Yoshida et al *(10) with permission from the American Physiological Society.*

be detected. The fibrillation studies proved that the ^{133}Xe was being washed out by flowing blood and was not leaving the heart by direct diffusion into the atmosphere. *The ^{133}Xe studies revealed that convective flow continued through the tissue both when retrograde flow was being collected and when the precapillary vasculature was completely embolized. It is likely that flow through venous level collaterals accounted for the washout.*

Venous retroperfusion studies

In these studies the potential for augmenting venous level nutrition of ischemic myocardium by retroperfusing the coronary sinus was evaluated (9). Potential nutritional exchange between venous conduits and myocardial cells was evaluated by ^{133}Xe washout. Following LAD occlusion, flow in the ischemic bed decreased from 0.86 ± 0.16 to 0.13 ± 0.02 ml/min/g. There was no appreciable effect of GCV occlusion on ^{133}Xe washout. However, retroperfusion of the coronary venous circulation with arterial blood at a pressure of approximately 60 mmHg significantly

enhanced washout in the ischemic region by 85% to 0.23 ± 0.03 ml/min/g ($p<0.005$). The effect of retroperfusion of the GCV on isotope washout from the ischemic myocardium can be seen in figure 7. Superimposition of ^{133}Xe washout curves recorded during LAD occlusion, LAD and GCV occlusion, GCV retroperfusion, and finally normal perfusion demonstrates sequential improvements in myocardial blood flow. The effect of direct injection of ^{133}Xe into the GCV after establishment of retroperfusion with arterial blood is shown in figure 8. Curve stripping yielded a very fast phase (assumed to be passage of the tracer through the vascular compartment) and a considerably slower late phase which was identical to that seen with washout of an arterial bolus from ischemic myocardium indicating that they both represent washout of the myocardial compartment. *The initial count rate of the slower phase, assumed to represent myocardial cell washout, averaged only 10.1 ± 1.5% of that following arterial injection indicating that the venous route was only 10% as efficient as arterial injection in depositing isotope into the tissue.*

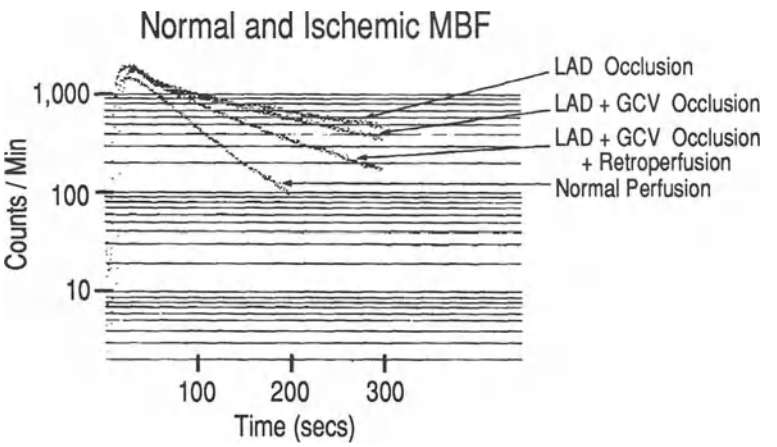

Fig. 7. Superimposed ^{133}Xe washout curves from one representative animal during normal perfusion, left anterior descending (LAD) occlusion, combined LAD and great cardiac vein (GCV) occlusion, and GCV retroperfusion with arterial blood following simultaneous LAD and GCV occlusions. For all curves an identical dose of isotope dissolved in saline was injected into perfusion tubing of a patent LAD. For the 3 curves recorded during LAD occlusion, the LAD was occluded 50 secs after ^{133}Xe injection to permit clearance of the isotope from the tubing and epicardial veins. Reprinted from Cohen et al *(9) with permission from the American Physiological Society.*

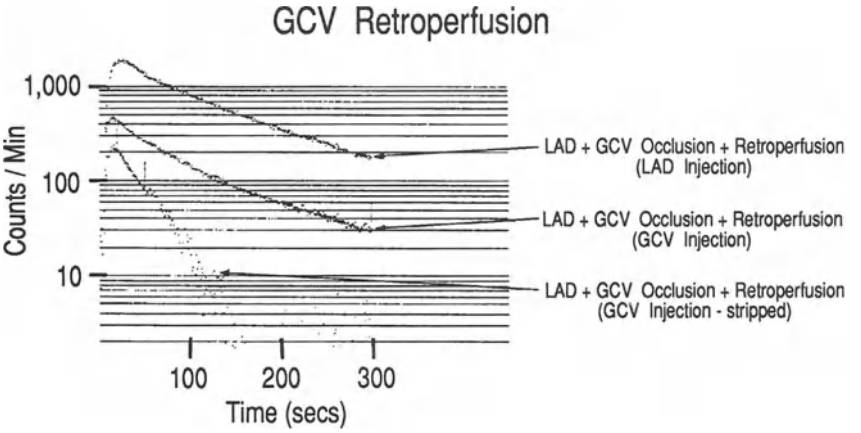

Fig. 8. Superimposed ^{133}Xe washout curves from the same animal as in figure 7 during retroperfusion of the great cardiac vein (GCV) with arterial blood following simultaneous left anterior descending (LAD) and GCV occlusions. The top washout curve was recorded after ^{133}Xe injection into the perfusion tubing of a patent LAD and then LAD and GCV occlusion and initiation of retroperfusion 50 secs later. The middle curve was recorded after isotope injection into the GCV perfusion circuit following LAD and GCV occlusions and commencement of retroperfusion. Because at least 2 components of this latter curve were recognized, the slower component designated by vertical lines above the curve was stripped off to reveal a very fast washout component, the bottom one of the 3 curves. The $t_{1/2}$ of the slower washout component of the middle curve following GCV isotope injection was virtually identical to that determined for the top curve following arterial isotope delivery. Reprinted from Cohen et al *(9) with permission from the American Physiological Society.*

Venous level collaterals: best explanation of the data

These studies reveal that radiolabeled microspheres failed to enter the field of the LAD when retrograde flow was collected from the LAD cannula. At the same time, however, an appreciable amount of diffusible ^{133}Xe could be washed out of the tissue or ^{86}Rb could be washed into the tissue. Obviously, there is a portion of the collateral nutrition to the ischemic zone which microspheres failed to measure. The most attractive explanation for this hidden flow are venous level collateral channels which perfuse the ischemic zone with venous blood from post capillary vessels. In support of this theory is the present demonstration of a very low resistance, venous level anastomotic network in the heart (9). When the LAD is occluded flow delivered by arterial level collaterals would be expected to drop to less than 20% of the preocclusion level. Yet GCV effluent was noted to continue at 44% of the preocclusion value. As our

resistance model predicts, the favorable pressure gradients during arterial occlusion draw venous blood from adjacent beds into the small veins of the ischemic zone.

The retrograde perfusion studies further reveal that perfusion of the venous anastomotic channels does provide some nutrition of the tissues. On a single pass, nearly 10% of the contained ^{133}Xe entered the tissue compartment. This exchange could occur directly across the walls of the small veins or some venous blood might be propelled retrogradely into the large capacitance of the capillary bed (14) with the mechanical action of the heart beat. The attenuation of ^{133}Xe washout by embolization of the microcirculation would support the latter theory as arteriolar embolization should reduce the amount of venous blood that could ebb and flow into the capillaries. Others have noted that monastral blue injected into the coronary sinus during retroperfusion appears in capillaries, venules, and arterioles of ischemic myocardium (15) and that simultaneous occlusion of the LAD and GCV results in a positive pressure gradient between the vein and artery, probably accounting for the selective staining of ischemic myocardium when monastral blue is introduced into the coronary sinus and the absence of staining of normally perfused myocardium (16). Finally Beyar *et al* (17) observed that coronary sinus occlusion in dogs resulted in bidirectional flow in the coronary artery distal to an occlusion, again supporting the hypothesis that venous blood can be propelled retrogradely into the capillary bed.

The coronary venous anatomy is elegantly demonstrated in monographs by James (18) and by Baroldi and Scomazzoni (19). Yet the physiological significance of this anatomy has remained virtually unexplored. To this end the simple resistance model of the venous level anastomoses was found to be invaluable to our understanding in this study. A primary assumption of the model was that the GCV drains exclusively the LAD territory. Hammond and Austen (20) measured oxygen saturations in isolated canine heart preparations and estimated that only 40% of LAD flow drained into the coronary sinus, while most of the remainder drained directly into right-sided chambers. However, Klocke and co-workers (21) using an in situ animal preparation concluded that two-thirds of LAD inflow drained into the GCV. Perhaps more importantly GCV effluent was virtually free of circumflex adulteration (22). In all but one of the experimental animals described above, the concentration of indocyanine green in the GCV was virtually the same as that in the LAD inflow.

Given these observations, the effect of LAD occlusion and subsequent release on GCV outflow was unexpected. Following LAD occlusion a meager collateral flow,

perhaps only 10-20% of normal antegrade flow, perfuses the LAD myocardium. In our experiments (10) retrograde flow, an estimate of arterial level collateral flow, averaged only 14.1 ± 6.8% of baseline LAD flow. Yet GCV outflow during LAD occlusion averaged 44% of preocclusion flow. These data are surprisingly similar to those obtained by Feldman *et al* (3) who monitored GCV flow during balloon occlusion of the LAD in man. Additionally we found that venous flow during arterial reactive hyperemia consistently fell short of peak LAD flow. During reactive hyperemia pressure gradients would favor flow through venous collaterals away from the study bed.

All of the above behavior cannot be satisfactorily explained unless it is postulated that the GCV has an additional source of venous blood during LAD oligemia and that drainage pathways other than the GCV are present during LAD hyperemia. Venous anastomoses and collaterals have been identified in latex and vinyl casts of the coronary circulation (7, 18, 19, 23), postmortem injection studies (24, 25, 26, 27), venographic examinations (28), as well as in vivo injections of fluorescent dyes (29). Furthermore, several physiological studies have demonstrated that venous flow can be shunted to alternate venous pathways if conditions of a primary pathway are changed (21, 30, 31). Hence we proposed a model postulating the existence of functional venous collaterals. Predictions based on the simple resistance model agreed almost perfectly with actual data (Figs. 3 and 4). The observation that the derived resistance of the venous collaterals (R_2) was as low as that of the main venous pathways (R_5) was truly surprising. This observation at first appears to be anomalous since coronary arterial collateral resistance is many-fold higher than arterial resistance. However, in their casts of human hearts Baroldi and Scomazzoni (19) observed that 63.6% of the specimens demonstrated numerous venous anastomotic vessels which were mostly of the same caliber as the main channels. The consequence of this low resistance is the substantial redistribution of flow that may occur in response to relatively small pressure gradients. Therefore, it is not surprising that venous collaterals become significant whenever LAD flow is disturbed.

Conspicuously absent from our model are vascular waterfalls (13). Bellamy reported that coronary flow ceased when there was still a positive pressure gradient for flow (32). Although the presence of such a critical closure has been challenged by Spaan (33), many believe that the phenomenon is real and that a waterfall of between 10 and 20 mmHg is present somewhere in the coronary bed (14, 34, 35, 36). In light of those studies it was surprising to find that GCV and LAD pressures equalized after

simultaneous occlusion of the GCV and LAD. An intervening waterfall should have kept these two pressures separate. Several explanations are possible. The first is that critical closing behavior may simply be an artifact of the preparations employed and really does not exist. In fact, all of the critical closing data obtained with perfused coronary branches could be explained by venous-level collateral connections which would keep the apparent outflow pressure close to the node pressure, NP in figure 2, rather than that in the coronary sinus. That would not, however, explain the data in studies where the entire left main coronary artery was perfused (14). A more likely possibility is that waterfalls cannot be maintained in stagnant columns. Although the pressure-flow curve for a perfused coronary artery projects to a positive pressure intercept, demonstration of zero flow at that pressure has been difficult (36, 37). It should be noted that the presence of a 10 mmHg waterfall in either the arterial or the venous segment does not greatly alter the model's behavior except for the double occlusion condition. For the sake of simplicity we chose not to include waterfalls since our simple model was adequate to explain all of the observed phenomena and any attempt to include waterfalls would have only introduced more assumptions.

Clinical implications of the presence of functional coronary venous collaterals

GCV drainage has often been used to sample metabolites from the LAD perfusion territory. It has also been used to monitor the amount of collateral flow following LAD occlusion during angioplasty (4), and to assess changing patterns of local perfusion following relief of coronary artery occlusion (3). Admixture of non-GCV blood with GCV efflux in the coronary sinus has always been recognized as a technical limitation of these techniques. But the present data reveal that the admixture from non-LAD sources during ischemic conditions will introduce an error of an unexpectedly large magnitude.

Retroperfusion of the heart

Pratt (38) was the first to suggest that retroperfusion of the capillary bed by introduction of arterial blood into the coronary sinus might have beneficial effects. He forced defibrinated arterial blood into the coronary sinus of freshly extirpated hearts and noted both ventricles continued to contract in a coordinated manner for up to 10 minutes. More recently there has been a resurgence in interest in retroperfusion of the coronary venous system. Following coronary artery occlusion, retroperfusion reportedly can enhance global cardiac function (5, 39, 40, 41, 42, 43, 44), regional

performance of ischemic tissue (5, 6, 39, 40, 41, 43, 44, 45, 46), diminish the magnitude of ischemic S-T segment elevation (39, 40, 41, 42, 43, 45, 47, 48), improve myocardial oxygenation (29, 40) and energy metabolism (29), prevent ischemic changes in ultrasonic backscattering properties of myocardium (46), and decrease the size of infarcts (5, 41, 42, 44, 48, 49).

Although some of the beneficial effects of coronary venous retroperfusion could possibly be related to mechanical factors (50) without any actual benefit to ischemic myocardium, investigators have reported that retroperfusion can increase blood flow to ischemic tissue by as much as 50-60% (6, 40, 45, 47, 51). However, in all but one of these investigations (40) radioactive microspheres were injected into the left atrium. It is not obvious how arterial-level collateral flow could be affected by retroperfusion. It seems unlikely that arterially-borne microspheres could have traveled retrogradely through veins to the microcirculation. Direct injection of microspheres (7, 52) macroaggregated albumin (53), or colloidal carbon (40) into the coronary veins leads to deposition in the ischemic myocardium. But again, all of these studies employed particulate matter which evidently became lodged in some unknown vascular bed. The precise relationship between deposition in the myocardium and nutritional support of the ischemic tissue cannot be inferred. Cibulski and colleagues (54) demonstrated that ^{43}K and ^{85}Kr injected into the coronary sinus could concentrate in the myocardium, especially after constriction of the coronary sinus. Markov *et al* (40) used ^{85}Kr clearance to document more than a 100% increase in washout from ischemic tissue following initiation of retroperfusion of the coronary sinus with arterial blood. Furthermore ^{85}Kr injected into the coronary sinus could be detected in sampled LAD blood.

Retroperfusion clearly improved nutritional blood flow to ischemic myocardium in our study as evidenced by the ^{133}Xe washout (Fig. 7) (9). The average $t_{1/2}$ decreased and therefore tissue blood flow increased by 85%. For these washout experiments the radioactive gas was injected only into the LAD perfusion line and therefore taken up only by tissue in the LAD perfusion territory. Hence the improved washout was directly related to the retroperfusion. A recent investigation using PET scans in dogs with coronary occlusions demonstrated that coronary sinus retroperfusion did indeed enhance nutrient blood flow to and improve myocardial metabolism of ischemic myocardium (55).

Although it seemed unlikely that retroperfusion may have somehow increased arterial-level collateral flow, the data could not exclude this possibility. To address this

issue ^{133}Xe was injected into the venous circuit after retroperfusion had been initiated. Only 45% of the injected isotope reached the ischemic region, the rest presumably having entered venous collaterals en route to the coronary sinus. More than 2/3 of the isotope entering the field washed out very quickly with a calculated flow of 2.4 ml/min/g, or 3 times the rate of washout during normal perfusion. This rapid washout phase probably represents exchange with the venous intravascular blood rather than the myocardial cells. Only about 10% of the injected isotope actually reached the myocardium, suggesting that retroperfusion is very inefficient as a nutritional source. These data confirm similar assertions based on coronary venous injections of microspheres (7, 52). Calculations suggest that only 14-25% of normal myocardial oxygen requirements could be met by coronary sinus retroperfusion (56). The inefficiency is in part related to the multipe arterioluminal, arteriosinusoidal, and Thebesian veins which probably drain 30% of arterial blood introduced into the coronary sinus and the numerous venous connections to other vascular territories which divert arterial blood before it can reach the ischemic area. If the retroperfusion is made more pulsatile as in synchronized retroperfusion systems better irrigation of the microcirculation would likely result in improved nutritional efficiency.

As noted above some recent studies have demonstrated bidirectional flow in the coronary artery distal to an occlusion when the coronary sinus is obstructed, thus in part explaining the ability of retroperfused blood to affect myocardium in the ischemic zone. But Taira and colleagues (29) have also demonstrated that the myocardium in the distribution of an occluded LAD in rats showed uniform NADH fluorescence (implying deficient tissue oxygenation and inhibited oxidative phosphorylation) except for nonfluorescent 140 μm-wide zones surrounding the epicardial veins, signifying continuing oxygenation of this peri-vein region. Therefore, the venous system can apparently function as an oxygen delivery and exchange circuit, supporting the use of retroperfusion for temporary, rapid, albeit partial, reoxygenation of ischemic tissue.

Given this extensive scientific background clinicians have begun to use venous retroperfusion in patients with coronary obstructive disease to support ischemic myocardium. Accordingly retroperfusion is being proposed as an immediate measure to salvage jeopardized myocardium in clinical myocardial infarction (5, 6) and to preserve myocardial integrity during cardiac surgery (7, 57). Additionally, retroperfusion has been found to be eficacious in individuals with unstable angina pectoris (58). Balloon inflation can be successfully prolonged during coronary angioplasty with fewer

symptoms and less myocardial dysfunction if retroperfusion is employed (59, 60, 61, 62, 63). Cardioplegia is now routinely introduced through the coronary sinus by some surgeons (64), and coronary venous bypass grafting has been performed in patients with diffuse arterial disease or very small distal coronary arteries not amenable to arterial grafting (64). Thus venous retroperfusion has become a recognized tool in the treatment of ischemic heart disease.

Is the hidden flow to the tissue nutritional?

One may now ask whether the hidden flow which persists following coronary artery occlusion is nutritional. Although this flow provides a measurable perfusion to the ischemic zone, it is deoxygenated venous blood that flows into the ischemic zone. As seen in the results summarized above, the hidden flow was much better at washing ^{133}Xe out of the tissue than it was at washing ^{86}Rb into the tissue. The xenon measurements indicated a flow of 0.11 ml/min/g while the rubidium experiments indicated a flow of only 0.04 ml/min/g. The most likely explanation is that much of the rubidium was extracted from the blood on its first pass through the coronary capillaries so that there was less to be deposited as it passed into the ischemic region via the venous collaterals. Thus while this flow may wash away some metabolites or bring pharmaceuticals to the ischemic zone, it will not bring much oxygen. Furthermore, since the hidden flow appears to have a maximal delivery of about 0.1 ml/min/g, a negligible effect on infarct size would be expected even if the hidden flow could be fully oxygenated. Therefore, in the usual situation this source of collateral flow to ischemic myocardium would not be expected to have very much effect. However, addition of arterial blood to the venous circuit transforms the latter into an accessory distribution pathway for arterial blood and has already yielded important clincial benefits.

In conclusion, the presence of a low resistance collateral interconnection between the coronary veins has been demonstrated. The finding that considerable flow can occur across this circulation in response to small pressure gradients and that these vessels are capable of limited molecular exchange with the myocardium sheds new light on many past observations and supports continuing and future efforts to exploit the coronary venous circulation for treatment of ischemic heart disease.

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MICROVASCULAR COLLATERALS IN THE CORONARY CIRCULATION

H. Fred Downey
Department of Physiology, Texas College of Osteopathic Medicine,
Fort Worth, Texas, U.S.A.

INTRODUCTION

The existence and functional importance of collateral arterial vessels in the coronary circulation is well recognized (1, 2). Since coronary collateral vessels can often be observed, both visually and angiographically, models of the coronary collateral circulation have described macrovascular, interarterial anastomoses originating and terminating proximal to the microcirculation (3, 4, 5). Experiments designed to estimate the pressure at the origin of collateral vessels lend physiological support to this model (4, 5). However, a growing body of evidence indicates that additional, microscopic anastomotic pathways contribute significantly to total coronary collateral blood flow. This chapter reviews evidence for microscopic coronary collateral vessels and attempts to place in some perspective their functional importance.

ANATOMICAL STUDIES

Casts, micro-angiography and histology: Figure 1 illustrates collateral pathways which have been described in reports of anatomical studies of the human and canine coronary circulations. These studies have consistently demonstrated abundant microvascular anastomoses in the coronary circulation, at both the arteriolar and capillary levels (6, 7, 8, 9). The preponderance of microvascular anastomoses are homocoronary and connect branches of the same artery, but intercoronary microvascular anastomoses also connect perfusion territories supplied by different parent coronary arteries. It is not surprising that these intercoronary microvascular anastomoses are difficult to visualize and identify (9), since the perfusion territory interface comprises a small portion of the vascular system and considering the difficulty in delineating the parent arteries of microvascular anastomoses.

Post-mortem injection of colored silicone elastomers and dyes: In a study design specifically to identify microvascular anastomoses at the perfusion territory interface,

Wolfgang Schaper and Jutta Schapter, (eds.), Collateral Circulation.

Factor and coworkers (10, 11, 12, 13) injected adjacent coronary perfusion territories of isolated canine hearts *post mortem* with differently colored silicone elastomers (microfil). They found that each color was restricted to its respective territory, and they described the microvascular anatomy at the interface between the coronary perfusion territories as a series of "hairpin capillary loops" formed by branching capillaries which always doubled back and ran parallel to the parent vessel. Since no vessels were found to contain both color elastomers, the investigators concluded that there were no microvascular connections at the interface of coronary perfusion territories. Along the interface an irregular series of peninsulas extended from one territory into the other, an arrangement thought to account for intermediate values of flow and other variables found in tissue at the border of an acutely ischemic region (14). Despite contrary evidence from earlier anatomical investigations, these studies are frequently cited to support the view that coronary arteries are functional end-arteries with no microvascular anastomoses and lacking any capability to produce a gradient of perfusion at the border of an ischemic region. However, the ability of the elastomers to intermingle in the presence of microvascular anastomoses has not been demonstrated, so perhaps the first color to reach a vessel prevented entry by the other color due to the relatively high viscosity of the elastomers. Thus, results produced by this technique may not be definitive.

In contrast to the absence of intercoronary mixing of different colored silicone elastomers, Przyklenk et al (15) observed microvascular mixing of differently colored, low viscosity dyes infused into neighboring coronary arteries of isolated canine hearts. Microscopic examination of tissue from the perfusion territory interface revealed that approximately 20% of the capillaries contained both color dyes, and this proportion of dually labeled capillaries was not altered by cautery of epicardial collateral vessels. Furthermore, there was a positive correlation between the number of dually labeled capillaries and blood flow measured in this region by in vivo administration of radioactive microspheres into one of the parent coronary arteries. Not only did this study demonstrate the presence of microvascular anastomoses between adjacent coronary perfusion territories, it suggested that these anastomoses might be functionally important in delivering flow to acutely ischemic myocardium near the interface.

Przyklenk et al (15) did not measure the width of the region containing capillaries with both colors, nor did they determine the origin of the microvascular anastomoses. Although they described the anastomoses as "capillary," since differently colored dyes

were observed in the capillaries, flow through precapillary, arteriolar anastomoses could have produced these dually labeled capillaries.

In vivo studies with colored microspheres: In a collaborative study with Dr. Karel Rakusan and Nick Cicutti of the University of Ottawa, we have developed a novel technique to determine whether microvascular elements at the interface of coronary perfusion territories are perfused by both parent arteries (16). This technique utilizes simultaneous in vivo infusions of non-radioactive, colored microspheres (E-Z Trac, Los Angeles, CA; 17) into neighboring coronary arteries of normally perfused, in situ, working, canine hearts. When preliminary experiments revealed significant numbers of microvessels at the interface containing both colors of microspheres, we quantitated the transmural morphology of this zone of dual arterial supply.

Following anesthesia and thoracotomy, the left anterior descending (LAD) and left circumflex (LCx) coronary arteries were isolated, and twenty gauge needles attached to syringe pumps were inserted 1-2 cm from their origins. Five milliliter suspensions containing approximately 30 x 106 red or blue microspheres were infused into the LAD and LCx at constant and identical rates for 10 min. In initial experiments 12 ± 1.9 μm microspheres were utilized, but in most experiments more uniform 10 ± 0.2 μm microspheres were used, since the larger microspheres caused a small but significant decline in ventricular function. Three to five transmural samples approximately 1.5 cm^2 in cross-sectional area were cut along the interface between the LAD and LCx. These samples were frozen for cryotomic preparation of 40 μm serial sections for microscopic and morphometric analysis.

Microscopy revealed clustering of microspheres in their perspective perfusion territories, i.e., red microspheres in the LAD region beyond the interface zone and blue microspheres in the LCx region beyond the interface zone. Collateral flow as conventionally described was nil in this preparation, since only rarely was a microsphere of an alternate color observed within a perfusion territory distant from the interface. At the interface, a region of variable width contained both colors of injected microspheres, and numerous microvessels within this interface region contained microspheres of both colors.

At the interface, two zones containing both colored microspheres were identified and measured. An *interface transition zone* (ITZ) was defined as the region having intermingling, differently labeled microvessels, although both colors were not

necessarily observed in the same vessel. This was the maximum distance which microspheres supplied by one coronary artery penetrated into tissue also perfused by the other coronary artery. A *boundary watershed zone* (BWZ) was defined as the region having microvessels each containing both colors. The BWZ was always narrower and located exclusively within the ITZ. The presence of bicolored aggregates in microvessels of the BWZ was interpreted as evidence that these vessels were perfused simultaneously by both the LAD and the LCx. Measurements of the BWZ ranged from 1745 μm to 4987 μm, with a mean width of 3151 ± 611 μm. The ITZ extended from 3519 μm to 7613 μm, with a mean width of 5251 ± 770 μm. The ITZ and the BWZ were 14% and 17% wider, respectively, in subepicardial tissue compared to subendocardial tissue.

Although a large number of colored microspheres was infused into each coronary artery to adequately label the interface zones, only a small percentage of coronary capillaries was obstructed. Based on Bassingthwaighte et al.'s (18) data on capillary density and length, we estimate that the number of capillaries embolized by infusing 30×10^6 microspheres each into the LAD and the LCx would be less than 4% of the total number of capillaries in their perfusion territories. This is consistent with the stable blood pressure, coronary blood flow, and heart rate we observed when the 10 ± 0.2 μm microspheres were infused.

The functional significance of a region at the interface perfused by two coronary arteries remains to be determined. From the widths of the ITZ and the BWZ measured in this study, it would appear that tissue protected by dual perfusion would constitute a relatively small part of area jeopardized by an acute, proximal coronary artery obstruction. On the other hand, occlusion of a small artery or arteriole near the interface should be readily compensated by the dual perfusion sources. This would minimize ischemic damage in the BWZ and, most likely, in the ITZ. Clearly, the possibility that the zones of dual perfusion expand under conditions of chronic myocardial ischemia merits investigation.

It should be appreciated that microvascular anastomoses exist between all branches of the coronary circulation, and not just at coronary perfusion territory interfaces. The colored microsphere technique, which requires infusion of the microspheres into relatively large parent coronary arteries is limited to examination of microvascular anastomoses at the interface of large perfusion territories. However, we believe that

microvascular anastomoses provide redundant sources of perfusion to protect microvascular elements throughout the coronary circulation, as illustrated in figure 1.

The colored microsphere technique for determining sources microvascular blood flow has the advantage of being applicable to investigation of the coronary circulation in the *in situ* working heart, since the microspheres remain trapped for subsequent analysis. In contrast, other approaches to this problem have required *in vitro* perfusion of the coronary circulation and immediate arrest of the circulation to localize the intravascular distribution of perfusion markers (10, 15). Furthermore, the colored microsphere technique allows investigation of interventions which might alter the locations and

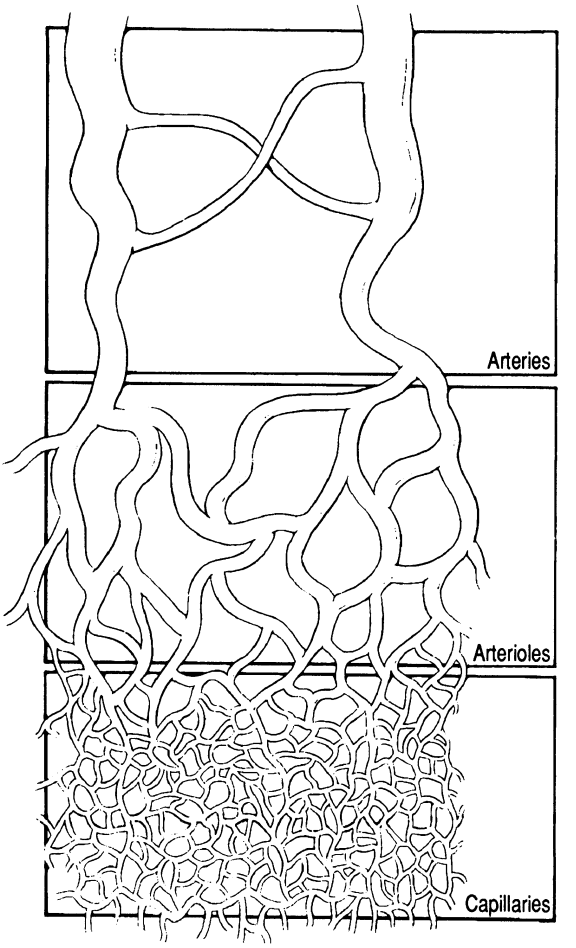

Fig. 1. Macro- and microvascular collateral anastomoses in the coronary circulation (adapted from Grayson et al (9)).

widths of the ITZ and BWZ by infusing additional, different colored microspheres after the zones have been first labeled under control conditions. Preliminary studies using this approach show that acute occlusion of one parent artery causes the dually perfused zones at the interface to shift several millimeters into the territory of the occluded artery (19).

PHYSIOLOGICAL STUDIES

Retrograde flow studies: Blood flowing outward from a coronary artery vented to atmospheric pressure distal to an obstruction is referred to as retrograde coronary flow, and this flow has frequently served as an index of coronary collateral flow (2). Eckstein (20) and Kattus and Gregg (21) postulated that retrograde flow exceeds true collateral flow since the resistance of the diverting cannula is less than that of the peripheral vasculature. On the other hand, Levy et al (22) and Cibulski et al (23) found that retrograde flow represented less than half of collateral flow estimated from clearance of diffusible indicators in hearts with acute coronary artery obstructions. Bloor and Roberts (24), however, suggested that vascular radioactivity accounted for the discrepancy between clearance and retrograde flow.

To resolve this question, we conducted studies to determine whether flow indicators could be taken up by collateral-dependent myocardium during retrograde flow diversion (25, 26). We reasoned that if retrograde flow accounts for all collateral flow, or overestimates collateral flow as suggested by some workers, no indicator would reach the ischemic myocardium. Studies were conducted in canine hearts with acute coronary artery occlusions and in canine hearts with chronic, Ameroid-induced coronary occlusions and well developed collateral vessels.

In hearts with acute coronary artery occlusions, regional collateral blood flow before and during retrograde flow diversion was measured with diffusible flow indicators, 42K and 86Rb, and with radioactive microspheres. In normal myocardium, the flow tracers were distributed uniformly across the ventricular wall. Occlusion of LAD markedly reduced the uptake of the flow tracers in the ischemic region, and before retrograde flow diversion, collateral flow in the ischemic region was distributed preferentially toward the subepicardium. Opening the retrograde flow cannula diverted collateral flow from the ischemic region at an average rate of 3.5 ml/min without altering systemic hemodynamic variables. Despite loss of this collateral flow, a

significant quantity of the isotope reached the acutely ischemic tissue; this radioactivity averaged 40% of that taken up during the control period. Diversion of retrograde flow did reverse the transmural gradient of radioactivity in the ischemic region, since uptake of the isotope in the outer two layers was reduced by 74%, whereas that of the inner layer was reduced only by 30%. Thus, retrograde flow appeared to constitute a larger portion of the potential collateral flow to the outer region of the acutely ischemic left ventricular wall. Results with radioactive microspheres were similar; 92% of the potential collateral flow to epicardial and mid-myocardial tissues was diverted, but only 29% of the flow to subendocardial tissue was diverted.

The results of these studies collaborated earlier findings that clearance of diffusible radioactive indicators exceeded the collateral flow measured retrogradely (22, 23), and the results are consistent with our view that significant collateral flow enters the collateral dependent region through microvascular anastomoses distal to significant hydraulic resistance. Microvascular collaterals were originally postulated by Levy et al (22) to explain the differences they observed between retrograde flow and clearance of radioactive rubidium. These channels should not contribute much to retrograde flow, since the resistance to antegrade flow through the downstream, extensive capillary bed would be less than that to retrograde flow through the upstream, sparse arteriolar network.

However, several other explanations for residual antegrade perfusion during retrograde flow diversion must be considered. First, perfusion of the ischemic region by vessels originating from the ventricular lumen might provide a source of non-divertable flow, especially if the luminal vessels do not anastomose at a high level with the coronary circulation. This explanation seems unlikely on the basis of failure by our laboratory (27) and others (28) to detect significant luminal flow into ischemic myocardium following acute coronary artery obstruction.

Another explanation for the radioactivity we detected in the LAD region and attributed to antegrade collateral flow is that tissue perfused by other arteries was included in samples presumed to be entirely of collateral-dependent myocardium. Kirk (29) used a "shadow technique" (30, 31) to exclude overlapping perfusion territories and claimed in a preliminary report that all apparent residual antegrade flow was, in fact, due to contamination from overlapping, normally perfused tissue. However, if tissue perfused by microvascular anastomoses along the perfusion territory interface accounts for the residual antegrade flow, this tissue would be identified as

containing contaminating overlap flow and would be excluded by the shadow technique, since, by definition, the technique excludes all tissue with a dual arterial supply. Our findings with colored microspheres support this view that tissue perfused by microvascular collaterals would, indeed, have been excluded by the shadow technique. Thus, the results of Kirk's application of the shadow technique may, in fact, be consistent with our notion that microvascular anastomoses are responsible for residual antegrade flow during retrograde flow diversion.

In acutely ischemic canine myocardium, the residual collateral flow reaching the collateral-dependent region during retrograde diversion of flow represents a small flow when expressed as flow per gram of ischemic tissue. On this basis, one might conclude that microvascular anastomoses, if they are responsible for the residual flow, are functionally unimportant. Since microvascular anastomoses connect arterioles and capillaries, it seems unlikely they would extent far beyond the periphery of the ischemic region. It is, thus, at the periphery of an ischemic region where the greatest benefit of microvascular anastomoses should be derived. With more distal sites of coronary artery occlusion, the periphery comprises a greater proportion of the mass of the acutely ischemic region, and studies have shown that the infarcted region, expressed as a percentage of the ischemic or "at risk" region, decreases as the total area at risk becomes smaller (32, 33, 34). Thus, these observations support a significant role for microvascular collaterals in protecting small regions of myocardium from ischemic damage.

We also examined the effect of retrograde flow diversion on collateral flow in hearts with chronic coronary artery occlusions (26). An Ameroid constrictor was placed on the LAD of adult mongrel dogs to induce extensive coronary collateralization. Six to nine weeks later the animals were anesthetized and regional blood flow was measured with radioactive microspheres before and during retrograde flow diversion from the LAD. Care was taken to insure that peripheral coronary pressure during retrograde flow diversion was reduced to 0 mmHg. A mean peripheral coronary pressure of 76 mmHg and a mean retrograde flow of 53 ml/min under control conditions demonstrated that the collateral circulation was well developed in these hearts. Despite diversion of a large retrograde flow and a 11 mmHg fall in aortic blood pressure, residual blood flow in the collateral-dependent region averaged 0.55 ml/min/g, 67% of the flow measured in this region under control conditions prior to diversion of retrograde flow. In similarity to the control condition, this residual collateral flow was distributed preferentially toward

the subepicardial tissue (0.66 ml/min/g) compared to the subendocardial tissue (0.48 ml/min/g), and this flow gradient steepened as indicated by an increase in the subepicardial/subendocardial flow ratio from 1.3 to 1.8. Thus, microvascular collateral anastomoses provide a greater proportion of residual collateral flow to the epicardial region in hearts with well developed collateral circulations, whereas microvascular anastomoses contribute more significantly to subendocardial flow in hearts with acute coronary artery occlusions.

In four of these experiments, 25 ± 5 μm diameter microspheres were injected along with 9 ± 1 μm diameter microspheres during diversion of retrograde flow to gain some information on the size of the collateral channels responsible for the residual antegrade flow. In each experiment, the residual collateral flows measured with the differently sized microspheres were nearly identical, so the microvascular anastomoses responsible for the residual flow observed during retrograde flow diversion must have been precapillary.

Coronary capillaries do interconnect to form networks (Fig. 1) (6, 9, 18), so capillary anastomoses provide a microvascular channel for collateral flow. Since the radioactive and colored microspheres we have used to examine microvascular collateral flow do not traverse coronary capillaries (35), this potential component of collateral flow was not reflected in our studies. Although capillary anastomoses probably contribute to microvascular collateral flow, we would expect the effective range of this collateral flow to be more limited than that carried through arteriolar anastomoses.

Recently reported studies from Bache's laboratory (36, 37), confirmed our observation of significant residual antegrade flow during retrograde flow diversion in hearts with well developed collaterals. Under control conditions, they measured residual flows of 51 to 64% of total flow in normally perfused myocardium not subjected to retrograde flow diversion. Administration of an α-adrenergic agonist decreased residual flow by 23%, whereas retrograde flow was reduced only 10%. Since microvascular collaterals contribute a small amount to retrograde flow as well as to residual flow (26), some of the small decrease in retrograde flow was likely due to reduced microvascular collateral flow. These findings indicate that microscopic collaterals are more responsive to α-adrenergic stimulation than are macroscopic collaterals.

Peripheral embolization: Schulz et al (38) and other investigators (39, 40) demonstrated that embolization of the coronary microvasculature caused a significant increment in retrograde flow. Since peripheral embolization prevented continued antegrade collateral flow, the residual collateral flow was converted to an additional increment of retrograde flow. The fact that peripheral embolization could increase retrograde flow strongly supports our earlier studies (25, 26) and those of Cibulski et al (23), which found evidence of residual collateral flow during retrograde flow diversion. It should be noted, however, that two studies failed to find an increment in retrograde flow after peripheral embolization (4, 41).

We extended the concept of peripheral embolization to include injections of different size microspheres to gain information on the diameter of microvascular collaterals in canine hearts with well developed coronary collateral circulations (42). Eight to ten weeks after surgical implantation of an Ameroid constrictor on the LAD, the dogs were anesthetized and the LAD was cannulated distal to the occluder. The coronary cannula was connected to a blood-filled, pressurized reservoir, so the LAD could be perfused when desired. A "T" in the perfusion line permitted diversion of coronary flow retrogradely through the outflow line whose height was varied as needed to keep LAD pressure at 0 mmHg during this maneuver. Aortic blood pressure was maintained at 100 mmHg throughout the experiment by withdrawing or infusing blood. After measuring retrograde flow, the LAD was perfused with blood from the reservoir and an occlusive dose of microspheres either 13 ± 0.5 μm or 84 ± 7 μm in diameter was administered into the coronary perfusion line. To minimize the possibility of microsphere emboli leaving the LAD bed through collateral vessels, coronary perfusion pressure was set at 90-95 mmHg during the embolization process. Embolization was considered complete when further administration of the microspheres had no further effect on antegrade flow in the LAD perfusion line. Retrograde flow from the LAD was again measured. In addition, retrograde flow from hearts embolized with 84 μm microspheres was examined microscopically for presence of the microspheres.

Coronary embolization with 13 μm diameter microspheres caused retrograde flow to increase by 43%. Since mean arterial blood pressure was not altered by coronary embolization, the increment in collateral flow following embolization reflected collateral flow which entered the collateral-dependent bed distal to significant vascular resistance and would have flowed antegradely during retrograde flow diversion had not

the 13 μm vessels been embolized. Thus, these results agree with observations of increased retrograde flow following peripheral embolization in hearts with acute coronary artery occlusions (38, 39, 40). The average increment in retrograde flow caused by coronary embolization with 13 μm microspheres in these hearts with well developed collaterals was 24 ml/min. This increment in retrograde flow was similar to the residual antegrade flow we observed previously in well collateralized hearts (25). However, the increment in retrograde flow caused by coronary embolization in these collateralized hearts was less than the 100 to 200% increase observed previously in heart without well developed collaterals (38). This suggests that the conductance of macrovascular collateral vessels increased more than that of microvascular collaterals during chronic coronary artery obstruction. Since microvascular collaterals would be most effective near the border of the collateral-dependent region, a greater increase in the conductance of the larger collaterals is required to perfuse the central portion of the collateral-dependent region.

In contrast to the increase in retrograde flow caused by coronary embolization with 13 μm microspheres, embolization with 84 μm microspheres caused no significant change in retrograde flow. From this data we concluded that collateral flow entering the collateral-dependent region through anastomoses greater than approximately 84 μm is completely diverted during the retrograde flow maneuver. Since coronary embolization with 13 μm microspheres increased retrograde flow, whereas embolization with 84 μm microspheres did not, a significant portion of the total collateral blood flow must enter the collateral-dependent region at the microvascular level through vessels less than 84 μm in these hearts with well developed coronary collateral circulations.

The finding that all collateral flow entering the collateral-dependent bed through anastomoses greater than 84 μm could be converted to retrograde flow agrees with our earlier analysis of the origin of residual antegrade coronary flow during retrograde flow diversion (26). That analysis suggested, however, that a significant portion of the microvascular collateral flow would contribute also to retrograde flow. If this were the case in the present investigation, some flow entering the collateral-dependent bed at sites less than 84 μm would flow retrogradely. Two lines of evidence from the current experiment support that notion. First, 84 μm microspheres were detected in all collections of retrograde flow. Secondly, the embolization was less complete following diversion of retrograde flow. Both of these findings can be explained by dislodgement of 84 μm emboli by a component of microvascular collateral flow. Thus, these findings

demonstrate that collateral flow vessels less than 84 μm contribute to retrograde flow as well as being totally responsible for residual antegrade flow in chronically collateral-dependent myocardium.

Scheel et al (43) reported similar experiments in canine hearts with acute coronary artery occlusions. Their approach was to inject sequentially occlusive doses of microspheres of increasing size into the same heart and examine incremental increases in retrograde flow. While it is not clear how further injections of larger microspheres could affect flow in a bed already obstructed by smaller microspheres, Scheel et al. concluded that microvascular collaterals which contribute to residual flow during retrograde flow diversion range up to 80 μm in diameter. This is similar to the our findings in hearts with well developed collaterals (42).

SUMMARY

Anatomical and physiological evidence supports the existence of microvascular collaterals in the coronary circulation. These microvascular anastomoses provide redundant sources of blood flow to microvascular elements throughout the myocardium. Furthermore, microvascular collaterals may respond differently or be more sensitive to physiological or pharmacological factors compared to macrovascular collaterals. Retrograde flow measurements are largely insensitive to flow through microvascular collaterals, and the "shadow technique" excludes myocardium supplied by dual sources of arterial blood through microvascular anastomoses. Studies of coronary collateral function should be designed to examine the function of the microvascular component of the coronary collateral circulation as well as the macrovascular component.

Acknowledgments: The author's research described in this chapter was supported by NIH grants HL-21657 and HL-35027 and by the Cardiology Fund.

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19

COLLATERAL DEVELOPMENT AND FUNCTION IN MAN

*Attilio Maseri MD, **Luis Araujo MD, * Maria Luisa Finocchiaro MD
* Institute of Cardiology, Catholic University, Rome, Italy
** Division of Nuclear Medicine, Department of Radiology, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A

The protective role of coronary collaterals in patients was questioned because collaterals were more frequently found in patients with myocardial infarction than in those without (1, 2). This apparent paradox is explained by the greater average frequency of coronary occlusion or very severe stenoses in patients with infarction than in those without (3).

A protective role, similar to that clearly demonstrated in experimental animals by gradual coronary occlusion (4, 5, 6, 7), was subsequently shown in patients (8, 9, 10, 11, 12). However, the collateral circulation present in patients with obstructive coronary artery disease could have various origins and the protective effect on myocardial ischaemia depends not only on its anatomical development, but also on changes in coronary vasomotor tone and possibly on coronary blood flow "steal".

In order to examine in detail the potential mechanisms of myocardial ischaemia that may operate in patients in the collateralised territories, in this chapter we will summarise the development of collateral circulation and review its protective effect.

DEVELOPMENT OF CORONARY COLLATERALS

Elegant animal studies in anaesthetised and chronic dogs have proven convincingly that 3-6 months following gradual coronary artery occlusion, collateral blood flow can provide very substantial compensation. During submaximal exercise in chronic dogs with gradual total coronary artery occlusion, regional myocardial blood flow (estimated by the microsphere technique) was found to be only slightly lower in collaterally perfused myocardium than in myocardium distal to a non-obstructed artery (7).

A series of studies have shown convincingly that collaterals may form from: 1) pre-existing end-to-end interarterial anastomoses which connect terminal branches of major coronary arteries; and 2) newly formed vessels establishing new communications

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between adjacent vascular territories at the capillary level, which subsequently dilate progressively.

The relative importance of these two modalities of collateral development in humans is unknown.

1) Intercoronary anastomoses (ranging in size from 50 to 200 μm in diameter) were demonstrated convincingly in human hearts without evidence of obstructive coronary disease in post-mortem studies by Baroldi and Scomazzoni (13) and Fulton (14) (Fig. 1).

Such anastomoses are present in variable numbers in different mammals (15, 16, 17, 18, 19). They are so numerous in guinea pigs that they can prevent infarction even during sudden proximal coronary occlusion of a major coronary branch. They are more numerous in dogs than in pigs, rats and rabbits, and practically absent in sheep. Their presence and number in different species, and possibly in different individuals, are likely to be genetically determined. The patency of such anastomoses requires blood flow through them which can develop only as a result of a pressure gradient occurring

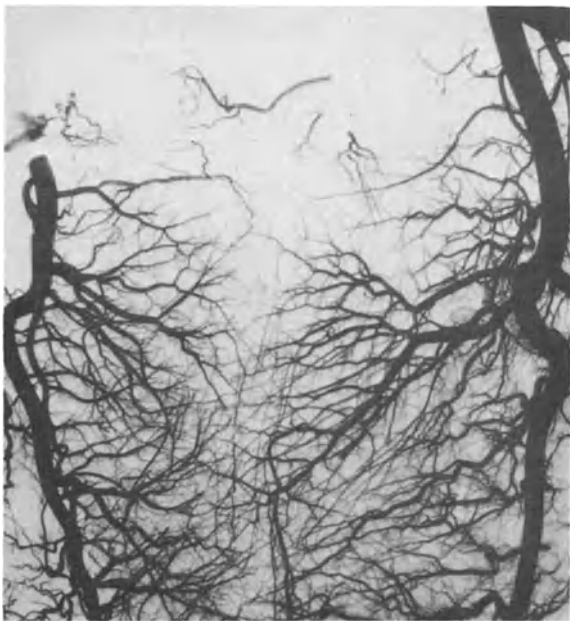

Fig. 1. Anastomotic communications in the interventricular septum, upper portion of a normal heart. Some course in a slightly downward direction across the septum, joining the anterior descending artery on the right to the posterior descending artery on the left. Some run nearly at right angles to this direction, and lie on the left side of the septum, magnification x 2. These vessels may be numerous but are usually small (from Baroldi et al (41)).

during some part of the cardiac cycle or created by alternating constriction of parent branches. Anastomoses gradually enlarge because of increased blood flow through them resulting from the development of a continuous pressure gradient. When they are intramyocardial their development may also be influenced by ischaemia in the surrounding tissue, but this is unlikely when they are far upstream in the ischaemic zone.

Vessel growth needs not only cell proliferation, that would result in a reduction of lumen diameter, but also a process of remodelling which would make vascular enlargement possible by controlled and graded destruction of the existing structure (19). Anastomoses can provide a very considerable amount of blood flow and in dogs reach a maximum development 3-6 months after a gradual coronary occlusion.

2) The development of collateral circulation can also occur in the absence of pre-existing anastomoses. This was clearly demonstrated following the procedure of mammary artery implants described by Vineberg in the myocardium of dogs with gradual total occlusion of a major coronary artery (20, 21, 22). The amount of collateral blood supply provided by such neoformed vessels was variable among different animal species but always rather small compared to that provided by interarterial anastomoses.

The neoformation of vessels that establish new communications between adjacent arterial territories and their development are likely to be determined and influenced by tissue ischaemia (23, 24, 25, 26). Vascular growth factors play an important role in the control of this mechanism (19, 27, 28, 29, 30).

The very variable ability to provide collateral compensatory blood flow in different species, and possibly among different patients, is likely to depend predominantly on the number, size and location of pre-existing intercoronary arterial anastomoses, and to a limited extent, by formation of new connections between previously separated arterial territories.

VARIABLE COLLATERAL BLOOD FLOW RESERVE IN PATIENTS

A marked variability in the degree of collateral development for a comparable severity of coronary artery obstructions is a common clinical observation. At one extreme, in some patients without signs of myocardial infarction, complete proximal occlusion of a major branch can be compensated for by angiographically visible collaterals to such an extent that maximal predicted heart rate can be attained during effort test without detectable signs of ischaemia.

At post-mortem, patients who had had stable angina were frequently found to have complete coronary occlusions but no signs of myocardial infarction (31, 32). In such patients and in those with good coronary flow reserve, the compensation provided by collaterals supplying the occluded artery branch is obviously good. However, in patients with a single mid- or distal coronary vessel occlusion or severe stenoses, coronary flow reserve, as assessed by exercise stress test, remains low even over a period of months, and they continue to experience angina with ischaemic ST segment depression on the ECG and positive myocardial scintigraphy at mild to moderate levels of effort.

In general, patients with coronary occlusion and good coronary flow reserve also have larger and more numerous collaterals and more rapid and intense collateral filling of the occluded vessel. Conversely, those with low coronary flow reserve have fewer and smaller angiographically visible collaterals with lower and less intense filling of the occluded vessel.

Differences in the number and size of pre-existing interarterial anastomoses would seem the most plausible explanation for the variable degree of collateral development observed in cross-species studies. However, the degree of collateral development in response to the development of coronary stenoses or occlusion in patients has not been specifically studied, and the causes of the variable compensation provided by collateral development are still speculative. Therefore, at the present time no information is available about:

1. the frequency of coronary occlusion without signs of infarction with very good collateral compensation;
2. the precise mechanism responsible for the lack of development of infarction in the presence of total coronary occlusion;
3. the causes of such variable collateral development and its compensatory function.

COLLATERALS IN PATIENTS WITH ISCHAEMIC HEART DISEASE

The role of collateral vessels and the factors associated with their development were examined in patients with chronic stable angina, variant angina or myocardial infarction and during acute coronary artery occlusion in patients undergoing angioplasty.

Coronary angiography is the technique used most frequently in clinical practice for the assessment of collateral vessels, but it has several limitations. At best it can only

define vessels ≥2-300 μm in diameter, while many arterial collaterals are much smaller. Second, collaterals may be present but invisible because they are not filled by dye in the absence of a sufficiently large pressure gradient. Collaterals that are invisible in basal conditions may be detectable during balloon angioplasty because of the transient reduction of coronary pressure distal to the occluded segment (10, 33).

Patients with chronic stable angina pectoris

The presence of collateral vessels is commonly related to the severity of the coronary artery stenoses. In contrast, the relation between collateral visualisation and angina is variable.

Cohen et al (34) studied patients with one-vessel disease, a history of angina pectoris ranging from 1 week to 36 months, no previous transmural myocardial infarction and normal left ventricular function. They showed that during coronary angiography the severity of the baseline lesion was the only independent variable associated with collateral filling and the best predictor of collateral flow reserve. In this group of patients there was no relation with age, gender, risk factors, proximal versus distal location of the lesion and duration of angina. In contrast, Piek et al (35) demonstrated that the angiographic appearance of collateral vessels significantly correlated not only with the severity of the stenoses before angioplasty, but also with the duration of angina. In particular, collaterals were related to a lesion severity ≥ 70% and duration of angina ≥ 3 months.

This discrepancy could be explained by a variable prevalence in these two studies of different types of angina, as severe limitation of effort tolerance would more likely be associated with critical coronary artery stenoses than angina with good effort tolerance. The correlation between chronic stable angina and collaterals could be an indirect one i.e. mediated by the severity of the coronary artery stenoses. However, as described above, the development of collaterals may be quite variable among patients with equally severe stenoses and angina of equally long duration.

Patients with variant angina

The presence of collaterals in variant angina is more frequently associated with ST depression than ST elevation (36, 37, 38). The possible protective role of collaterals during epicardial coronary artery spasm was conclusively demonstrated following ergonovine-induced coronary artery spasm, by a transient visualisation of collateral vessels from non-spastic arteries associated with ST-segment depression (39).

These findings indicate that during coronary artery spasm collateral flow through pre-existing vessels could prevent transmural myocardial ischaemia, resulting in a lesser degree of ischaemia associated with ST-segment depression.

Patients with myocardial infarction

The available data suggest that collaterals have a protective role in the development of infarction, its outcome and post-infarction ventricular function.

Development of myocardial infarction. Post-mortem and angiographic studies show that coronary occlusion can also develop in patients in the absence of infarction and in the presence of collaterals (40, 41, 42).

Hansen et al (11) demonstrated that in patients with coronary artery occlusion, good collaterals were associated with the most severe degree of coronary artery disease. In addition, they found an association with a lower incidence of Q-waves on the ECG and a history of previous myocardial infarction and diskynesia at ventriculography. Survival rates after 10 years were 51.5% in patients with good collaterals and 34.5% in those with poorly developed collaterals.

Watt et al (43) reported three cases in which occlusion of the left main coronary artery was associated with a well preserved left ventricular function. All three patients had dominant right coronary arteries and prominent epicardial and septal collateral vessels to the territories normally supplied by the left anterior descending and circumflex arteries.

Outcome of myocardial infarction. Piek and Becker (44) studied the relationship between collateral development and the type and size of myocardial infarction in human hearts at post-mortem. They observed that hearts with subendocardial infarction had a greater number of microspheres in the collateral-dependent area compared to those with transmural infarcts. Moreover, the border zone of myocardium at risk (i.e. the area of the occluded artery containing viable myocardium) was smaller in hearts with a transmural infarct than in those with a subendocardial infarct. This study suggests that collateral development plays an important role in determining infarct type and in preserving the viability of the border zone of the myocardium at risk.

These conclusions were confirmed by a study of patients in whom thrombolytic therapy had failed to induce reperfusion within 90 minutes of its administration. In patients with angiographically visible coronary collaterals at the onset of myocardial

infarction, significantly lower values of peak serum creatine kinase and CK-derived infarct size estimates were observed compared to a group of patients without collaterals (12)

Formation of left ventricular aneurysm and improvement of left ventricular function. In acute myocardial infarction, several studies showed a significantly protective role of collaterals against the formation of left ventricular aneurysm (45, 46).

Significant improvement in regional and global ventricular function was observed in patients with good collaterals and successful reperfusion (45, 47). The improvement in ventricular function was unrelated to the time lapse between the occurrence of myocardial infarction and reperfusion and may also be observed after "late reperfusion" (47, 48). In addition, other studies showed there was a tendency towards improved left ventricular function in patients with well developed collaterals and no reperfusion (12, 49, 50).

It is likely that collateral blood flow maintains myocardial viability in the infarct-related arterial bed for a prolonged period after an acute myocardial infarction, and the restoration of anterograde blood flow favours an improvement in ventricular function.

Percutaneous transluminal coronary angioplasty

Coronary artery occlusion during balloon inflation in patients undergoing angioplasty offers a good clinical model with which to study the development of collateral vessels and their protective role.

Coronary occlusion induced by balloon inflation demonstrated the ability of the collateral circulation to limit myocardial ischaemia. Compared to patients without visible collaterals those with good collaterals showed a lesser degree of ECG and ventriculographic signs (shift of ST-segment, new ventricular hypocontractility or fall in ejection fraction) of myocardial ischaemia during balloon inflation (10, 35, 51). Furthermore, Cohen and Rentrop (10) reported the occurrence of anginal pain in all patients with poor collateral filling (grade 0-1) but only in 36% of patients with good collaterals (grade 2-3). These observations suggest that the collateral circulation can protect the myocardium at risk of ischaemia from the very onset of coronary occlusion.

HAEMODYNAMIC DETERMINANTS OF COLLATERAL BLOOD FLOW

For any degree of development of collateral vessels, whatever their origin, the amount of blood they carry to the jeopardised coronary vascular bed is determined by

their vascular resistance and by the pressure gradient between their origin and termination. The pressure at the termination of collateral vessels depends on post-stenotic coronary artery pressure.

The development of an epicardial coronary artery stenosis causes the post-stenotic pressure to decrease and in turn the trans-stenotic pressure drop allows flow to be maintained across the stenosis in spite of the increased resistance to flow. The decrease in post-stenotic pressure becomes established as a result of dilation of arteriolar vessels sufficient to compensate for the increased resistance produced by the epicardial stenosis (with a proportionate reduction of coronary flow reserve). The pressure distal to a total occlusion produced suddenly, i.e. before any collateral blood flow develops, is likely to be close to the peripheral coronary pressure in resting conditions. This pressure, measured in the cannulated distal end of a severed coronary branch, is about 30% of pre-stenotic pressure and indicates the order of magnitude of the initial pressure gradient between the origin of pre-existing anastomotic vessels and the vascular territory beyond the occlusion. A critical stenosis is required in order to determine a sizeable pressure drop, but the tighter the stenosis and the higher the flow, the closer the initial post-stenotic pressure will be to peripheral coronary pressure. During the progressive increase of a stenosis of a major epicardial coronary artery, the trans-stenotic pressure gradient (i.e. the post-stenotic pressure drop) at rest is minimal until the lumen is reduced by about 90% and increases markedly with flow. The development of collaterals increases post-stenotic coronary pressure progressively, thus gradually restoring coronary flow reserve.

Measurements of post-stenotic coronary artery pressure in humans (52) were performed at the time of by-pass surgery, but only after the insertion of the graft and re-establishment of cardiac rhythm. The mean values reported for critical (91-99%) or total stenoses were 29 and 22 mmHg respectively. These values represent the integrated results of the severity of the stenoses and collateral blood flow compensation, and provide only a rough idea of the range of pressure gradient that could exist in patients between normal and post-stenotic vascular beds. Unfortunately, the effects of cardiac arrest during extracorporeal circulation (associated with the by-pass procedure) on collateral flow and post-stenotic pressures, are unknown.

COLLATERAL BLOOD FLOW STEAL

The phenomenon of subclavian blood flow steal was inferred from the clinical observation of deficits occurring in cerebral function in patients with total occlusion of the subclavian artery during intense dynamic exercise of the arm, which was perfused by collaterals originating from the carotid and vertebral arteries.

In analogy to this phenomenon, in the late 1960s Fam and McGregor (53) introduced the concept of collateral blood flow steal for the coronary circulation. The authors assumed that arteriolar vessels distal to a critical coronary stenosis were maximally dilated so that flow through them was strictly dependent on the perfusion pressure generated by collateral flow. Pharmacologically or metabolically induced coronary dilation could not affect the collateralised vascular bed because it was already maximally dilated, but it could cause a decrease in pressure at the origin of collaterals if the upstream resistance in the parent coronary artery was significant following arteriolar coronary vasodilation.

This can be the case when collaterals originate distal to a critical coronary artery stenosis and even in normal arteries, if the pressure gradient along their course proximal to the origin of collaterals is significant and increases during high flow. In both cases in order for steal to occur, it is necessary that the perfusion pressure in the collateralised territory is so low that it cannot be reduced further or cannot be reduced in the same proportion to the reduction at the origin of collaterals in the parent vessel because the arteriolar vessels in the collateralised bed are already maximally dilated.

In a recent study using positron emission tomography, we measured regional myocardial perfusion with ^{15}O-labelled water at rest and during the dipyridamole test, in 6 patients with single occlusion and angiographically visible collaterals. All 6 patients had a history of chronic stable angina ≥ 6 months, none had evidence of prior myocardial infarction and all had normal left ventricular function. The exercise stress test was positive in 5 patients, but in one patient with complete occlusion of a proximal right coronary artery, it was negative at maximal predicted heart rate. The dipyridamole test caused myocardial ischaemia in all, as evidenced by an increased uptake of ^{18}F-deoxy-glucose in the collateralised myocardium immediately after the test (Fig. 2).

Myocardial ischaemia induced by dipyridamole was associated with a lesser increase of transmural blood flow to the collateralised myocardium compared to those

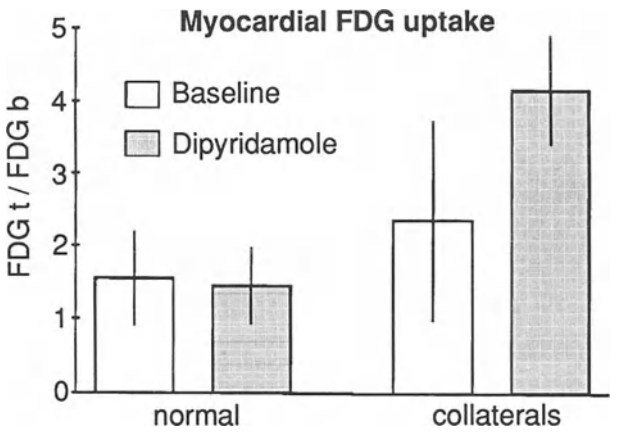

Fig. 2. This graph shows the results of myocardial 18F-2-deoxy-glucose (FDG) uptake at baseline and after vasodilation induced by dipyridamole infusion, in patients with single vessel occlusion, no infarction and good collateral circulation to the territory at risk. Normal myocardium (normal) shows low FDG uptake as characteristic of the fasting state. Note a significant metabolic change in the collateralised myocardium (collaterals) after dipyridamole infusion indicative of myocardial ischaemia. (FDGt: FDG concentration in tissue (myocardium); FDGb: FDG concentration in arterial blood).

territories supplied by angiographically normal arteries (Fig. 3); but flow to the cardiac wall perfused by collaterals did not decrease below resting levels in any patients.

These data suggest that in this group of patients, the arteriolar vessels in the collateralised vascular bed were not fully dilated at rest. They were dilated by dipyridamole and this dilation caused a drop of perfusion pressure in the collateralised vascular bed as well as an increase in collateral flow. Dipyridamole therefore caused myocardial ischaemia in the collateralised territory, but not through blood flow steal from the normally perfused vascular bed which gave origin to the collaterals. The dipyridamole-induced arteriolar dilation actually increased the driving pressure across the collaterals, but the increased collateral flow was insufficient to prevent a drop of perfusion pressure in the collateralised vascular bed.

In the absence of detectable collateral blood flow steal, the myocardial ischaemia in the collateralised cardiac wall which was detected by ^{18}F-deoxy-glucose uptake (and also by ECG changes and angina in 3 patients and by angina alone in the other 3 patients), was caused by transmural blood flow steal. As arteriolar vessels in subepicardial regions became dilated by the drug, post-stenotic perfusion pressure decreased too much to adequately perfuse subendocardial regions, which then became ischaemic. The increased collateral blood flow was insufficient to maintain an adequate

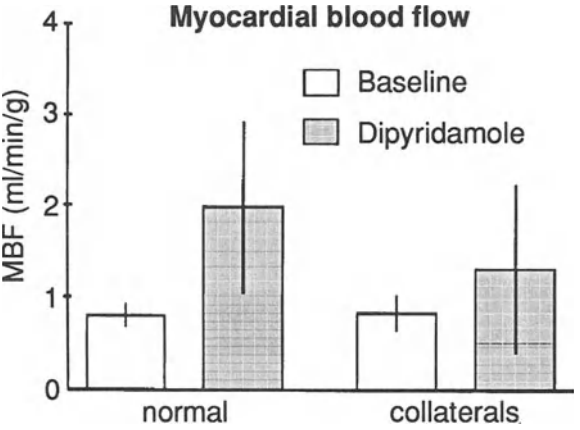

Fig. 3. This graph shows the results of transmural myocardial blood flow (MBF) in the same group of patients as in figure 2. MBF at baseline was normal and similar in both normal and collateralised myocardial areas (normal - collaterals) but a markedly blunted vasodilatory response is noted after the administration of dipyridamole. This correlated with an enhanced glucose uptake in the territory at risk (see Fig. 2).

post-stenotic perfusion pressure. Therefore a transmural (vertical) blood flow steal developed with subendocardial ischaemia caused by increased flow through subepicardial layers.

TRANSIENT IMPAIRMENT OF COLLATERAL BLOOD FLOW

The haemodynamic determinants of coronary collateral blood flow, i.e. driving pressure and resistance, can be influenced by transient changes in vasomotor tone which could cause episodes of myocardial ischaemia.

In patients with chronic stable, predominantly effort related angina, a possible modulation of collateral blood flow by vasomotor tone is suggested by both clinical observation and pharmacological studies. Such modulation may not necessarily exist in previously healthy animals subjected to gradual coronary occlusion.

The interpretation of clinical observations is usually complicated by the frequent coexistence of multiple alterations, each of which by itself could represent a plausible cause of ischaemia. However, sometimes by careful selection of cases clinical investigators are able to make use of special models of disease created by nature. Such models may lend themselves to the dissection of single pathogenetic components of the disease (out of multiple and complex abnormalities), which can cause or contribute to cause myocardial ischaemia. Patients with chronic stable angina who have a single complete coronary artery occlusion with coronary collaterals, no evidence of infarction and no other epicardial coronary stenoses or spasm, represent one such singular human model of ischaemic heart disease.

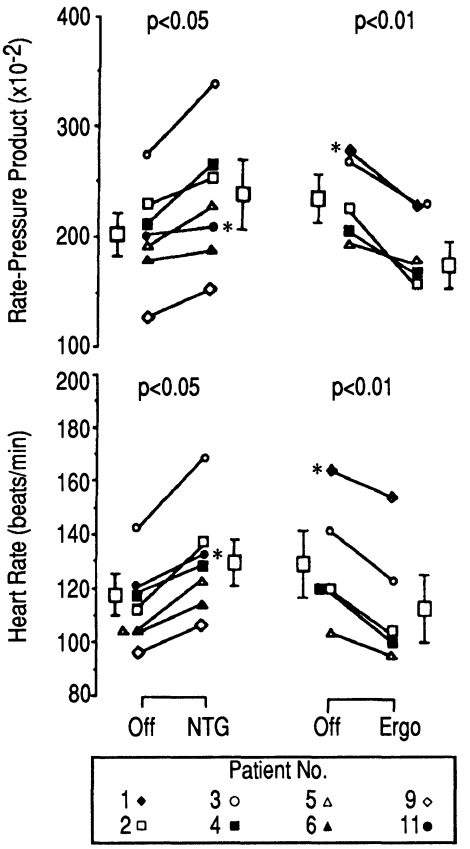

Fig. 4. Heart rate and rate-pressure product at a 1-mm depression of the ST segment during exercise testing in patients with chronic stable angina and a single complete coronary artery occlusion. Exercise stress testing was performed when patients were off therapy (Off), after sublingual administration of 0.5 mg of nitroglycerin (NTG), and after the intravenous administration of ergonovine (Ergo). Asterisks indicate negative results on exercise tests. Both the heart rate and rate-pressure product at a 1-mm depression were consistently higher after nitroglycerin and lower after ergonovine administration. The large open squares and bars indicate mean (±SD) values.

Clinical observations

The clinical observation of a marked variability in the anginal threshold of patients with a single coronary artery occlusion, coronary collaterals, no previous infarction and no other coronary stenoses, prompted a study which explored the possible role of transient functional impairment of collateral blood flow. These patients had no anatomical substratum for dynamic modulation of coronary stenoses, hence residual coronary flow reserve could only be modulated by constriction of vessels providing collateral flow. This possible mechanism of ischaemia is indicated by three lines of evidence obtained in a group of patients with this model of human disease (54).

(i) Exercise stress testing repeated in the presence of coronary constrictor or dilator drugs proved that a marked variability in ischaemic threshold could be induced pharmacologically (Fig. 4).

(ii) Ambulatory monitoring during ordinary daily life proved that most ischaemic episodes occurred at values of heart rate much lower than those which were attained during exercise before the development of ischaemic changes on the ECG. In addition, some ischaemic episodes occurred at a heart rate of 20 or more beats lower than other episodes or lower than episodes of prolonged tachycardia during ordinary physical activity which were not associated with any signs of ischaemia (Figs. 5 and 6).

(iii) Administration of ergonovine during angiography caused a marked reduction of collateral filling, without any evidence of epicardial coronary artery spasm, suggesting that collateral blood flow can be impaired by constrictive stimuli which do not cause substantial constriction of epicardial coronary arteries (Fig. 7).

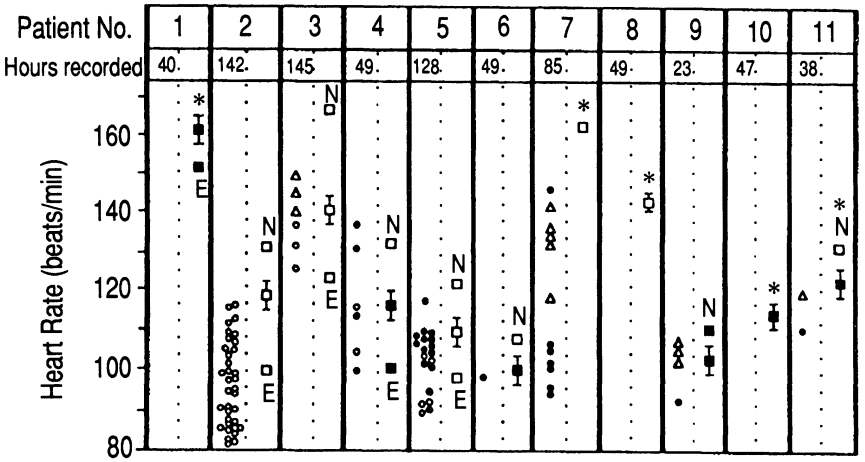

Fig. 5. Heart rate during ambulatory ECG monitoring and exercise testing in the same group of patients shown in figure 4. For each patient the left side of the column contains Holter-monitoring data, and the right side exercise-testing data. Solid symbols indicate the presence of anginal pain. Circles indicate episodes of ischaemia during Holter monitoring, and triangles episodes of sinus tachycardia unaccompanied by ST-segment depression or angina. Squares indicate exercise tests when the patients were not receiving therapy (with range bars), after the sublingual administration of nitroglycerin (N), and after the intravenous administration of ergonovine (E). Asterisks indicate negative exercise-test results. The variations in the heart rate at a 1-mm depression of the ST-segment during daily life and during effort tests performed after the administration of nitroglycerin or ergonovine are considerable, particularly in some patients (No. 2, 5, and 7).

Pharmacological studies

Intracoronary infusion of serotonin in patients with variant angina caused myocardial ischaemia by complete occlusion of an epicardial coronary artery segment (Fig. 8). However, in patients with chronic stable angina and stenoses comparable in severity to those of patients with variant angina, serotonin also caused myocardial ischaemia, but at a 10-fold higher dose and with only minimal constriction of the stenoses. Diffuse constriction of angiographically visible distal vessels and with a marked decrease in collateral vessel filling were present, in those patients with a total coronary occlusion (Fig. 9) (55).

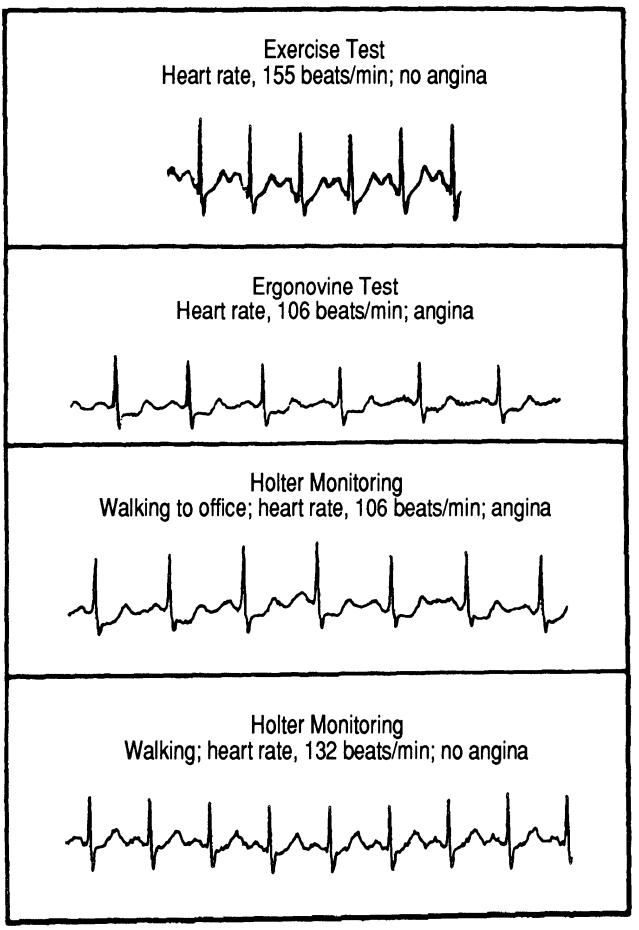

Fig. 6. Electrocardiograms during exercise testing when the patient (corresponding to patient No.7 in figures 5 and 6) was receiving no therapy, during intravenous administration of ergonovine at rest, and during ambulatory ECG monitoring when receiving no therapy.
During ambulatory ECG monitoring and the administration of ergonovine, angina and ischaemic ST-segment changes developed at a heart rate much lower than the rate achieved on other occasions during ECG monitoring or exercise testing when no angina or ST-segment changes occurred.

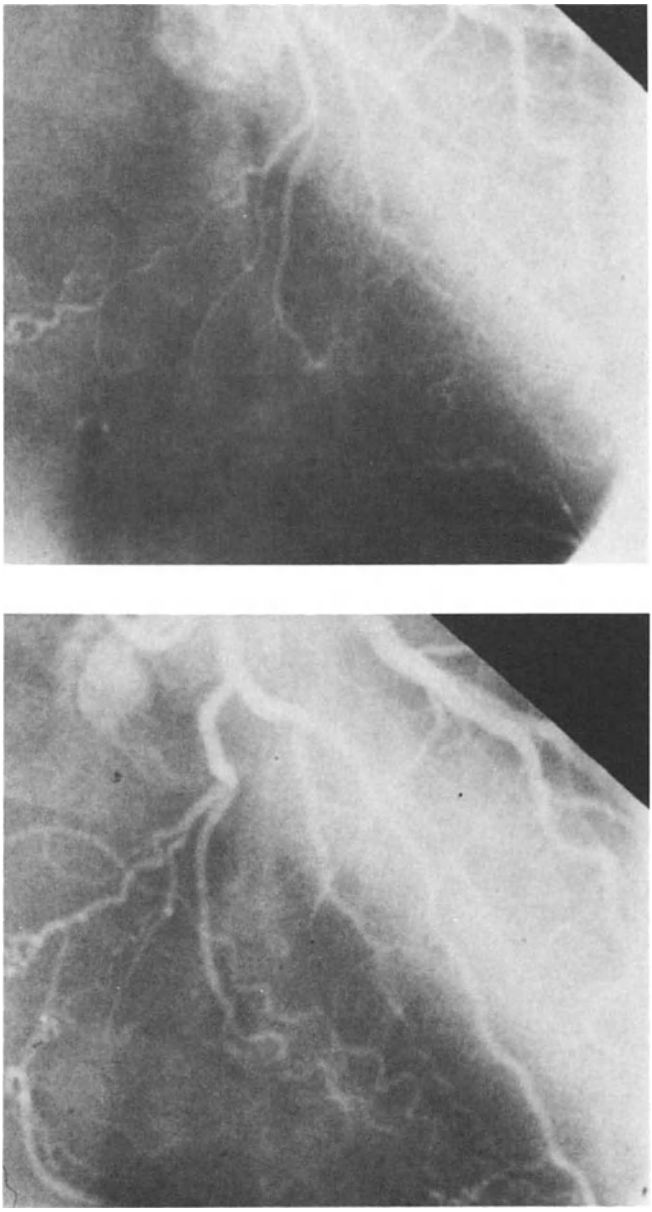

Fig. 7. Angiographic findings in a patient with stable angina pectoris under basal conditions (top) and during an episode of angina and ST-segment depression after the intracoronary administration of ergonovine (bottom). The calibre of the collateralised vessel and of visible collateral vessels decreased after the administration of ergonovine.

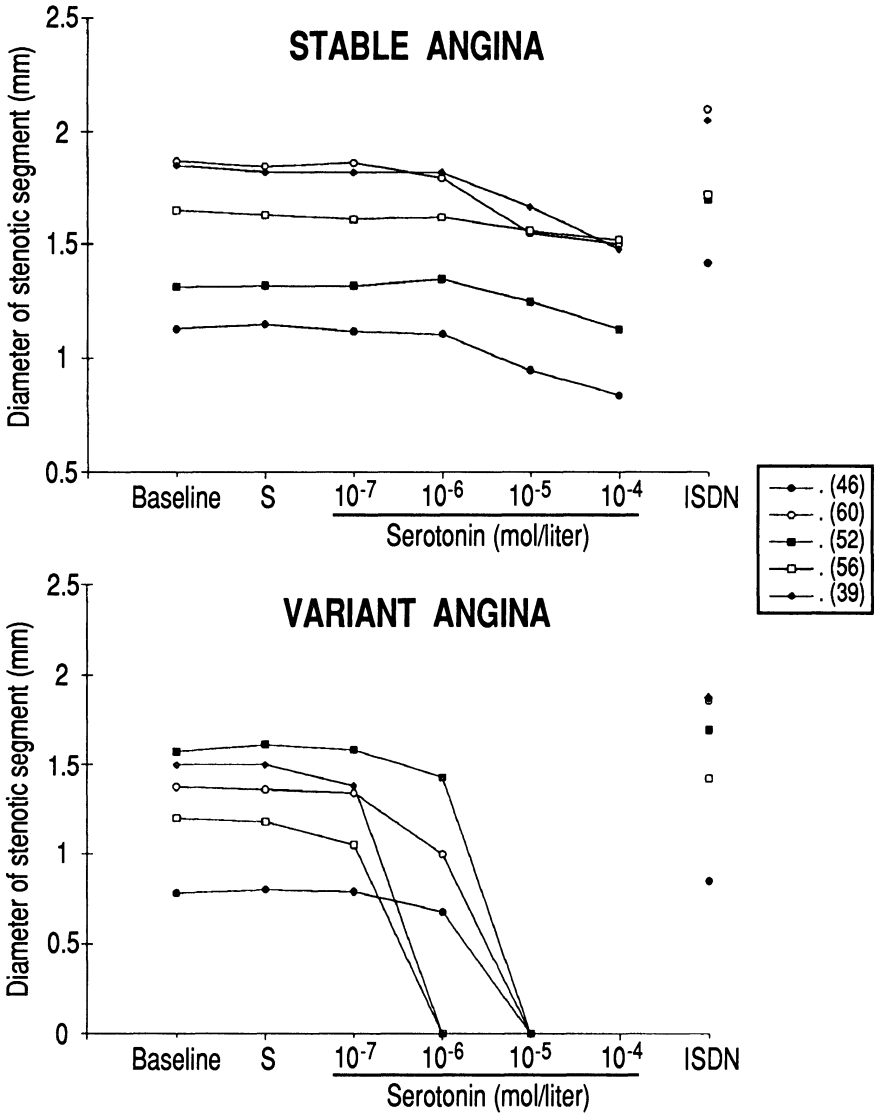

Fig. 8. Dose-response curves in stenotic segments from patients with stable angina and patients with variant angina after a two-minute infusion of saline (S), two-minute infusion of increasing concentrations of serotonin, and an injection of isosorbide dinitrate (ISDN).

The keys indicate patient numbers and (in parentheses) the percent severity of stenoses at baseline. In patients with variant angina, complete occlusion occurred at a concentration of 10^{-6} or 10^{-5} mol of serotonin per liter, whereas in those with stable angina there was only a progressive reduction in diameter.

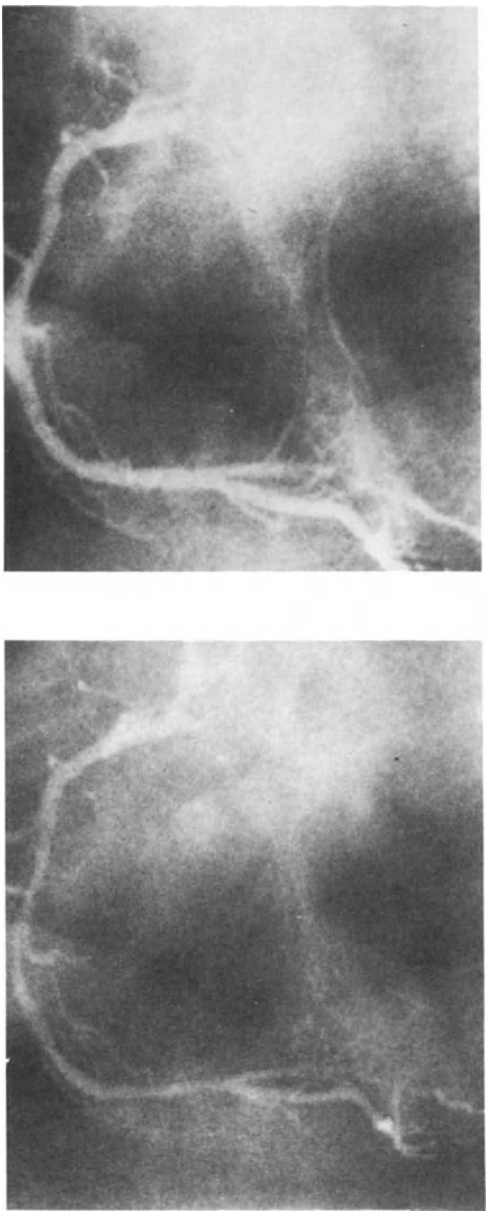

Fig. 9. Angiographic finding in a patient with stable angina at baseline (top) and after intracoronary infusion of 10^{-4} mol of serotonin per liter at 1 ml per minute for two minutes (bottom). Markedly reduced collateral filling of the left anterior descending coronary artery and moderate patchy constriction of the right coronary artery are evident after the infusion of serotonin.

However, in patients with atypical chest pain, 10-fold higher doses of the drug gave systemic effects, but failed to cause signs of ischaemia.

Thus, in patients with a chronic form of ischaemic heart disease, serotonin (at doses in the range that can be produced in the coronary arteries by platelet aggregation) can also impair collateral blood flow independently of its effects on proximal coronary artery stenoses.

Mechanisms of transient impairment of collateral blood flow

A transient reduction of collateral blood flow can be caused by:

(i) a reduction of the driving pressure between the origin and the termination of collaterals;

(ii) an increased resistance across the collaterals themselves, due to a selective vasoconstriction;

(iii) a combination of both.

In turn a reduction of collateral driving pressure can be caused by:

(i) a drop of pressure at the origin of collaterals, due to a significant increase of resistance of upstream vessels or to an increase of flow not associated with a proportionate reduction of upstream vascular resistance;

(ii) an increase of pressure at the termination of collaterals due to a significant increase of peripheral coronary resistance in the collateralised territory;

(iii) a combination of both.

The results of the clinical studies reported above do not allow any inference as to whether the coronary artery vasoconstriction responsible for myocardial ischaemia, occurred selectively in collateral vessels, at proximal or distal sites. It is possible that the impairment of collateral blood flow which causes ischaemia, is part of a generalised distal coronary vessel constriction which causes ischaemia also in patients without collaterals during intracoronary NPY infusion (56) and in dogs during intracoronary endothelin infusion (57). Evidence of possible dysfunction of distal vessels, even in patients without critical epicardial coronary stenoses, is growing (58, 59, 60). This constriction may occur in either pre-arteriolar vessels which, because of their extramyocardial portion or size, are not directly involved in the metabolic autoregulation of blood flow, or in arteriolar vessels which are directly involved in the continuous matching of blood supply to myocardial metabolism (58).

The mechanisms responsible for the abnormal vasoconstrictor response of reactive coronary vessels and collaterals in patients may be multiple. Defective endothelial function, hyperreactivity of smooth muscle to constrictor stimuli, abnormal neural stimuli as well as abnormalities of the arteriolar metabolic control, alone or in combination, can be responsible for a transient impairment of collateral blood flow at the site of pre- or post-collateral vessels or in the collaterals themselves.

The alterations causing ischaemia by distal coronary vessel constriction are not necessarily the same in all patients, and may not exist or be the same in experimental animals.

CONCLUSIONS

The development of coronary collaterals in humans in response to epicardial coronary stenoses and occlusion is quite variable. The reason for this variability is likely to be related to the amount of pre-existing interarterial anastomoses between major coronary branches, but it may also depend on ischaemia-mediated neoformation of vascular connections at the capillary level and on their subsequent growth.

The extent of collateral blood flow compensation is indicated by the restoration of post-occlusive or post-stenotic coronary perfusion pressure towards its original values. When post-occlusion or post-stenotic pressure drop occurs because of distal arteriolar vasodilation, it may not be fully compensated for by increased blood flow through collaterals, therefore subendocardial layers become hypoperfused and ischaemia develops.

For any given degree of collateral development, its compensatory function can be modulated by changes in vasomotor tone in pre-, post- or collateral vessels due to abnormalities of the endothelium, hyperreactivity of the smooth muscle to constrictor stimuli and to abnormal neural stimuli.

The precise understanding of the mechanisms responsible for small and/or collateral coronary vasoconstriction causing or contributing to cause myocardial ischaemia, would open the way to the development of specific antivasoconstrictor agents. This appears to be an important line of research because at present, in order to prevent occasional episodes of local coronary constriction, we are forced to use drugs that persistently reduce the vasomotor tone in the whole body. This approach, which is the only option at present, may not fully succeed in its purpose either because the local constrictor stimuli or response to these are particularly strong or because the reduction

of systemic vasomotor tone causes reflex vasoconstriction by feed-back control mechanisms and this feed-back is greater on coronary vessels.

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