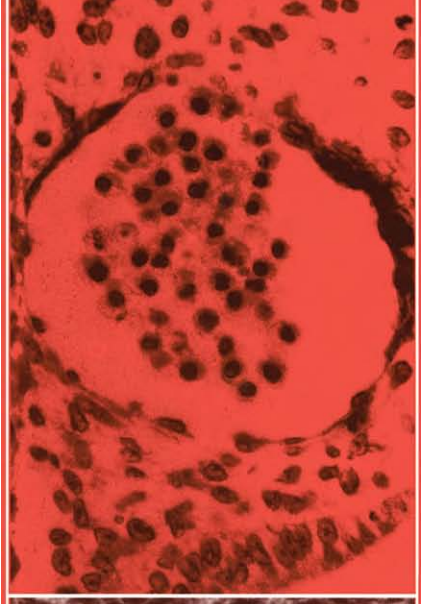

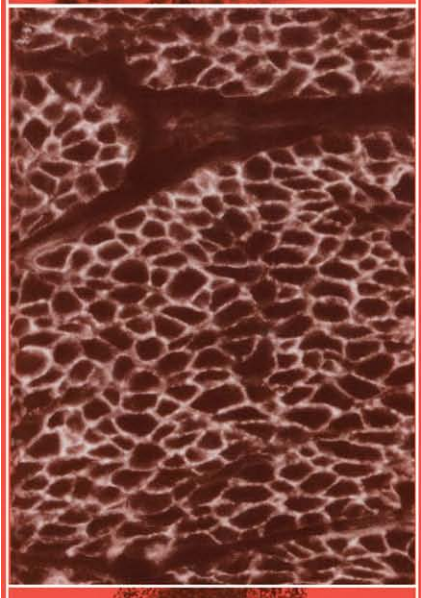

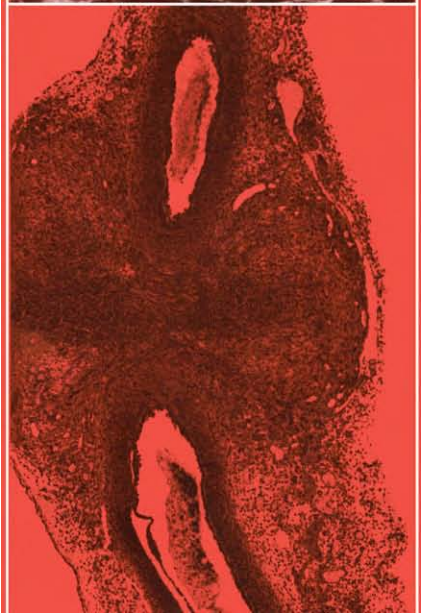

The Vascular Smooth Muscle Cell

Molecular and Biological Responses to the Extracellular Matrix

EDITED BY

Stephen M. Schwartz

AND

Robert P. Mecham

The Vascular Smooth Muscle Cell

Biology of Extracellular Matrix Series

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THE VASCULAR SMOOTH MUSCLE CELL

Molecular and Biological Responses to the Extracellular Matrix

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Preface

The theme of this book is a question: What is a smooth muscle cell? Until the 1970s, the answer would have been quite clear. Most of our knowledge of the biochemistry of the vessel wall was based on studies of vascular smooth muscle. Smooth muscle biochemistry was easy because the vessel wall is made up largely of smooth muscle. If one takes a typical artery, scrapes off the endothelium, and then strips off the adventitia, what remains is arguably a pure population of smooth muscle cells. Such a preparation is even cleaner, in terms of cell type, than the usual biochemist's "stew" made from liver. As a result, the kinds of biochemistry that could be done in that era led to a great deal of knowledge about the chemical composition of vascular smooth muscle cells.

The situation changed dramatically around 1973. In that year, studies by Eric Jaffee on the endothelium, studies by Gordon and Julie Campbell working on smooth muscle, and work by Russell Ross on smooth muscle led to practical techniques for culturing each of these cell types. The immediate result was a change in emphasis by biochemists interested in the vessel wall. Cultured smooth muscle cells proved to be a great disappointment. As quickly noted by the Campbells, the cultured smooth muscle cell lost most of the features so carefully characterized by the biochemist using freshly obtained smooth muscle. *In vitro,* smooth muscle cells lost most of their contractile proteins. Thus, the hope of understanding smooth muscle contractile mechanisms using passaged cell lines turned out to be futile. Even criteria for distinguishing "fibroblasts" from smooth muscle cells have been unclear; as discussed in the chapter by Desmoulière and Gabbiani, cells usually thought of as fibroblasts can express "smooth muscle" proteins.

In contrast, the cultured endothelial cell offered tremendous insight into endothelial biology. Whole new fields of biology have been derived from studies of this cell in culture. We now know, for example, a great deal about the adherence molecules used by endothelial cells and leukocytes to provide the adherence necessary for the inflammatory response. We also know a great deal about molecules produced by endothelial cells to act as vasoregulators for smooth muscle function. Finally, there has been extensive characterization of the molecules involved in angiogenesis, the very process of forming new blood vessels. Endothelial biology has progressed to the point where ambitious clini-

cal trials based on our understanding of the role of these molecules in various vascular diseases are already underway.

That ultimate goal, the use of vascular biology to understand vascular disease, is obviously a driving force toward understanding smooth muscle biology as well. It is at least arguable that a greater proportion of human disease is dependent on smooth muscle function than on endothelial function. For example, in the United States, as in most of Europe, atherosclerotic vascular disease is the single most prevalent killer. Based on early work by Haust and later work over many years by Robert Wissler and by Russell Ross and his collaborators, it is widely believed that the smooth muscle cell is a central target of atherosclerosis. Indeed, at times the debate over atherosclerosis has become so extreme that investigators have questioned the role of macrophages! Similarly, if we include the complications of hypertension and exclude infectious diseases, it is likely that hypertension is, worldwide, a more important disease than atherosclerosis. The belief that the genes for familial hypertension will turn out to be genes endogenous to the vessel wall, particularly genes that control vessel wall mass, is widespread.

Now, 20 years after the advent of smooth muscle culture, the object of this book is to explore what we believe is the beginning of a new era. That era will be driven by new techniques and new tools. The tools obviously are the tools of molecular biology. It is our opinion, however, that molecular biology provides more than tools. The electron microscope changed our concept of biology, but molecular biology has had an even more revolutionary effect. New kinds of questions can be asked, or at least old questions can be addressed much more explicitly. Examples of the impact of molecular biology are the chapters included in this book devoted to the identity of smooth muscle cells. These include the chapters by Glukhova and Koteliansky, McQuinn and R. Schwartz, Small and North, Periasamy and Nagai, and S. M. Schwartz and colleagues, as well as the overviews by the Campbells and Owens.

These chapters really debate the identity of the smooth muscle cell itself. What do we mean by a smooth muscle cell? In the opening paragraph, we referred to the fact that biochemists used the medium of the blood vessel, that is, what was left after removing endothelium and adventitia, to study "smooth muscle." The assumption was that this was a homogenous tissue. If one contrasts the homogeneity of the medium of an artery with the heterogeneity even of the liver, one is immediately struck by the simplicity of the artery wall. Histochemical studies, by Gabbiani and Frid among others, suggest that there is more than one kind of smooth muscle cell. Thus, there is debate about whether these kinds of smooth muscle cells represent distinct lineages, as are found in the immune system, or adaptive forms of a single cell

type. The answer to that question is likely to depend on the discovery of the transacting factors responsible for controlling sets of smooth-muscle-specific genes. That topic, in today's terms, becomes promoter analysis, and the chapters by McQuinn and R. Schwartz and by Periasamy and Nagai begin to address this issue for two genes in particular: smooth muscle α-actin and myosin. While we have emphasized the molecular biology of the expression of smooth muscle cell proteins, the identification of proteins specific to the smooth muscle cell is likely to be important for another field not discussed in the present edition of the book, that is, the nature of the smooth muscle contractile apparatus. We chose not to include this topic because it is well covered in other books. Nonetheless, the fields will converge: as more is understood about the control of expression of smooth muscle proteins, and more proteins are identified in terms of their specificity to the smooth muscle cells, we will undoubtedly learn more about how these proteins work together. The chapter by Small and North attempts an early bridging of this gap by considering how smooth muscle cell-specific proteins interact.

Before attempting to define a vascular smooth muscle cell, one needs to ask how vessels are organized. The opening chapter describes the morphogenesis of the tunica media. Not surprisingly, this is a very new field. Historically, embryologists have been much more interested in the origins of endothelium than in the origins of smooth muscle. The field of vascular developmental biology was greatly accelerated by the discovery in the late 1980s of tools that can be used to identify early endothelial cells. Those tools, especially antibodies able to recognize quail endothelium that could be used in chick-quail chimeras, led to a great deal of understanding of how vessels are developed. The essence of the story is that we know that endothelial precursors are present as early as gastrulation and that these precursors migrate widely across the body to form the primitive vascular system. This topic, as discussed in the chapter by Gittenberger-de Groot and her colleagues, leads to a fundamental understanding of how the pattern of the vasculature is organized. Obviously, however, those endothelial cells could not function without smooth muscle coats. The question of how the smooth muscle coats arise is only now beginning to be explored. Mediators, and even adherence molecules, involved in the migrations necessary to form the coats are not known. The chapter by Glukhova and Koteliansky begins to approach the subject by asking about the kinds of smooth muscle molecules, including matrix molecules and adherence molecules, that are expressed during vessel wall development.

Finally, the book progresses to an attempt to begin looking at the pathology of smooth muscle cells. It was somewhat difficult to decide

how to assemble a set of chapters dealing with smooth muscle pathology. A discussion of the role of smooth muscle cells in hypertension or atherosclerosis alone could be the subject of a whole series of books. Our decision was to focus on basic processes, that is, the processes proposed to underlie smooth muscle pathology. By and large, those are the processes of smooth muscle cell hyperplasia and hypertrophy. We chose to approach this issue as a problem for traditional pharmacology; that is, what agonists and receptors control smooth muscle replication and growth? A great deal of our knowledge of this subject has been based on the balloon injury model in the rat, a topic which is explored well in the chapter by Reidy. However, emphasis on the rat model may be an oversimplification.

The issue of whether smooth muscle replication is important, however, turns out to be much more complex than usually proposed. The chapter by S. M. Schwartz and coauthors points out that attempts to identify smooth muscle replication at critical points in human disease have generally failed. This does not mean that smooth muscle replication is not important; however, we do not know whether replication occurs in human disease and the debate over the importance of proliferation is critical. We do know from studies in other animals and in cell culture that a larger and more complex set of molecules has been implicated in smooth muscle replication. The chapter by Jackson is very important for readers interested in potential targets for pharmacological intervention in processes believed to be involved in smooth muscle replication. Similarly, the critical overview by Ross should give the reader a balanced point of view from the individual most responsible for the current paradigm of vascular response to injury.

In summary, we may assume that over the next few years, not only will the identity of genes specific to the smooth muscle lineage be made clear, but the mechanisms controlling the expression of these genes will be identified. That identification, in turn, will help us to define what we mean by "smooth muscle cell." We expect that this same process will lead to new insights into vascular developmental biology and vascular pathology.

Finally, the editors thank the authors of this book. Most of the authors have written quite new chapters, and much of the discussion and many of the ideas have not been presented elsewhere. Similarly, asking the prestigious authors who have written our overviews to be concise and to present critical views is an unusual request. We thank them not only for the briefness of their articles but also for the precision of their arguments.

Stephen M. Schwartz

Development of the Vessel Wall: Overview

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I. Differentiation, Dedifferentiation, and Phenotypic Modulation of Smooth Muscle Cells

Jarmolych *et al.* (1968) and Fritz *et al.* (1970) first used the term "dedifferentiation" when describing the outgrowth of smooth muscle cells from pig aorta media explants in culture. They suggested that mature smooth muscle cells dedifferentiated in the first few days in culture to modified smooth muscle cells, then to fibroblast-like cells, then finally to primitive cells, whereupon they rapidly proliferated.

When we first described the growth of enzyme-isolated smooth muscle cells in primary culture (Campbell *et al.*, 1971) we also used the term "dedifferentiated" to describe the changes observed in these cells. However, in 1979 when we realized that these changes were often spontaneously reversible or could be made so, we adopted the term "phenotypic modulation" (Chamley-Campbell *et al.*, 1979). This was because the literature indicated that many differentiated cells

can alter their character when the environment changes. These alterations in character are called modulations of the differentiated state and involve reversible interconversions between phenotypes. Modulations in cell phenotype may occur as a result of cell interactions, alterations of extracellular matrix, or in response to other signals such as hormones.

Smooth muscle cells are capable of expressing a range of phenotypes. At one end of the spectrum of phenotypes is the smooth muscle cell whose function is almost exclusively that of contraction ("contractile" state). This function is reflected structurally with 80 to 90% of the cell volume occupied by contractile apparatus (Gabella, 1984). Organelles such as rough endoplasmic reticulum, Golgi, and free ribosomes are few in number and are located in the perinuclear region. Cells of the taenia coli, vas deferens, circular muscle of the ileum, and smaller muscular arteries express this phenotype (see Gabella, 1984). These muscles contain relatively little connective tissue (e.g., volume of extracellular space is about 30% in taenia coli, and 10 to 15% in the circular muscle of the ileum), and much of this matrix is synthesized by constituent fibroblasts. The smooth muscle cells therefore require little synthetic function. At the opposite end of the spectrum of phenotypic expression is the muscle cell whose function is almost exclusively that of synthesis ("synthetic" state). In line with other cell types actively engaged in the production of extracellular matrix (such as fibroblasts), the cytoplasm contains few filament bundles but large amounts of rough endoplasmic reticulum, Golgi, and free ribosomes. Cells in the synthetic state are seen in development and repair (Campbell *et al.*, 1981).

It must be emphasized that the contractile and synthetic states just described represent, to our mind, the extreme ends of a continuous spectrum of phenotypes with most smooth muscle expressing an intermediate morphology. For instance, the volume of the connective tissue containing extracellular space in large mammalian elastic arteries is 50 to 60% and smooth muscle is the only cell type present (Pease and Paule, 1960; Karrer, 1961; Paule, 1963). This cell must, therefore, not only be responsible for contraction/relaxation but the normal turnover of extracellular matrix components. Again this is reflected structurally with 60 to 70% of the cell volume occupied with myofilaments in the adult animal (Gerrity and Cliff, 1975; Olivetti *et al.*, 1980).

We have suggested that most of the characteristics of smooth muscle cells in atherosclerotic plaques and myointimal thickenings can be accounted for by normal phenotypic changes of the average cell in the

adult arterial wall, **complicated by special local conditions**. We also argue that vascular smooth muscle cells in culture undergo similar changes in phenotypic expression and biology and are thus an appropriate model for cells attempting to effect repair.

II. Phenotypic Changes in Vascular Smooth Muscle Cells in Culture

Smooth muscle cells enzyme dispersed from their tissue of origin and plated in primary culture are ribbon or fusiform in shape and phase dense during the first 3 days (Chamley *et al.*, 1973, 1974; Chamley and Campbell, 1975a,b). In the case of smooth muscle from some visceral organs such as the taenia coli, gizzard, vas deferens, or ureter, the cells are also spontaneously contractile (Chamley *et al.*, 1973; Purves *et al.*, 1974; Campbell *et al.*, 1971, 1974). There are only a few reports of spontaneous contractions in single vascular smooth muscle cells in culture, but in the first 1 or 2 days of primary culture the cells can be stimulated to contract with vasoactive agents (Chamley *et al.*, 1977; Mauger *et al.*, 1975; Ives *et al.*, 1978). "Contractile" smooth muscle cells do not in general proliferate even though they are continuously exposed to serum mitogens in the culture medium from day 0. After about 5 days, most cells have spread, become less phase dense, and resemble fibroblasts. All spontaneous or inducible contractions cease.

Ultrastructurally, smooth muscle cells isolated from the aorta of mature animals in the first 3 days of culture closely resemble the cells of the intact tissue with a high volume density of myofilaments ($V_v$myo) as measured using ultrastructural morphometry. However, after 5 days in culture, the $V_v$myo decreases significantly to resemble the cells during development or regeneration ("synthetic" state). Concomitant with this change, the α-actin mRNA as a percentage of total actin mRNA significantly decreases from about 84 to 54%. The α-actin protein also decreases from 92 to 70%. The latter is not sufficient to result in an observable decrease in staining with fluoresceinated α-actin antibodies, and this fact has led some researchers (see Schwartz *et al.*, this volume) to believe phenotypic change has not occurred, when in reality this parameter is not a definitive indicator. Indeed, as many contributors to this volume have pointed out, α-actin is not even a definitive marker of smooth muscle lineage since it occurs in many nonmuscle cell types. Staining with antibodies to specific myosin isoforms, however, decreases dramatically on phenotypic change (Gröschel-Stewart *et al.*, 1975; Larson *et al.*, 1984; Rovner *et*

al., 1986; Kawamoto and Adelstein, 1987; Periasamy and Nagai, this volume). One day after phenotypic change is complete the cell number in the culture begins to increase as the cells proliferate logarithmically in the presence of serum mitogens. This continues until confluency is reached (Chamley-Campbell *et al.*, 1981; Campbell *et al.*, 1989b).

If more than five cumulative population doublings are undergone to achieve confluency, the smooth muscle cells remain indefinitely in the "synthetic" state and are thus termed "irreversible synthetic" (Campbell and Campbell, 1987; Campbell *et al.*, 1989b). This does not mean that they are transformed since with continued passaging they become senescent, that is, are unable to proliferate further. This exhaustion of the replicative capacity is characteristic of normal diploid cells, and indeed senescence is a characteristic of many smooth muscle cells in advanced human lesions (Abdulla *et al.*, 1984; Ross *et al.*, 1984). Senescent smooth muscle cells from other tissues also have a low $V_v$myo (Cliff, 1970; Martin *et al.*, 1983; Campbell and Campbell, 1984). "Irreversible synthetic" state smooth muscle cells in culture produce up to 43-fold the amount of collagen as "contractile" state cells (Ang *et al.*, 1990). The type of collagen also changes, with an increased proportion of collagen type I similar to that found in plaques. The amount of glycosaminoglycans (particularly chondroitin sulfate) increases 5-fold with change in smooth muscle phenotype in culture, similar to that seen in lesions (Merrilees *et al.*, 1990). Indeed, in human lesions, senescent smooth muscle cells are often surrounded by a dense capsule of glycosaminoglycan-containing proteoglycans. "Irreversible synthetic" cells also bind, internalize, and degrade more of the atherogenic lipoprotein β very low density lipoprotein than "contractile" state cells, resulting in an overaccumulation of lipids (Campbell *et al.*, 1986).

Vascular smooth muscle cells in culture undergo many other changes to resemble the smooth muscle cells of plaques. These include an increase in the vimentin to desmin ratio (Skalli *et al.*, 1986), an alteration in the metavinculin to vinculin ratio (Birukov *et al.*, 1993), the form of caldesmon expressed (Glukhova *et al.*, 1987; Ueki *et al.*, 1987; Birukov *et al.*, 1993), the form of fibronectin (Glukhova *et al.*, 1989), and the expression of HLA-DR after stimulation with γ-interferon (Hansson *et al.*, 1988; Warner *et al.*, 1989). Platelet-derived growth factor (PDGF)-A and PDGF-B genes are also expressed (Sjölund *et al.*, 1988; Majesky *et al.*, 1988; Valente *et al.*, 1988) with a change in smooth muscle phenotype.

III. Reversibility of Smooth Muscle Phenotypic Change

It is important to note that a change in smooth muscle phenotype in culture is reversible once cells have achieved confluency as long as they have undergone fewer than five cumulative population doublings. Under these circumstances, the $V_v$myo of the cells, the percentage α-actin mRNA, and staining with myosin antibodies return to near original levels (Campbell *et al.*, 1989b). *In vivo*, a similar reversible phenotypic change is observed in the smooth muscle cells which form the myointimal thickening in the ballooned carotid artery both ultrastructurally (Manderson *et al.*, 1989; Campbell *et al.*, 1988) and with markers for smooth muscle differentiation such as α-actin, myosin, tropomyosin, and desmin (Kocher *et al.*, 1991; Desmoulière and Gabbiani, this volume). Indeed, a change in phenotype of mature smooth muscle cells appears to be a normal response of the artery wall to injury or disruption. This causes the cells to switch from their normal function of contraction and maintenance of vessel wall tone to one of repair of the wound by synthesis of matrix and/or proliferation. Once the wound is healed, the cells return to their previous phenotype and function.

IV. Does Change in Phenotype Imply Replication?

Change in phenotype of smooth muscle cells does not necessarily imply cell replication, and indeed it is possible to maintain smooth muscle cells quiescent in the synthetic phenotype simply by the exclusion of mitogens. Also, "synthetic" state cells which have undergone multiple divisions become senescent, that is, are **unable** to replicate. Smooth muscle cells with a low $V_v$myo are also capable of producing large amounts of matrix and this, rather than division, may be the functional demand placed on the cell.

It is also important to recognize that not all smooth muscle cells have to undergo a significant change in phenotype to divide. In 1974, we (Chamley and Campbell, 1974) observed and filmed spontaneously "contractile" visceral smooth muscle cells in culture undergoing division. These cells stopped contracting for only a few minutes during the actual process of cytokinesis. However, it must be emphasized that this phenomenon is rarely observed, and in the majority of cases when one sees a dividing smooth muscle cell *in vitro* or *in vivo* it is not contractile and has a low $V_v$myo.

V. Why Do the Majority of Contractile Smooth Muscle Cells Undergo a Change in Phenotype prior to Mitosis?

To understand this, it may be helpful to look at other differentiated muscle systems. The adult mammalian ventricular myocyte is considered incapable of repairing local injuries, and a connective tissue scar forms at the site of injury (Fanburg, 1970; Bing, 1971; Rumyantsev, 1977, 1981). This loss of regenerative ability is due to an irreversible withdrawal of the cardiac myocyte from the cell cycle (Ingwall, 1980) and the fact there are no satellite cells present as in skeletal muscle (Rumyantsev, 1979; Campion, 1985). Cardiac muscle of developing animals, however, can divide but this requires some loss of Z-disc material and myofibrillar disintegration (Oberpriller and Oberpriller, 1971; Rumyantsev, 1972, 1974, 1982; Erokhina and Rumyantsev, 1983). Thus whether or not cells are capable of division appears related to their degree of differentiation or development of specialized characteristics.

Similarly, in "contractile" smooth muscle cells the dense packing of myofilaments may hamper cytokinesis. Disorganization and/or loss of myofilaments as occurs with phenotypic change may allow the necessary shape change to occur.

VI. Cells with an Undifferentiated Appearance in Mature Blood Vessels

There are many reports of "poorly differentiated" (Lee *et al.*, 1970), mesenchyme-like (Wilcox *et al.*, 1988), and fibroblast-like (Geer and Haust, 1972) cells and smooth muscle cells of altered phenotype in the intima of human arteries (Mosse *et al.*, 1985b, 1986; Orekhov *et al.*, 1984; Babaev *et al.*, 1988, 1990). Their origin currently is not known but there are several possibilities.

1. They may be mature smooth muscle cells which have temporarily altered phenotype to perform a specific function such as repair after local injury.
2. They may be aged, senescent smooth muscle cells.
3. They may be a subset of smooth muscle cells (see Schwartz *et al.*, this volume).
4. They may be derived from blood-borne cells.

VII. Origin of Intimal Smooth Muscle Cells

As discussed earlier, we and Gabbiani's group have shown that when one balloon deendothelializes a vessel, smooth muscle cells un-

dergo a change in phenotype in order to migrate, proliferate, and synthesize matrix in the repair of the vessel wall. Proliferation ceases after about 2 weeks, and 8–18 weeks later the smooth muscle cells in the neointima return to approximately the same phenotype as prior to injury in relation to $V_v$myo and other markers of differentiation such as α-actin, tropomyosin, myosin, and desmin. Indeed, there is reexpression of the differentiated phenotype as early as 5 days after injury when animals are treated with heparin (Clowes *et al.*, 1988). However, we would predict from cell culture studies already described that if there are many cell divisions in forming the neointima then the cells may stay in an "undifferentiated" phenotype ("irreversible synthetic") (see Campbell *et al.*, 1989b). These cells would then constitute a permanent subset.

Alternatively, Schwartz *et al.* (1985; see also this volume) suggested that the cells which form a myointimal thickening after balloon injury of adult rat arteries may be derived from a subpopulation of embryonic stem cells residing within the media. They argued that cells from the neointima of the ballooned arteries (after multiple passaging in culture) are epitheloid, express the PDGF-B gene, and secrete large amounts of PDGF-BB, but have little or no PDGF α-receptor mRNA. They noted that this is similar to medial smooth muscle cells multipassaged from very young rats, but unlike those from adult rat arterial media which express abundant PDGF α-receptor mRNA but little PDGF-B mRNA and little or no PDGF-BB (Walker *et al.*, 1986; Majesky *et al.*, 1992). Despite the large differences in PDGF-B mRNA levels of passaged "stem" cells compared with adult medial smooth muscle cells, Majesky *et al.* (1988) found only similar low levels of PDGF-B transcripts in intact aorta from newborn and adult rats. Indeed, primary cultures of neointimal smooth muscle cells from adult rats or medial smooth muscle cells from newborn rats had low levels of PDGF-B mRNA that were similar to those in the intact artery, suggesting that the earlier observation could represent induction of gene expression *in vitro*.

In 1992, Majesky *et al.* constructed and screened a smooth muscle cDNA library for molecular markers of the "fetal-neointimal" SMC phenotype. Two cDNA clones were identified, and elevated levels of the two mRNAs were maintained in cultures of neointimal (but not medial) smooth muscle cells in culture. DNA sequence analysis indicated that the cDNA clones encoded rat tropoelastin and α_1 procollagen (type I). However, our studies indicate that upon phenotypic change to the "synthetic" state in culture, adult rabbit medial smooth muscle cells express 20- to 30-fold α_1 and α_2 procollagen (type I) and 5-fold α_1 procollagen (type III) as when they are in the "contractile" state (Campbell *et al.*,

1989a; Tachas *et al.*, 1990). Fibronectin and elastin mRNA are also increased by 15- to 20-fold. Further screening of Majesky's smooth muscle cDNA library by use of a subtracted probe enriched in fetal-neointimal-specific sequences identified a third extracellular matrix protein, osteopontin (Giachelli *et al.*, 1991). In addition to calcified tissues, sources of osteopontin include normal cells in the kidney, inner ear, placenta, decidua, brain, and bone marrow, and all transformed cells thus far examined regardless of origin synthesize significantly higher levels of osteopontin than their untransformed counterparts (Craig *et al.*, 1988). In this regard it is interesting to note that, like transformed cells, fetal-neointimal cells have a mechanism for growth that does not require either exogenous PDGF or fibroblast growth factor (Bondjers *et al.*, 1991).

A. Myofibroblasts

In 1979, Guido Majno wrote an amusing and well-worth reading story of his first experiments in 1958 which led to the description of myofibroblasts. While it is now fully established that these cells play important roles in wound contracture, their origin has not been unequivocally determined (Majno *et al.*, 1971; Gabbiani *et al.*, 1971, see Desmoulière and Gabbiani, this volume). The early experiments on granulation tissue fibroblasts (myofibroblasts) were made using the croton oil-induced granuloma pouch which is a highly vascular tissue. This raised the possibility that myofibroblasts were not of fibroblastic origin but were "synthetic" state smooth muscle cells derived from surrounding blood vessels. Campbell and Ryan (1983; see also Mosse *et al.*, 1985a) addressed this problem by studying the origin of myofibroblasts in the avascular capsule which surrounds free-floating intraperitoneal blood clots, concluding that these myofibroblasts were derived from peritoneal macrophages. Since there is evidence of a blood monocytic origin of peritoneal macrophages, this raised the "old" question of whether some circulating mononuclear leukocytes can develop into fibroblasts (or myofibroblasts) within tissues.

B. Can Hematogenous Mononuclear Leukocytes Develop into Fibroblasts/Smooth Muscle Cells?

In 1904, Maximov reported that mononuclear white blood cells in sterile inflammatory exudate in rabbits undergo "transformation" into fibrocytes. In 1928 he observed a similar transformation in buffy coat cells grown in tissue culture. There have been many reports of buffy coat cells (Allgöwer and Hulliger, 1960; Carrel and Ebeling, 1926; Fischer, 1925), cultured peritoneal exudates (Kouri and Anceta, 1972), and marrow cells in diffusion chambers (Berman and Kaplan,

1959) transforming into fibroblasts. However, few studies on the transformation of hematogenous cells into fibroblasts have appeared in the literature since 1965 when Russell Ross and his student Jack Lillywhite reported the fate of buffy coat cells in subcutaneously implanted diffusion chambers. Their studies "did not rule out the possibility of conversion of hematogenous cells to fibroblasts," but showed that contamination by extraneous connective tissue cells while withdrawing blood could account for the apparent transformation of cells (Ross and Lillywhite, 1965). Their findings were further substantiated by studies of wound healing in parabiotic rats (Ross *et al.*, 1970).

This hypothesis is, of course, relevant to the stem cell proposal of Schwartz and colleagues (this volume) for if hematogenous cells are capable of differentiation they may provide an alternative source of some smooth muscle cells in the intima of arteries. As pointed out by Jackson (this volume), one potential problem with cultured cells is that they are usually the progeny of a small proportion of cells in the tissue of origin. He cites his own study where after enzymatic dispersion of the rat aorta, only 9% of the cells originally in the tissue survived in primary culture. Since Schwartz *et al.* (this volume) did not see the dramatic differences in intimal cells as a population *in vivo* or in primary culture but only after passage five, it is possible that the "fetal-intimal" cells are not typical of the intima but are a subset within the developing intimal thickening.

VIII. Conclusions

There is no doubt that smooth muscle, as multifunctional mesenchyme, is capable of regeneration (see Wissler, 1968). Depending on the functional demands placed on the smooth muscle cell, it can exist in a whole spectrum of phenotypes. When the prime function is contraction the cytoplasm is filled with myofilaments, and when it is repair (synthesis of matrix and/or cell division) the morphology changes to fit the altered functional demand. There is no argument that smooth muscle cells are heterogeneous, but whether this heterogeneity extends to a subset of "stem" cells within the arterial media, preferentially stimulated to migrate and proliferate following injury, has yet to be proven. Logistically, at least, this does not appear necessary as mature smooth muscle cells can readily regenerate.

The Schwartz and Campbell hypotheses are not mutually exclusive. Perhaps there is a small number of mature smooth muscle cells in the artery wall which are transiently in the "synthetic" phenotype because of normal turnover or because they are effecting local repair. Upon balloon injury, these cells would be first to respond as they

would not need to change phenotype, thus the neointimal thickening would consist primarily of these cells. In the special environment of the neointima, the cells of altered phenotype may be susceptible to changes in gene expression, many of which do not become manifest until after multiple passage in culture.

Alternatively, both we and Schwartz may be wrong/part right, with some of the proliferating cells of the neointima not derived from the media at all, but from blood-borne hematogenous mononuclear leukocytes transformed into myofibroblasts.

IX. Questions Which Need to Be Answered

1. Do neointimal smooth muscle cells, once they have morphologically returned to the contractile phenotype (i.e., more than 8 weeks after injury), still exhibit changes in gene expression when passaged in culture or is this phenomenon confined to cells of the 2-week neointima?
2. Do species other than the rat share these distinctive changes in cultured neointimal cells 2 weeks after injury?
3. Is there evidence of smooth muscle "stem" cells in nonvascular tissues?
4. How does the replicative history of a smooth muscle cell influence its gene expression?
5. Can cells from the blood become proliferative myofibroblasts and contribute to the formation of neointima?

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Smooth Muscle Cell Differentiation during Early Development and during Intimal Thickening Formation in the Ductus Arteriosus

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I. Introduction

The impetus for studying the development of vascular smooth muscle comes from two facts. First, most of the pathology of blood vessels involves the formation of a special vascular layer, the intima. We still do not know why or exactly how this layer is formed. Second, the smooth muscle cells of the intima often have a different phenotype *in vitro* compared with the medial smooth muscle cells. The intimal phenotype resembles the immature, also called pup or fetal phenotype, in the expression of several mRNAs like platelet-derived growth factor

(PDGF)-B chain and the absence of PDGFα receptor mRNA (Majesky *et al.*, 1988; Majesky and Schwartz, 1990). Elastin, cytochrome P450IA1, and procollagen type I are other specific genes characteristic for the immature phenotype (Giachelli *et al.*, 1991; Majesky *et al.*, 1992). *In vivo*, intimal cells overexpress some of the same genes and fail to express many of the genes of differentiated medial smooth muscle (see chapters by Schwartz *et al.*; Glukhova and Koteliansky; and Demoulière and Gabbiani). The expression of genes characteristic for the immature phenotype during development, in the intimal cells of full grown vessels, implicates that the developmental processes occurring during smooth muscle cell differentiation may be important for understanding vascular pathology.

This chapter reviews current knowledge of the developing smooth muscle cell. We start with the very early differentiation and gene expression, followed by the expression of smooth muscle α-actin during early development. Subsequently, the formation of the tunica media of the vascular wall and the possible role of neural crest cells in this process are discussed. Finally, typical characteristics of the intimal and medial smooth muscle cells of the ductus arteriosus are reviewed, as this vessel is unique in developing physiological intimal thickening.

II. Regulation of Smooth Muscle Cell Differentiation and Analogies to Skeletal Muscle

In differentiation the expression of each gene is determined by the interaction of regulatory proteins in the cell at any time given. To remain in the differentiated stage, uninterrupted expression of negative and positive regulators is essential (Blau and Baltimore, 1991). There are only scant data about controlling mechanisms in smooth muscle cell differentiation. In contrast, skeletal muscle differentiation at the level of transcription has been extensively studied. Since many cytoskeletal proteins are expressed in both, it is feasible that skeletal muscle cells and smooth muscle cells have common elements that control the expression of these proteins (VanNeck *et al.*, 1993). In skeletal muscle cells the members of the MyoD family are master regulatory genes controlling myogenesis (Weintraub *et al.*, 1991). They are characterized by a helix-loop-helix motif that is responsible for dimerization, and, depending on this dimerization partner, activity can be controlled. The MyoD function is a very ancient and highly conserved function, but each organism has its own answer to the questions of how, where, and when a muscle cell is to be generated. MyoD expression starts in a very early stage. In *Caenorhabditis elegans* the capacity for transcription of MyoD is observed as early as

the 28-cell stage. The protein, stained by an antibody against MyoD, is observed only a short period after this at the 100-cell stage. During mouse development, myogenin, one of the other members of the MyoD family, appears in the somites before MyoD, but MyoD appears before myogenin in the limb bud. Therefore it is postulated that different members of this gene family are responsible for initiating different types of muscle lineages during development (Sassoon *et al.*, 1989; Lassar *et al.*, 1989).

MyoD can be suppressed by a dominant suppressor as the inhibitor of differentiation (ID) protein. Upregulation of the latter is stimulated by growth factors. From this ID gene a smooth muscle-specific variant has been found (Kemp *et al.*, 1991; Pauly *et al.*, 1992), substantiating that control mechanisms in skeletal and smooth muscle cells are comparable. Although VanNeck and colleagues (1993) found that rat aortic smooth muscle cells did not contain MyoD homologous mRNA, their experiments with exogenous MyoD suggested that endogenous MyoD might be present. Furthermore, in the presence of FGF, expression of the endogenous MyoD in smooth muscle cells was prevented by either the absence of necessary cofactors or the presence of inhibitors.

In addition to the helix-loop-helix family, genes with a helix-turn-helix motif as the homeobox genes may play a role in smooth muscle cell differentiation. Patel and colleagues (1992) cloned a homeobox transcription factor, called GAX, from vascular smooth muscle cells. GAX was identical to the mouse Hox 1.11 gene. In adults, transcripts were only detected in aortic smooth muscle cells and lung. They were undetectable in cardiac or skeletal muscle, visceral smooth muscle cells, and other tissues, including brain, and they concluded that this gene is specific for vascular smooth muscle cells in the adult. In the embryo, expression was detected in the neural tube with a sharp expression boundary occurring at an anterior position in the myelencephalon, in the third and fourth branchial arches, and in vessels leading from the heart. The authors suggested that this gene probably has multiple roles during development whereas in the adults it participates in the control of vascular smooth muscle differentiation and proliferation.

III. Smooth Muscle α-Actin Expression during Early Development

Until now, two different origins of smooth muscle cells have been reported in literature. First, smooth muscle cells can originate from the ventrolateral mesoderm in *Xenopus laevis* (Saint-Jeannet

et al., 1992). Second, the mesectodermal-derived neural crest cells are reported to give rise to smooth muscle cells in pharyngeal arteries in avian embryos (Kirby and Bockman, 1984). This has been confirmed with retroviral neural crest-tracing techniques (Noden *et al.*, 1995).

One of the most characteristic features of the smooth muscle cell is the presence of smooth muscle α-actin. In *X. laevis*, smooth muscle α-actin is a marker for the ventrolateral mesoderm. The ventrolateral mesoderm gives rise to the lateral plate mesoderm, which contributes to cardiovascular formation, in the endothelial (DeRuiter *et al.*, 1992), smooth muscle, and cardiomyocyte lineage (Saint-Jeannet *et al.*, 1992). Mesodermal induction experiments have shown that with bFGF or low concentrations of XTC-MIF, which is thought to be a member of the transforming growth factor β (TGF-β) family (Woodland, 1989), the mesoderm differentiates into lateral plate mesoderm and activates smooth muscle α-actin expression. Dorsal structures, i.e., somites and notochord, without smooth muscle α-actin expression are induced by activin, another member of the TGF-β family, and high concentration of XTC-MIF. Therefore, it is concluded that as early as in the gastrula stage, the smooth muscle cell precursors are regionalized and differentiate from the ventrolateral mesoderm (Saint-Jeannet *et al.*, 1992).

Smooth muscle α-actin mRNA marks the onset of differentiation of cardiac cells. In early stages from stage 37/38 onward in the *X. laevis* embryo (Saint-Jeannet *et al.*, 1992) and from stage 9 (Hamburger–Hamilton) in the chicken embryo (Ruzicka and Schwartz, 1988), smooth muscle α-actin is observed in the developing cardiac cells of the complete heart tube. In the chick embryonic heart, in contrast to the smooth muscle cells, the smooth muscle α-actin gene is downregulated from stage 12 (Hamburger–Hamilton), whereas the cardiac α-actin and skeletal α-actin transcripts accumulate. The outflow tract of the heart is the last segment with smooth muscle α-actin gene expression, which correlates with the finding that the outflow tract originates in a stage 12 (Hamburger–Hamilton) avian embryo as the last developing segment of the heart (DelaCruz *et al.*, 1977; DeRuiter *et al.*, 1993).

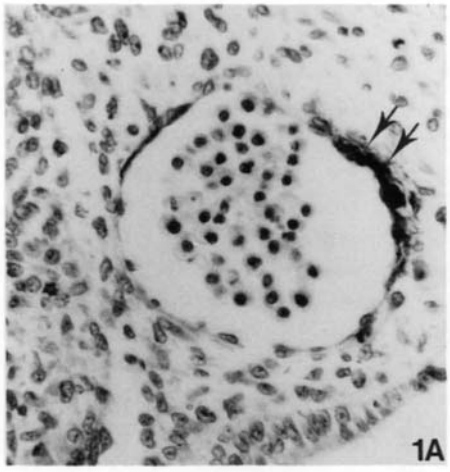
Although smooth muscle α-actin is the most widespread used smooth muscle cell marker, its specificity is not restricted to smooth muscle cells. As discussed earlier and more extensively in the chapter by Glukhova and Koteliansky, smooth muscle α-actin is a marker for the cells of the ventrolateral mesoderm in early stages (Saint-Jeannet *et al.*, 1992). It has been found that smooth muscle α-actin mRNA marks the onset of differentiation of cardiac cells (Ruzicka and

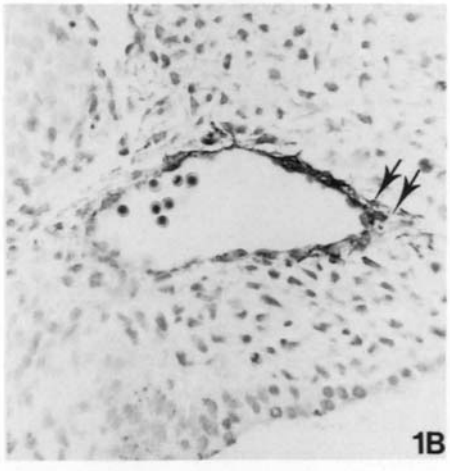
Schwartz, 1988). Also, nonmuscle cells as myoepithelial cells and myofibroblasts (Gown, 1990) express smooth muscle α-actin. During development, smooth muscle α-actin appears before skeletal and cardiac α-actin, and in the heart the presence of smooth muscle α-actin marks different stages of development. Combined with the observations that fibroblasts (Darby *et al.*, 1990) and endothelial cells (Arciniegas *et al.*, 1992) can be induced to express smooth muscle α-actin in processes such as wound healing or under *in vitro* conditions, these results postulate a new role for smooth muscle α-actin. In addition to being a marker for smooth muscle cells, smooth muscle α-actin also marks a certain differentiation state of cells from ventrolateral mesoderm origin. Various stimuli can induce cells from this origin to reexpress their differential phenotypes. This theory would clarify some of the mysteries around the origin of the myofibroblasts (Gown, 1990) and give new perspectives in the question whether a endothelial cell might transdifferentiate into a smooth muscle cell and vice versa. Likewise, the questions around the origin of the myoepithelial cells, being ectodermal or mesenchymal, should be reviewed bearing this hypothesis in mind.

IV. Development of the Tunica Media of the Vascular Wall

Actin expression in relation to the developing tunica media of the vascular wall is first observed at day 10 of the developing rat embryo. With HHF35, an antibody that recognizes muscle-specific α- and γ-actin isotypes, positive cells were found in the mesoderm surrounding the dorsal aorta (DeRuiter *et al.*, 1991). This expression of muscle-specific actins appears before mesenchymal condensations, hence, before a histological detectable tunica media (Fig. 1A). It was also found that the actin-positive cells did not surround the endothelium of the vessel evenly (Figs. 1A and 1B), but that actin-negative areas were present, which were thought to be important sites in the formation of new vessels as well as sites from which already developed vessels disappear. After the main events in vascular remodeling have been completed, α-actin expression spreads along the complete vascular system (Fig. 1C).

Unpublished results of our group show that the ascending aorta and pulmonary trunk exhibit special behavior in smooth muscle cell differentiation. In human fetuses, smooth muscle cells of these vessels express cytokeratins 8, 18, and 19 in late stages of development (Fig. 2A) in contrast to the descending aorta and the ductus arteriosus that have lost this expression (see also chapter by Glukhova and Koteliansky). In avian embryos the ascending aorta and pulmonary





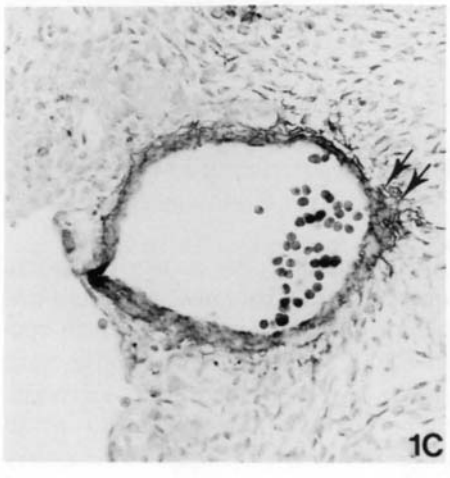


FIG. 1. Media formation in the rat embryo. (A) At 11 days of gestation, an uneven distribution of actin expression (arrows) is observed in the dorsal aorta (312×). (B) In the 13-day-old embryo, mesenchymal condensations start to develop (arrows) and actin expression is spreading around the dorsal aorta (195×). (C) A medial layer is now present around the complete dorsal aorta. The initial side of actin expression (arrows) can still be recognized in the 14-day-old embryo (175×).

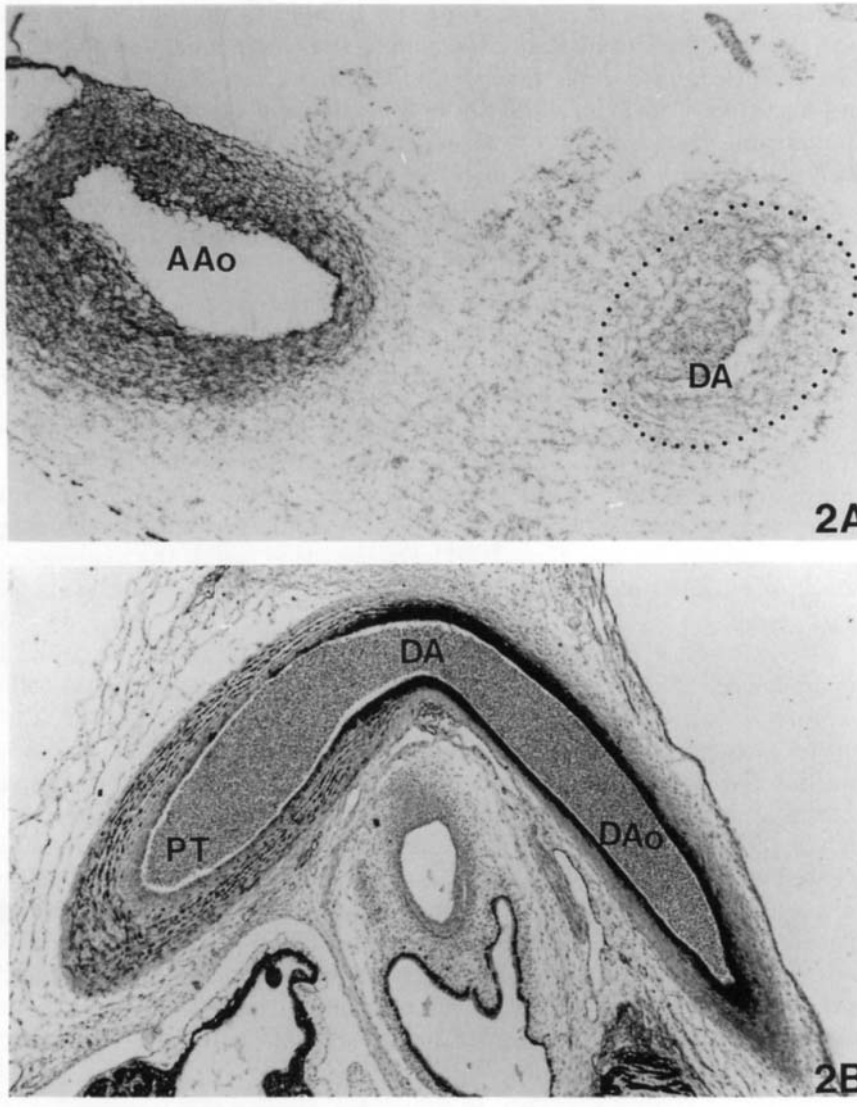


FIG. 2. (A) The ascending aorta (AAo) of a 13-week-old human fetus expresses cytokeratin 8 in high levels, while only some cells in the ductus arteriosus (DA) are positively stained (29×). (B) A patchy actin expression is found in the outer media of the pulmonary trunk (PT); the inner media is negative in a HH37 chick embryo. The DA and descending aorta (DAo) show strong staining of the inner media, whereas the outer media is negative (49×).

trunk are also special. Muscle-specific actins are present in a weak and patchy staining in the outer media; the inner media is negative. The smooth muscle cells, however, of the inner media of the descending aorta, carotid artery, ductus arteriosus, and coronary arteries of comparable stages stain very strongly for these muscle-specific actins, whereas the outer media is negative (Fig. 2B). This staining pattern seems to be stage dependent. This has been described for a number of smooth muscle cell differentiation markers in the outflow tract of the chick heart (Burke *et al.*, 1994).

From our observations, it can be concluded that not every smooth muscle cell contains muscle-specific actins. Whether this difference in actin staining reflects differentiation profiles or differences in cell lineage (neural crest vs mesoderm) requires further study.

During development of the aortic media, the smooth muscle cells differentiate from an immature to a differentiated phenotype. The relative contents of smooth muscle α-actin, smooth muscle myosin heavy chains, *meta*-vinculin, and 150-kDa caldesmon increase during maturation (Glukhova *et al.*, 1991). On the other hand, the expression of splice variants of fibronectin A-FN and B-FN decreases (Glukhova *et al.*, 1990).

PDGF-B has a regulatory role during the formation of micro- and macrovessels (Holmgren *et al.*, 1991). Microcapillary endothelial cells expressed both the PDGF-B chain and the PDGF-β receptor. Endothelial cells of the larger vessels did not express the PDGF-β receptor, but the smooth muscle cells and fibroblast-like cells surrounding intermediate and large blood vessels readily had detectable levels of PDGF-β receptor. The authors suggested that these data demonstrated a switch in the PDGF-B signaling pathway from an autocrine to a paracrine mechanism to stimulate the growth of surrounding mesenchymal stromal cells containing the PDGF-β receptor. Consistent data were found during angiogenesis in neoplasia and cell repair after blood vessel injury. We therefore assume that PDGF-B plays an important role in the process of media formation in the developing embryo as well.

V. Smooth Muscle Development and Neural Crest Cells

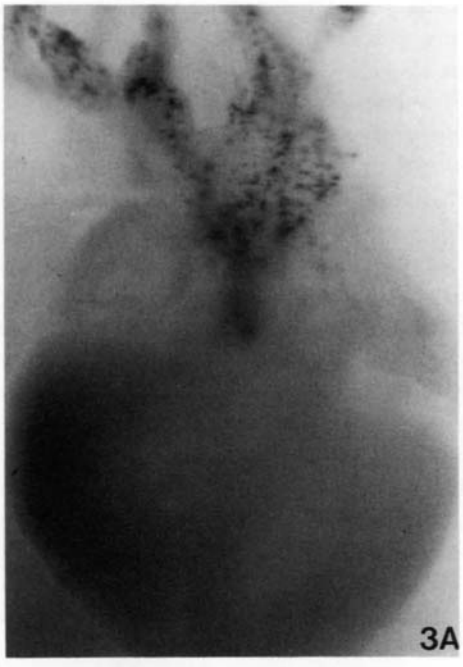
Until now, research concerning the origin of smooth muscle cells has not been conclusive. Actin staining has shown that the mesenchymal cells in the ventrolateral mesoderm are positive, but these also give rise to the developing heart and thus the cardiac muscle phenotype (Saint-Jeannet *et al.*, 1992). The contribution of migrating neural crest cells further complicates the story.

It is evident that smooth muscle cell differentiation is not dependent on neural crest cells because the umbilical artery and vein develop a tunica media (DeRuiter *et al.*, 1991) whereas neural crest cells are not reported there (Kirby and Bockman, 1984; Bronner-Fraser, 1993). In the pharyngeal arch arteries, smooth muscle cells are observed before neural crest cells arrive (Rosenquist and Beall, 1990). The cardiac neural crest, comprising the neural crest cells at the level of the otic placode and the rhombomeres 1–3, gives rise to ectomesenchymal and ganglionic derivates. The ectomesenchymal neural crest cells mainly contribute to the aortopulmonary septum of the outflow tract of the heart, to the carotid and ultimobranchial body, and to the mesenchymal cells of thymus and parathyroids. Neural crest cells are also reported to contribute to the wall of the pharyngeal arteries (Kirby and Bockman, 1984).

Unpublished data from retroviral marking of embryos by Noden and our group show that neural crest cells are extensively present in the wall of the great arteries (Figs. 3A and 3B). Some of these retrovirus-marked neural crest cells are positive for smooth muscle α-actin. There are, however, also cells in the vessel wall that are retrovirus positive and smooth muscle α-actin negative (Fig. 3C). It is not known whether these cells will differentiate at a later stage into smooth muscle cells. It is also possible that these cells induce differentiation of mesenchymal cells into smooth muscle cells or regulate as suggested (Rosenquist and Beall, 1990) elastin formation. Further research with lineage markers as retroviruses may answer questions with regard to the actual role of neural crest cells in smooth muscle cell formation in more detail.

VI. Smooth Muscle Cells of the Ductus Arteriosus

Another unsolved problem concerning smooth muscle cell differentiation is the differentiation of the vessel wall into an elastic or muscular vessel. This question cannot be answered by only taking into account the location of the vessel. This is exemplified by the ductus arteriosus. The ductus arteriosus is a muscular artery connecting the elastic aorta and the elastic pulmonary artery. The typical muscular appearance of the ductus, with a different elastin pattern as compared to the adjoining elastic arteries, as studied by a standard elastic staining (resorcin–fuchsin) (Fig. 4), is not noted during early stages of development of the media. In the rat, distinct morphological differences between the ductus arteriosus and aorta can be observed after 15 days of gestation (unpublished observations). The unique elastin metabolism in the ductus was confirmed by studies of cultured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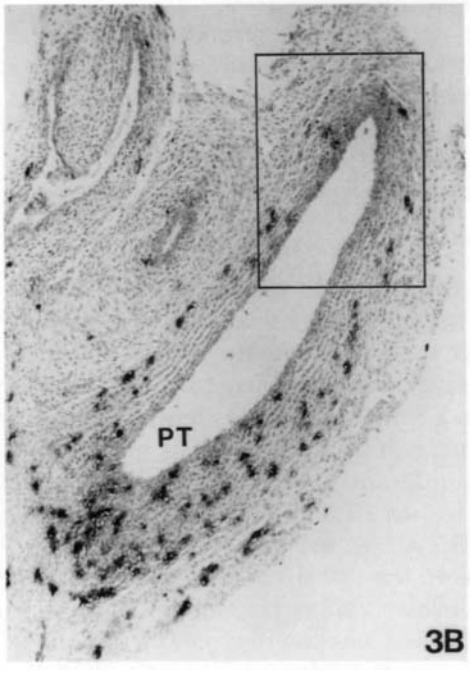
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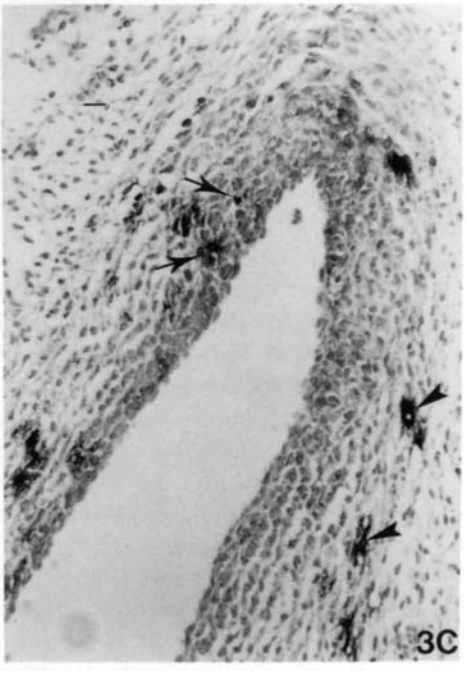
3C

lamb ductus arteriosus smooth muscle cells. Rabinovitch and colleagues (1988) showed that these cells, compared with aortic and pulmonary artery smooth muscle cells, produced a unique protein of 52 kDa, which was later identified as a post-translational product of tropoelastin (Rabinovitch *et al.*, 1990). A specific role of elastin is also observed in the abnormal ductus arteriosus. Normally, the ductus arteriosus closes shortly after birth. When the ductus remains patent until 3 months after birth, it is clinically called a persistent ductus arteriosus. This persistent ductus arteriosus is characterized by a subendothelial elastic lamina in close adherence to the endothelium (Gittenberger-de Groot, 1977; Slomp *et al.*, 1992) in addition to the often fragmented internal elastic lamina on the borderline of intimal cushion and media. Likewise, the intimal cushions normally formed during late gestation to prepare for its postnatal closure (Gittenberger-de Groot, 1979; Gittenberger-de Groot *et al.*, 1980c) are often abnormal in the persistent ductus arteriosus. The elastic fibers in the intima can be abundant and well organized in the persistent ductus arteriosus, whereas they are small and disorganized in the normal ductus.

Several causes for persistent ductus arteriosus may exist. A direct known cause, an *in utero* rubella infection, shows a persistent ductus arteriosus with a histology reminiscent of an immature ductus arteriosus. This rubella ductus lacks intimal cushion formation (Gittenberger-de Groot *et al.*, 1980b). Another cause is genetically based. A large family with patent ductus arteriosus and unusual facial phenotypes has been described in Scotland. The segregation pattern suggests that this is caused by autosomal dominant inheritance with incomplete penetrance with respect to the persistent ductus arteriosus (Davidson, 1993). Moreover, in the dog, a genetic cause of persistent ductus arteriosus is known (Patterson *et al.*, 1971). This variant, however, has no or only slight aberrant intimal cushion formation (Gittenberger-de Groot *et al.*, 1985).

The formation of intimal cushions in the normal ductus arteriosus is also of interest because it may be a model for the intimal formation

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FIG. 3. Retrovirus-labeled neural crest cells are extensively present (A) along the arterial pole of a stage 30 (Hamburger–Hamilton) chick heart. (B) Section through the wall of the pulmonary trunk (PT) showing extensive neural crest contribution (70×). (C) Both actin positive (arrows)- and actin negative (arrowheads)-labeled neural crest cells are present in the wall of the pulmonary trunk (195×).

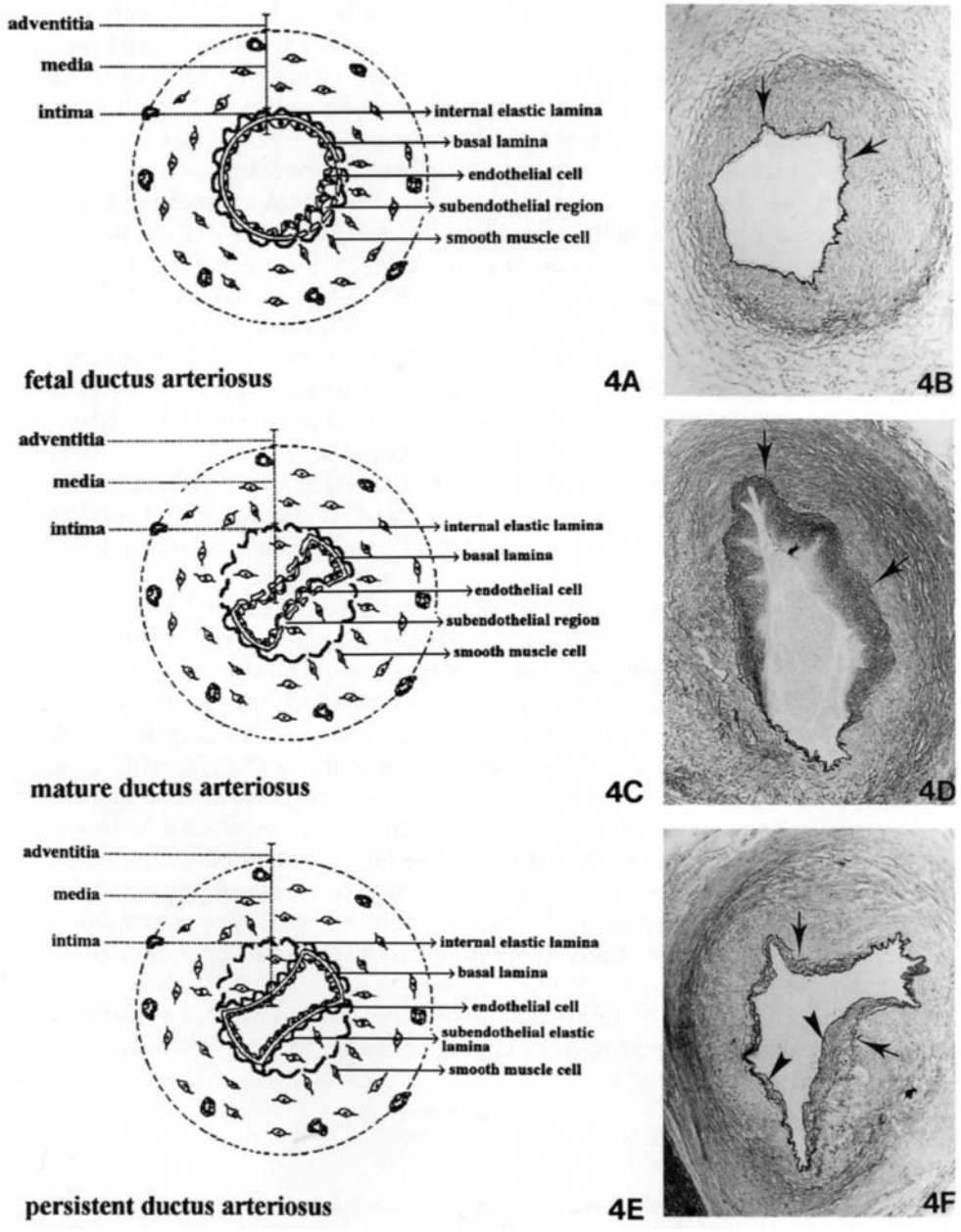

FIG. 4. (A) Fetal, (C) neonatal, and (E) persistent ductus arteriosus based on tissue sections of a (B) fetal, (D) neonatal, and (F) persistent ductus arteriosus stained with resorcin–fuchsin. Note the internal elastic lamina (arrows) at the border of the intima and media in each of these vessels. An additional subendothelial elastic lamina (arrowheads) is found in the persistent ductus arteriosus.

inherent to arteriosclerosis (Gittenberger-de Groot *et al.*, 1985; Slomp *et al.*, 1992). Ductus arteriosus intimal formation starts before birth in the fetal period with lifting of endothelial cells (Fig. 5A), accompanied by an accumulation of glycosaminoglycans and especially hyaluronan in the subendothelial region (Fig. 5B) (DeReeder *et al.*, 1988; Slomp *et al.*, 1992). Smooth muscle cells migrate through the fragmented internal elastic lamina into this subendothelial region (Gittenberger-de Groot *et al.*, 1985). This process, with lifting endothelial cells and glycosaminoglycan accumulations, continues until after birth (Slomp *et al.*, 1992). In cultured lamb ductus endothelial cells, hyaluronan production was 10-fold compared with that in the pulmonary artery or aorta endothelial cells; this was partly regulated by TGF-β (Boudreau and Rabinovitch, 1991, Boudreau *et al.*, 1992). In cultured lamb ductus smooth muscle cells, production of hyaluronan was endothelium dependent (Boudreau and Rabinovitch, 1991). Under the same circumstances, aortic smooth muscle cells from the same animal produced significantly less hyaluronan. Hylauronan is able to bind huge amounts of water. By this hygroscopic capacity it creates a environment well suited for smooth muscle cell migration (Toole, 1982). Migration studies with aorta and ductus smooth muscle cells have indeed shown that hyaluronan stimulates the migration of ductus smooth muscle cells and that migration was blocked completely by the addition of antibodies against a hyaluronan-binding protein (Boudreau *et al.*, 1991). Smooth muscle cell migration in the ductus is also regulated by extracellular matrix compounds such as fibronectin, laminin, and collagens and their receptors. Adhesion of ductus arteriosus smooth muscle cells and aortic smooth muscle cells to these components is regulated by the integrin β1 (Clyman *et al.*, 1990a,b, 1992), whereas migration over these substrates is αvβ3 dependent (Clyman *et al.*, 1992) (see the chapter by Glukhova and Koteliansky for a further discussion of integrins in development of blood vessels).

Migration of ductus arteriosus smooth muscle cells through a three-dimensional network of elastic membranes (Fig. 6) is associated with a reduced amount of elastin-binding protein in combination with increased fibronectin synthesis. In aortic smooth muscle cells, this situation is mimicked by adding chondroitin sulfate, a glycosaminoglycan rich in *N*-acetylgalactosamine. These molecules effectively shed the elastin-binding protein and also upregulate fibronectin synthesis (Hinek *et al.*, 1991, 1992). Together with an increase in hyaluronan, production of chondroitin sulfate is enhanced during intimal cushion formation (De Reeder *et al.*, 1988; Boudreau and Rabinovitch, 1991; Slomp *et al.*, 1992). These results suggest that chondroitin sulfate-in-

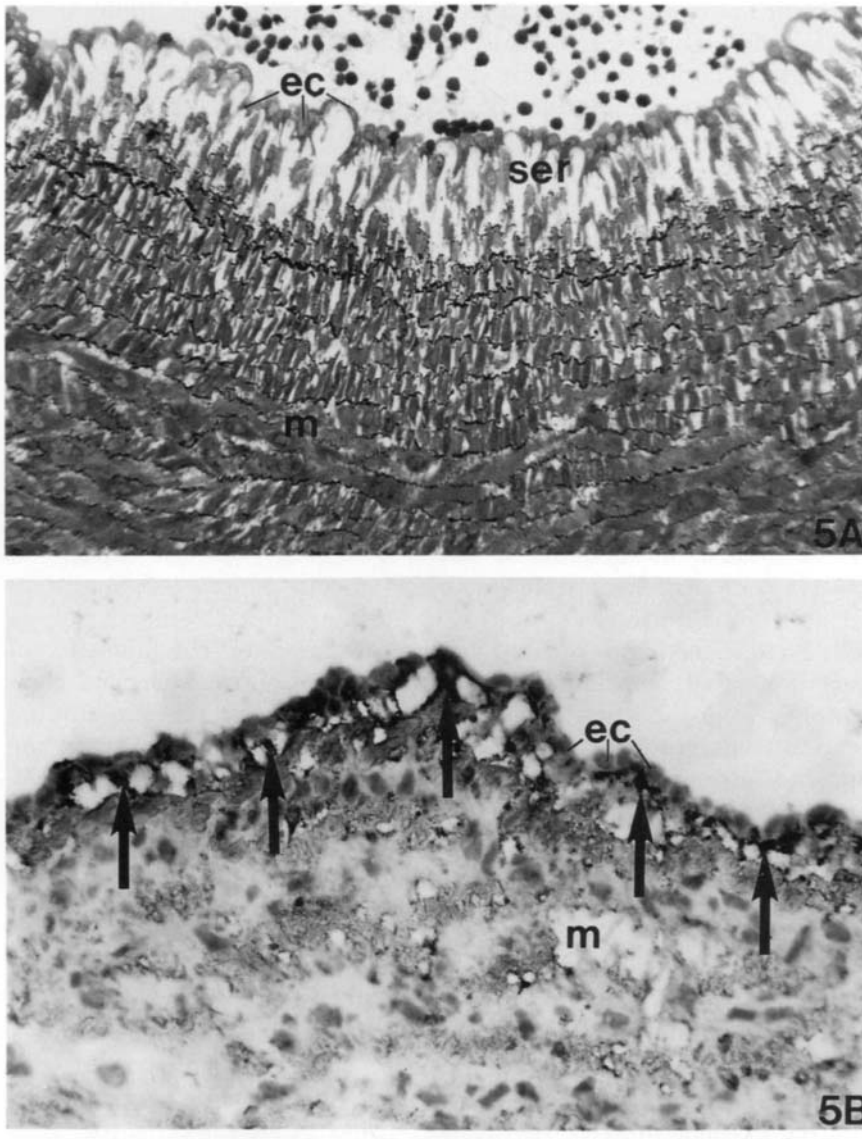

FIG. 5. (A) Initial stages of intimal formation in the rabbit ductus arteriosus, showing lifting endothelial cells and a wide subendothelial region (325×). (B) Subendothelial accumulations of hyaluronan (arrows) in the dog ductus arteriosus (455×). ec, endothelial cells; ser, subendothelial region; m, media

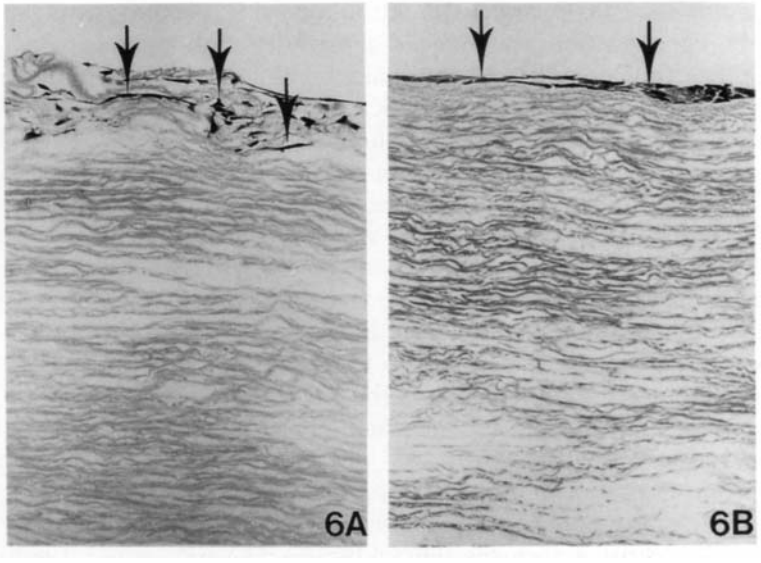

FIG. 6. (A) Lamb ductus arteriosus smooth muscle cells (arrows) during 24 hr of culture on elastic membranes are seen to migrate through these membranes, whereas (B) the smooth muscle cells of the aorta (arrows) remain on top of the elastic membranes under similar conditions (60×).

duced release of the elastin-binding protein is critical in promoting smooth muscle cell migration associated with intimal thickening in the ductus arteriosus and perhaps also in vascular disease (Hinek *et al.*, 1992).

VII. REGULATION OF SMOOTH MUSCLE CELL FUNCTION EXEMPLIFIED IN THE DUCTUS ARTERIOSUS

One of the most intriguing questions in ductus arteriosus smooth muscle cell behavior is what causes postnatal contraction. Several possibilities have been suggested to play a role in ductus arteriosus closure: an oxygen response, involvement of cytochrome P450, TXA_2, acetylcholine, norepinephrine, histamine, 5-hydroxytryptamine, and bradykinine (reviewed by Clyman, 1987). Relaxation of the ductus arteriosus is associated with reduced plasma adenosine levels and can be induced by prostaglandins (Clyman, 1987). Hypoxia induces increased prostaglandin synthesis in ductus arteriosus smooth muscle and endothelial cells (Rabinovitch *et al.*, 1989). Endogenous relaxation of the ductus arteriosus can be induced by at least two

prostaglandins: PGE_2 and PGI_2. Although PGE_2 production is far less than PGI_2 production, the marked sensitivity of the ductus arteriosus to PGE_2 indicates that this must be the main endogenous prostaglandin that regulates patency of the vessel (Clyman, 1987).

For a definitive anatomic closure after initial contraction it is important that the ductus arteriosus has formed intimal cushions that lack a subendothelial elastic lamina (Gittenberger-de Groot *et al.*, 1980a). In atherosclerotic vessels, where the presence of a subendothelial elastic lamina was associated with limited progression of the atherosclerotic changes (Sims *et al.*, 1993), it has been suggested that this lamina provides a barrier to the entry of macromolecules and cells from the lumen to the intima. This lamina does not prevent the responsiveness of the ductus to pharmaca as the prostaglandin inhibitor indomethacin. It has also been shown that the persistent ductus arteriosus will contract after indomethacin treatment, but when treatment is stopped the lumen of the vessel will reopen. Also, the opposite is seen with PGE_1 treatment to enlarge the persistent ductus arteriosus (Gittenberger-de Groot *et al.*, 1980b). It is also possible that lifting of the endothelium from the underlying internal elastic lamina will diminish the relaxing influence of NO produced by the endothelial cells (Coceani *et al.*, 1994). This could be a mechanism that prepares the ductus arteriosus for closure after birth when the endogenous PGE_2 effect, which is superior to the NO effect, is lost.

VIII. Conclusions

Initial steps in smooth muscle cell differentiation start in very early stages. The different types of cardiovascular cells originate from the ventrolateral mesoderm (Xenopus) or the splanchnic mesoderm (Aminotes). This ventrolateral mesoderm can be regionalized using smooth muscle α-actin as a marker. Unlike the endothelial cells and cardiomyocytes, smooth muscle cells remain positive for smooth muscle α-actin expression. Smooth muscle cells, however, are not unique in expressing smooth muscle α-actin. Other cells, most probably also from ventrolateral mesoderm origin, express smooth muscle α-actin as well, suggesting that smooth muscle α-actin marks a certain differentiation state of cells of ventrolateral mesoderm origin or perhaps a functional state required in many cells during development.

Actin expression in relation to the developing tunica media is associated with remodeling of the vascular system. There are, however, still actin patterns that are not well understood. They may be related to different influences on smooth muscle differentiation in the vascu-

lar wall, because until now it is not known exactly which cells contribute to the development of vascular smooth muscle cells. In this respect the role of the neural crest cells is not clear as well. Some of the neural crest cells differentiate into smooth muscle cells, but others may have an inductive role. An inductive role may also be exerted by the endothelial cells. Almost no data are present about the influence of the endothelial cells on the surrounding mesenchyme and their role in media formation.

Smooth muscle cell characteristics in relation to intimal thickening formation can easily be studied in the ductus arteriosus because physiologically this vessel forms intimal thickening during the second trimester of pregnancy. It has been shown that ductus arteriosus smooth muscle cells have a great migratory capacity compared to aortic smooth muscle cells, and that migration of ductus smooth muscle cells is dependent on extracellular matrix molecules and their receptors. Functionally ductus smooth muscle cells are special as well because this vessel closes shortly after birth. The exact mechanism of both functional and anatomical closure is not known. A well-developed intimal cushion without a subendothelial elastic lamina is necessary for anatomical closure. The age-dependent extreme sensitivity of ductus arteriosus smooth muscle cells for prostaglandins suggests at least a role of prostaglandins in ductus smooth muscle cell relaxation before birth.

Despite many questions remaining unsolved concerning the origin of smooth muscle cells and smooth muscle cell behavior during intimal thickening formation, this chapter has attempted to present the current knowledge in this field.

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Integrins, Cytoskeletal and Extracellular Matrix Proteins in Developing Smooth Muscle Cells of Human Aorta

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I. Introduction

The term "smooth muscle cell" refers to a somewhat heterogeneous group of cells. "Typical" smooth muscle cells are those found in the muscular layers of blood vessels and of internal organs. However, vascular and visceral smooth muscle cells are not identical, they differ from each other by structural and functional criteria. In addition, some cells that are usually not considered as smooth muscle have certain features characteristic of vascular and visceral smooth muscle cells. Examples include perycites, myofibroblasts, stromal cells of different glands (reviewed by Sappino *et al.*, 1990; Gown, 1990; see also chapter by Desmoulière and Gabbiani). The phenotype of smooth

muscle cells can change in certain disease states, i.e., in atherosclerosis, hypertension, and neoplasia, when smooth muscle cells appear less differentiated (reviewed by Schwartz *et al.*, 1986, 1990; Thyberg *et al.*, 1990; Glukhova *et al.*, 1991a; Bondjers *et al.*, 1991; Koteliansky *et al.*, 1992a). This phenotypic diversity may result from different embryonic origin of all these smooth muscle and "smooth muscle-related" cells and/or, at least in some cases, reflect a marked phenotypic plasticity intrinsic for this cell type. In other cell types, especially skeletal muscle, it is now commonly accepted that the differentiated state is reached and maintained due to regulation by a combination of cell intrinsic and extrinsic controlling mechanisms (Blau and Hughes, 1990; Blau and Baltimore, 1991). Early in embryogenesis, a small number of smooth muscle-specific regulatory "master" genes analogous to members of the MyoD family (reviewed by Weintraub *et al.*, 1991) could induce and further maintain smooth muscle differentiation program. Thus, smooth muscle cell intrinsic controlling mechanisms may support a cell autonomous direction of differentiation. On the other hand, during development and in disease the cell intrinsic program can be modulated by extrinsic factors, including direct cell–cell interactions, cell–extracellular matrix interactions, and diffusing soluble substances. The molecular mechanisms of smooth muscle development are poorly understood and have only recently became a subject of extensive investigation.

To the extent that we can define the smooth muscle cell, the definition is based on the expression of the cytoskeletal and contractile proteins. Specific organization of the cytoskeleton and contractile apparatus is one of the most characteristic features of smooth muscle cells that determine their morphological and functional properties, i.e., the phenotype. During their entire life span, smooth muscle cells are surrounded by the extracellular matrix, which is assumed to play a critical role in the regulation of gene expression and cytodifferentiation; it serves for cell–cell communication and for deposition of growth factors and other substances able to interfere with smooth muscle cell functions (Bissell and Barcellos-Hoff, 1987; reviewed by Hay, 1991).

This chapter summarizes current experimental data concerning the changes in organization of cytoskeletal and extracellular matrix components during arterial smooth muscle development. The major concept, which we will try to illustrate here, is that phenotypic transitions of smooth muscle cells occurring during embryogenesis, postnatal development, and in certain disease states are accompanied by reciprocal instructive interactions between cell and the extracellular matrix. Smooth muscle cells produce matrix molecules, organize interstitial matrix and basement membrane, and the matrix in turn

has its influence on cell differentiation. Changes in cytoskeletal organization and signal transduction pathways induced by the extracellular matrix components and mediated by specific integrins can modify gene expression and have a strong effect on cellular differentiation properties.

II. Development of Human Aorta

The main feature of artery wall structure is the organization of cells into three distinct layers. In the adult, an innermost layer, the tunica intima, is made up of two cell types: the endothelial cells which line the lumen and smooth muscle cells. The tunica intima is surrounded by tunica media which contains only smooth muscle cells. The tunica media, in turn, is surrounded by the tunica adventitia which represents the layer of connective tissue containing fibroblasts, histiocytes, nerve endings, etc. With the exception of components of the nervous system and other neural crest cell derivatives, the cells of the vessel wall entirely arise from the mesoderm (see chapter by Gittenberger-de Groot *et al.*). In the embryo, cells originating most probably from the ventrolateral portion of mesoderm give rise to smooth muscle precursor cells destined for myogenesis (Saint-Jeannet *et al.*, 1992). The endothelium may stimulate the organization of smooth muscle and adventitial fibroblast precursors into characteristic multilayer structures. Figure 1 summarizes the main steps of late human aorta development. By 9 weeks of gestation, the major part of the aortic wall is tunica media which consists of several layers of immature smooth muscle cells. These cells proliferate and have fibroblast-like morphological properties, i.e., a poorly developed contractile apparatus, no dense bodies and dense plaques, extensive rough endoplasmic reticulum, and prominent Golgi complex. The tunica intima of a 9-week-old fetal aorta includes the luminal endothelial layer and subjacent discontinuous, poorly formed internal elastic membrane. During further prenatal development, the media progressively thickens, its smooth muscle cells get more differentiated, and the internal elastic membrane becomes a continuous structure separating intima from media. In the aorta from a 22- to 25-week-old fetus, smooth muscle cells already have an elongated shape as in the adult aorta, but they are still immature with respect to their ultrastructure (Nikkari *et al.*, 1988). During the last weeks of prenatal development and several months of the postnatal period, medial smooth muscle cells finish their maturation process, but no principal changes occur in the structural organization of the aortic wall. The number of smooth muscle cell layers in the wall does not increase (Wolinsky and Glagov, 1967;

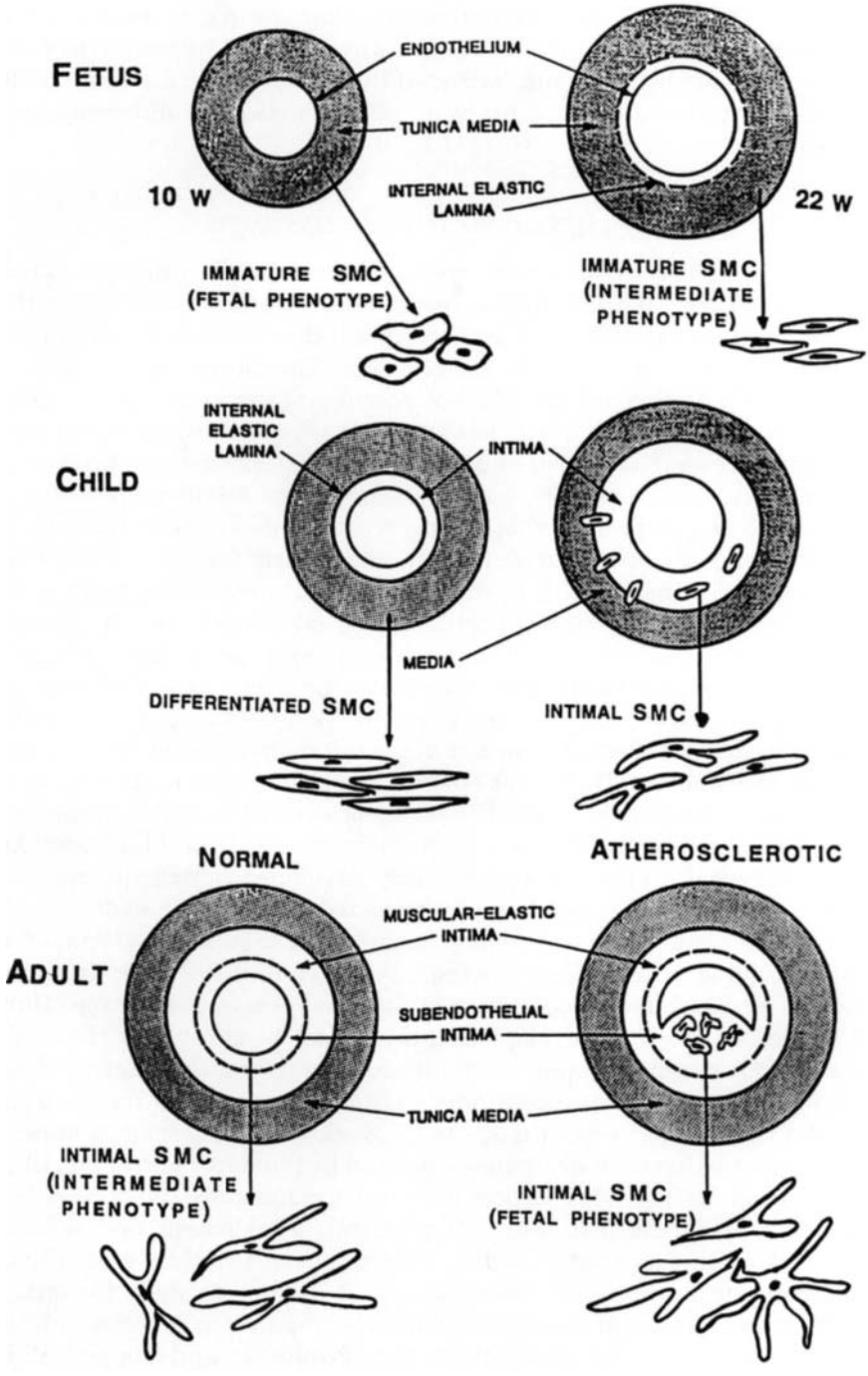

FIG. 1. Late development of the human aorta.

Dilley and Schwartz, 1989). Further thickening of the media is the result of an increasing cell number and cell mass as well as the production of extracellular matrix components. The intima remains extremely thin and contains practically no smooth muscle cells in fetal aorta, as well as in the child's aorta during several years of postnatal life. Intimal thickenings start to develop at certain points of the aorta at the age of 3–6 years, when smooth muscle cells appear in the space between the internal elastic membrane and endothelium (Haust, 1983). Gradually the intimal layer thickens, and in certain adult arteries, thickness of the tunica intima may exceed that of the tunica media. In the adult aortic intima (Fig. 1), two layers of smooth muscle cells can be distinguished: muscular elastic intima, a layer adjacent to the media, and hyperplastic or subendothelial intima, a layer adjacent to the lumen (Haust, 1983). The morphological properties of the cells from these two layers are very different (Orekhov *et al.*, 1984, 1986; Rekhter *et al.*, 1991). Smooth muscle cells of muscular elastic intima are similar to contractile well-differentiated cells of tunica media: they have a spindle-like shape and are organized in the layers separated by elastic membranes. On the contrary, the subendothelial intima is composed of smooth muscle cells of various shapes and the network of stellate cells predominates (Rekhter *et al.*, 1991). By some morphological criteria, these cells resemble the cells of fetal arteries (Kocher and Gabbiani, 1986; Mosse *et al.*, 1985; Nikkari *et al.*, 1988).

Accumulation of smooth muscle cells and of extracellular matrix in the intima continues during life and is a part of the aging process. In atherosclerosis, focal thickening of the subendothelial intima results in the formation of atherosclerotic plaques (Fig. 1) which contain enormous amounts of extracellular matrix, lipid deposits, a varying proportion of blood-borne cells, and smooth muscle cells which are very heterogeneous with respect to their phenotypic characteristics (Gown *et al.*, 1986, Jonasson *et al.*, 1986; Katsuda *et al.*, 1992; Frid *et al.*, 1992).

III. Developmental Changes in Expression of Cytoskeletal and Contractile Proteins

The expression of cytoskeletal and contractile proteins in smooth muscle cells is developmentally regulated and provides a good biochemical parameter that can be useful in the analysis of the phenotypic changes that smooth muscle cells undergo during development and in adult aorta. In smooth muscle cells, similar to the other types of muscle, the microfilament system (actin filaments and numerous

TABLE I

ACTIN FILAMENT-ASSOCIATED PROTEINS OF THE SMOOTH MUSCLE CELL CYTOSKELETON AND CONTRACTILE APPARATUS

Proteins	Muscle-specific isoform(s)	Alternatively spliced variants	Genomic organization
Contractile proteins			
Actin	α-sm, γ-sm	–	Multigenic
Myosin heavy chains	SM-1, SM-2	+	Multigenic
Contraction regulatory proteins			
Tropomysin	α-sm	+	Multigenic
Caldesmon	h-caldesmon	+	One gene
Calponin	+	+	?
Actin filament severing proteins			
Gelsolin	+	+	One gene
Actin filament cross-linking (bundling) proteins			
α-Actinin	+	+	Multigenic
Filamin	+	?	Multigenic
Fimbrin	+	–	One gene
Proteins at the cytoplasmic face of dense plaques			
Vinculin	γ-Vinculin, *meta*-Vinculin	+	One gene
Talin	–	–	One gene
Paxillin	–	?	?
Tensin	–	?	?
Phosphoglucomutase	+	–	Multigenic

associated proteins) serves as a basis for the contractile apparatus (see chapter by Small and North). The main microfilament-associated proteins of smooth muscle cells are listed in Table I. In addition to the major contractile proteins actin and myosin, smooth muscle cells contain numerous proteins involved in the regulation of contraction, such as caldesmon, calponin (calmodulin- and F-actin-binding proteins, regulate actin–myosin interactions), and tropomyosin (strengthens microfilaments); in regulation of microfilament stability, such as gelsolin (fragments microfilaments); in organization of the microfilament network, such as filamin (cross-links microfilaments), α-actinin, and fimbrin (bundle microfilaments); and a set of proteins involved in the attachment of microfilaments to plasma membrane, such as vinculin, talin, paxillin, and tensin (reviewed by Stossel *et al.*, 1985; Takahashi *et al.*, 1986; Burridge *et al.*, 1988; Turner and Burridge, 1991; Sobue and Sellers, 1991; Lees-Miller and Helfman, 1991). The major proteins of the intermediate filament system in smooth muscle cells are desmin and vimentin (Gabbiani *et al.*, 1981; Osborn *et al.*, 1981; Schmid *et al.*, 1982; Kocher *et al.*, 1984, 1985). Cytokeratins 8, 18,

and 19 are transiently expressed in developing smooth muscle and in pathological states in adult (Van Muijen *et al.*, 1987; Brown *et al.*, 1987; Bader *et al.*, 1988; Gown *et al.*, 1988; Jahn and Franke, 1989; Glukhova *et al.*, 1991b).

Many contractile and cytoskeletal proteins have muscle-specific variants that can be used as smooth muscle cell phenotypic markers: α- and γ-smooth muscle actins (Vandekerckhove and Weber, 1979; Fatigati and Murphy, 1984; Gown *et al.*, 1985; Skalli *et al.*, 1986; Owens *et al.*, 1986; Owens and Thompson, 1986), smooth muscle myosin heavy chains SM-1 and SM-2 (Rovner *et al.*, 1986a,b; Nagai *et al.*, 1989; Borrione *et al.*, 1989b; Sartore *et al.*, 1989), *meta-* and γ-vinculin (Glukhova *et al.*, 1986; Gimona *et al.*, 1987; Belkin *et al.*, 1988a), desmin (Gabbiani *et al.*, 1981; Osborn *et al.*, 1981; Schmid *et al.*, 1982), heavy (h)-caldesmon (Owada *et al.*, 1984; Bretscher and Lynch, 1985; Ngai and Walsh, 1985; Dingus *et al.*, 1986; Glukhova *et al.*, 1987), smooth muscle α-tropomyosin (Fatigati and Murphy, 1984; Wieczorek *et al.*, 1988), and calponin (Takahashi *et al.*, 1986, 1987; Gimona *et al.*, 1987; Takahashi and Nadal-Ginard, 1991). Theoretically, an ideal smooth muscle marker should appear in all smooth muscle cells regardless of their differentiation level, i.e., it should be expressed as soon as the cell is committed to the smooth muscle lineage, it should be present through differentiation and maturation, and it should not disappear from smooth muscle cells involved in various pathological processes (atherosclerosis, neoplasia). Early expression of the marker would be especially important for studies of cell lineages and interrelationships between different smooth muscle cells and smooth muscle cell-like cells. Up until now, none of the smooth muscle markers have been proven to be really smooth muscle specific. α-smooth muscle actin is transiently expressed in striated muscles during myogenesis in chicken and rat embryos (Ruzicka and Schwartz, 1988; Babai *et al.*, 1990) and can be induced in cultivated fibroblasts (reviewed by Sappino *et al.*, 1990). *meta*-Vinculin is present in cardiac muscle and in platelets (Belkin *et al.*, 1988a,b; Turner and Burridge, 1989). Desmin is a major intermediate filament protein in striated muscle and it is expressed only in the portion of vascular smooth muscle cells in the large arteries (Gabbiani *et al.*, 1981; Osborn *et al.*, 1981); smooth muscle myosin heavy chains were detected in vascular endothelial cells (Borrione *et al.*, 1989a).

In addition to a general smooth muscle-specific regulatory system, expression of each protein might be regulated by its own peculiar mechanism; therefore, in order to determine the smooth muscle cell differentiation state, it is important to use a set of smooth muscle markers instead of relying on a single one. The proportion of the

smooth muscle-specific variant and its nonmuscle counterpart is also a significant parameter in characterization of the smooth muscle cell phenotype.

The relative content of α-smooth muscle actin, smooth muscle myosin heavy chains, and smooth muscle variants of vinculin, caldesmon, and calponin was analyzed in human aortic smooth muscle cells during prenatal development, in the child, and in different layers of normal and atherosclerotic adult vessels. Data are presented in Table II. In general, during prenatal development and postnatal growth the relative content of smooth muscle-specific variants of contractile and cytoskeletal proteins in aortic medial cells increases, whereas in the intima thickenings, especially in the atherosclerotic plaque, smooth muscle cells switch again to the expression of nonmuscle variants. Similarly, during the development of rat aorta, an increase in α-smooth muscle actin, desmin, and smooth muscle myosin heavy chain content was observed (Kocher *et al.*, 1985; Owens and Thompson, 1986), whereas in the experimental intima thickening and during aging the proportion of desmin and α-smooth muscle actin drops (Kocher *et al.*, 1984, 1985; Nikkari *et al.*, 1990). The expression

TABLE II

EXPRESSION OF CYTOSKELETAL AND CONTRACTILE PROTEINS IN DEVELOPING AND ADULT HUMAN AORTIC SMOOTH MUSCLE CELLS[a]

Source of aortic smooth muscle cells	sm-MHCs			nm-MHCs	m-Vn	h-Cd	Cp
	α-A	SM-1	SM-2				
Fetal media							
8–10 weeks old	0.4	0.8	0.1	2.3	0.1	0.1	–
22–25 weeks old	0.8	1.1	0.4	1.6	0.2	0.4	–
Child media							
2–3 months old	n.d.[b]	1.3	0.5	1.3	0.2	0.4	n.d.
6 months old	1.0	1.0	1.1	1.0	0.7	0.5	+
18 months old	n.d.	n.d.	n.d.	n.d.	0.9	0.9	n.d.
Adult media	1.0	1.0	1.0	0.1	1.0	1.0	+++
Subendothelial intima							
Adult, normal	1.0	n.d.	n.d.	+	0.2	0.3	+
Atherosclerotic plaque	0.4	n.d.	n.d.	+	0.1	0.3	+

Note: α-A, α-smooth muscle actin; sm-MHCs, smooth muscle myosin heavy chains SM-1 and SM-2; nm-MHCs, nonmuscle myosin heavy chains; m-Vn, *meta*-vinculin; h-Cd, heavy caldesmon; and Cp, calponin. 1, level obtained for the smooth muscle cells of adult normal aortic media.

[a]The table summarizes data published by Glukhova *et al.* (1988, 1990 a,b), Frid *et al.* (1992), and Frid *et al.* (1993).

[b]Not determined.

of smooth muscle markers was not uniform in developing rat arteries; more desmin-positive smooth muscle cells were identified in the aorta toward the iliac arteries than at the arch (Osborn *et al.*, 1981; Schmid *et al.*, 1982).

Developmental changes in the expression of myosin heavy chains have received extensive study. At least four different types of myosin heavy chains are expressed in vascular smooth muscle cells: two smooth muscle specific, SM-1 and SM-2, and two nonmuscle (Rovner *et al.*, 1986a,b; Nagai *et al.*, 1989; Borrione *et al.*, 1989b; Kuro-o *et al.*, 1989, 1991; Zanellato *et al.*, 1990a; Eddinger and Murphy, 1991; Kawamoto and Adelstein, 1991; Babij *et al.*, 1992). During development of human aorta the proportion of smooth muscle variants increases; however, SM-1 appears rather early and its content remains more or less constant throughout the fetal period and after birth, whereas SM-2 content increases only in the postnatal period (Frid *et al.*, 1993). Of two nonmuscle myosin heavy chains, one seems to be expressed constitutively whereas expression of the other decreases during development, mainly in the postnatal period, and in the adult is expressed only in rare clusters of medial and intimal smooth muscle cells (Frid *et al.*, 1993).

Quantitative analysis revealed a somewhat asynchronous pattern of expression of smooth muscle-specific contractile and cytoskeletal proteins, α-smooth muscle actin and smooth muscle myosin heavy chain SM-1 are expressed at a rather high level already in the fetus, whereas the expression of *meta*-vinculin, h-caldesmon, smooth muscle myosin heavy chain SM-2, and calponin increases dramatically only in the postnatal period. These results reveal two steps of the smooth muscle cell maturation process, prenatal and postnatal, and reflect the existence of groups of proteins, the expression of which may be regulated coordinately by different genetic mechanisms.

IV. Changes in Expression of Extracellular Matrix Proteins in Development

Major components of smooth muscle extracellular matrix include collagens, noncollagenous glycoproteins, and proteoglycans (reviewed by Mayne, 1986; Rosenbloom, 1987; Majack and Bornstein, 1987; Shekhonin *et al.*, 1987; Wight, 1989; Timpl, 1989; Hynes, 1990). Recent work has revealed a surprising diversity of these molecules, arising either from multiple genes or from alternative splicing. Importantly, two compartments, interstitial matrix and basement membrane, should be distinguished in the smooth muscle extracellular matrix. Table III summarizes the major matrix constituents of vas-

TABLE III

EXTRACELLULAR MATRIX PROTEINS OF THE SMOOTH MUSCLE

Proteins	Composition and variants
Basement membrane components	
Laminins (heterotrimers)	Chains: α1 (400 kDa), β1 (225 kDa), γ1 (205 kDa), β2(190 kDa) possible variants: α1-β1-γ1 and α1-β1-γ1
Type IV collagen (heterotrimer)	2 × α1(IV) (185 kDa), α2(IV) (170 kDa)
Entactin/nidogen (monomer)	150 kDa
Heparan sulfate proteoglycan	Core protein (400 kDa), three heparan sulfate side chains
Interstitial matrix components	
Fibronectins (dimers)	Twenty different combinations of the three alternatively spliced segments (ED-A, ED-B, and the IIICS region) may be produced
	$ED\text{-}A^{+}ED\text{-}B^{+}$ IIICS 120 95 89 65 0
	$ED\text{-}A^{+}ED\text{-}B^{-}$ IIICS 120 95 89 65 0
	$ED\text{-}A^{-}ED\text{-}B^{+}$ IIICS 120 95 89 65 0
	$ED\text{-}A^{-}ED\text{-}B^{-}$ IIICS 120 95 89 65 0
Vitronectin (monomer)	75 kDa
Thrombospondin (trimer)	3×180 kDa
Type I, III, V collagens (trimers)	2× α1(I) (95 kDa), α2(I) (95 kDa) 3 × α1(III) (95–110 kDa) 2 × α1(V) (115 kDa), α2(V) (125 kDa)
Type VI collagen (trimer)	α1(VI) (140 kDa), α2(VI) (140 kDa), α3(VI) (140 kDa)
Elastin (fiber)	n × 72 kDa
Tenascin (gexamer)	6 × 190–320 kDa
Osteopontin	41 kDa

cular smooth muscle. The majority of extracellular matrix components are very large molecules. Sequence analysis has revealed that certain structural domains are shared by different proteins (reviewed by Engel, 1991). Apparently, such a chimeric structure may result from exon shuffling. Moreover, multiple functional domains have been identified in individual glycoproteins such as fibronectin and laminin. The most important domains are responsible for interaction with other extracellular matrix components, the cell surface and matrix assembly.

Fibronectin mediates cell attachment and migration during embry-

onic development and wound healing by providing a substrate for moving cells (reviewed by Hynes, 1990; Glukhova and Thiery, 1993). Multiple forms of fibronectin are generated by the alternative splicing of a single gene transcript and are believed to have different properties in modulating cell adhesion and affecting the assembly of specialized fibronectin matrices (reviewed by Schwarzbauer, 1991). The fibronectin molecule is built up by a series of homologous repeats, types I, II, and III; the sets of repeats form functional domains. Human fibronectin has three sites of alternative splicing: ED-A, ED-B, and the IIICS region. ED-A and ED-B are type III repeats encoded by single exons which are included or skipped in a tissue-specific manner (reviewed by Hynes, 1990). These two exons are always excluded from fibronectin mRNA in hepatocytes, where plasma fibronectin is produced, and are included in fibronectin mRNA in some cells which are known to produce and assemble fibronectin matrices.

Because of their localization in the close proximity to the major cell-binding site of the fibronectin molecule, ED-A and ED-B were suggested to play roles in adhesion and migration of the cells and in organization of the extracellular matrix (reviewed by Schwarzbauer, 1991). The IIICS region contains two additional cell-binding sites (reviewed by Yamada, 1991). ED-A has been shown to directly promote cell adhesion and thus to represent an additional cell-binding site whose expression may be modulated depending on the pattern of mRNA splicing (Xia and Culp, 1994).

The most studied laminin molecule, so called laminin 1 (Burgesson *et al.*, 1994), isolated from embryonic rodent tumor cells is a trimer containing two distinct 200-kDa chains, β1 and γ1, and one 400-kDa α1 chain (reviewed by Timpl, 1989). The three chains are connected by disulfide bonds and form a cross-like structure with one long and three short arms. Several other members of the laminin family have been described (reviewed in Burgesson *et al.*, 1994). These include merosin or laminin 2, containing the α2 chain, a homolog of the α1 chain; laminin 3, containing the β2 chain which is homologous to β1 (Leivo and Engvall, 1988; Hunter *et al.*, 1989a,b; Ehrig *et al.*, 1990); and others. Laminin variants exhibit a tissue-specific distribution. The α1 chain is present in basement membranes of endothelial, epithelial, and smooth muscle cells, whereas the α2 chain is found in striated muscles and in peripheral nerves. The γ1 chain has a widespread distribution and seems to be present in the majority of basement membranes. The β1 chain is also detected in the basement membrane of many cell types, whereas the β2 chain is limited to a small subset of basement membranes and is found in the myotendinous junctions, synaptic sites in muscles, glomeruli in the kidney, and in smooth mus-

cle (Leivo and Engvall, 1988; Ehrig *et al.*, 1990; Engvall *et al.*, 1990; Hunter, 1989b; Sanes *et al.*, 1990a,b; Glukhova *et al.*, 1993).

In general, fibronectin and laminin variants which exist in aortic media at the early fetal step of development (9–10 weeks of gestation) are progressively substituted by those characteristic of mature adult medial smooth muscle, whereas the protein variants expressed in the fetal tissue reappear in the intimal thickening (Table IV). Fibronectin molecules containing ED-A and ED-B are present in arterial media from 10-week-old fetuses and then disappear by 25 weeks of gestation (Glukhova *et al.*, 1990b). Starting from this period, medial smooth muscle cells are surrounded by matrix containing fibronectin that lacks both extra domains. Smooth muscle cells from intimal thickenings secrete fibronectin containing ED-A instead of ED-B (Glukhova *et al.*, 1989, 1990b). Adult smooth muscle cells from uterus, digestive tract, bladder, etc. produce ED-A-containing fibronectin (Glukhova *et al.*, 1990b). Expression of the fibronectin variants by smooth muscle cells also can be changed during development cardiac hypertrophy in rat induced by pressure overload. It was shown that very early after aortic stenosis, smooth muscle cells of coronary arteries were able to reexpress the mRNAs encoding the fetal (EIIIA- and EIIIB-containing) variant of fibronectin (Samuel *et al.*, 1991). Similar data were obtained in a study of ED-A-containing fibronectin expression in pulmonary arteries from patients with pulmonary hypertension. ED-A-containing fibronectin was detected only in some regions

TABLE IV

DEVELOPMENTAL CHANGES IN THE EXPRESSION OF FIBRONECTIN AND LAMININ VARIANTS AND INTEGRINS IN HUMAN AORTIC SMOOTH MUSCLE CELLS[a]

Source of aortic smooth muscle cells	Fibronectin variants	Laminin variants	Integrin subunits
Fetal media			
8–10 weeks old	ED-A$^+$ED-B$^+$	α1-β1-γ1	β1, α1, α5
25–27 weeks old	ED-A$^-$ED-B$^-$	α1-β1-γ1, α1-β2-γ1	β1, α1 ↓, α3, α5
Adult aortic media	ED-A$^-$ED-B$^-$	α1-β2-γ1	β1, α1 ↑, α3 ↑, α5 ↓
Subendothelial intima			
Adult, normal	ED-A$^+$ED-B$^-$	α1-β1-γ1, α1-β2-γ1	β1, α1 ↓, α3 ↓, α5
Atherosclerotic plaque	ED-A$^+$ED-B$^-$	α1-β1-γ1, α1-β2-γ1	β1, α1 ↓, α3 ↓, α5

Note: ED-A and ED-B, alternatively spliced extra domains; a plus sign, domain is absent in fibronectin molecule; a minus sign, domain is present in fibronectin molecule. α1, β1, γ1, and β2 are laminin chains. Integrin subunit detected at increased (↑) or decreased (↓) levels as compared with previous stage of development.

[a]The table summarizes data published by Glukhova *et al.* (1989, 1990 a,b), Belkin *et al.* (1990), and Glukhova *et al.* (1993).

of media and intima of normal pulmonary arteries, but was strongly expressed in neointima of hypertensive vessels (Botney *et al.*, 1992).

During embryonic development, laminin appears in the smooth muscle of the large arteries later than fibronectin (Risau and Lemmon, 1988). In the 6-day-old chick embryo, dorsal aorta already contains several layers of fibronectin-producing cells, but laminin appears only at day 8. In aortic media of human 10-week-old fetuses, α1, β1, and γ1 chains of laminin are expressed; by 27 weeks of gestation, the β2 chain, in addition, is present, whereas in adult arterial media only α1, β2, and γ1 chains are revealed (Glukhova *et al.*, 1993). In the intimal thickenings, the β1 chain of laminin reappears (Table IV). Since laminin is a trimer, conceivably α1-β1-γ1 protein variant during development is substituted by the α1-β2-γ1 form characteristic of mature smooth muscle, whereas in the intimal smooth muscle cells of normal arteries and in atherosclerotic plaques, the α1-β1-γ1 laminin coexists with the α1-β2-γ1 variant.

The α1-β1-γ1 variant of laminin was demonstrated to play an important role in the maintenance of the differentiated smooth muscle cell phenotype in culture (Hedin *et al.*, 1988). *In vivo*, in aortic smooth muscle, the α1-β1-γ1 laminin is expressed during the entire maturation period, whereas the α1-β2-γ1 laminin appears at the late stage of smooth muscle cell maturation and appears to be the only laminin variant associated with differentiated smooth muscle cells in adult media. Finally, in the intimal thickening, where the smooth muscle cells are less differentiated, the β1 chain is again expressed. Basement membrane in fetal smooth muscle cells is only starting to be formed, whereas in the intimal smooth muscle cells, as revealed by numerous ultrastructural studies, it becomes discontinuous and not as well organized as in the medial cells. Interestingly, the β1 chain-containing laminin is revealed when the basement membrane appears to be imperfect, whereas the β2 laminin is a constituent of a well-formed basement membrane of differentiated smooth muscle cells. In this context, it is challenging to speculate that laminin variants are differently involved in the control of smooth muscle cell proliferation and differentiation. Indeed, laminin and laminin fragments were shown to be mitogenic for fibroblastic cell lines (Panayotou *et al.*, 1989). The mitogenic activity of laminin may be inhibited if laminin forms complexes with other components of basement membrane, collagen type IV and nidogen (Panayotou *et al.*, 1989). Thus, the mitogenic activity of laminin may be unmasked when the basement membrane of smooth muscle cells is not completely assembled or is partially destroyed (as it is in the intimal thickening). The mitogenic activity of laminin is based on the presence of EGF-like motifs.

α1, β1, and γ1 chains of laminin contain 16, 13, and 11 EGF-like repeats, respectively (reviewed by Engel, 1991). Importantly, laminin chains contain different amounts of adhesive sequences. For example, the β2 chain contains three Leu–Arg–Glu sequences which are involved in the interaction with cell surface. The β1 chain, to which the β2 chain is most closely related, does not contain the Leu–Arg–Glu motif (Hunter *et al.*, 1989b). The Arg–Gly–Asp sequence is present only in the α1 chain, and the Tyr-Ile–Gly–Ser–Arg sequence is present in the β1 chain. All of these structural differences can provide different functions for laminin variants in smooth muscle cell development. However, *in vitro* experiments would be important to determine whether different laminin variants could differentially influence smooth muscle cell functional properties, i.e., motility, proliferation, and cytoskeleton organization.

It is worth mentioning that in addition to major constituents, some minor components of the basement membrane may affect the smooth muscle cell phenotype. It has been reported that patients that develop benign smooth muscle tumors associated with Alport syndrome, a hereditary defect of basement membranes, harbor deletions that desrupt paired α5(IV) and α6(IV) collagen genes (Zhou *et al.*, 1993). Thus type IV collagen variants may be involved in the control of visceral smooth muscle cell proliferation and differentiation even though α5(IV) and α6(IV) collagen chains are not abundant in the basement membrane.

In summary, the expression of fibronectin variants and laminin chains in vascular smooth muscle cells is developmentally regulated, and smooth muscle cells change their spectra of fibronectin variants and laminin chains at least twice, during prenatal development between 10 and 22 weeks of gestation and when recruited into intimal cell population. The expression of fibronectin variants and laminin chains is in good correlation with phenotypic transitions of smooth muscle cells. Differentiated smooth muscle cells produce ED-A- and ED-B-negative fibronectin and the β2 chain containing laminin variant, whereas immature fetal or intimal smooth muscle cells produce fibronectin containing ED-A or both ED-A and ED-B, and the β1-containing laminin form.

V. Integrins in Developing Smooth Muscle

Cell surface receptors for extracellular matrix proteins, integrins (reviewed by Hynes, 1987, 1992; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991), are directly involved in mechanisms that regulate smooth muscle cell migration, anchorage of cells during vaso-

constriction, interaction of smooth muscle cells with basement membrane, and integration of extracellular matrix components and the cytoskeletal framework. Integrins are heterodimers consisting of an α subunit linked noncovalently to a nonrelated β subunit (Table V). So far, 8 β subunits and 1 6 α subunits have been described (references

TABLE V
INTEGRINS AND THEIR LIGANDS

Integrin	Subunits	Ligands	Binding Site	Presence in vascular smooth muscle
β1	α1	Laminin, collagens		+
	α2	Laminin, collagens	DGEA	+[a]
	α3	Fibronectin, laminin, collagens	RGD	+
	α4	Fibronectin, VCAM-1	EILDV	+[b]
	α5	Fibronectin	RGD	+
	α6	Laminin		–
	α7	Laminin		+[a]
	α8	Fibronectin, vitronectin		+
	α9	Tenascin		+
	α_v	Fibronectin, vibronectin	RGD	+[a]
β2	αLFA-1	ICAM-1, ICAM-2		–
	αMac-1	C3b component of complement fibrinogen, ICAM-1		–
	αx	Fibrinogen	GPRP	–
β3	αIIb	Fibronectin, fibrinogen, vitronectin, von Willebrand factor, thrombospondin	RGD, KQAGDV	–
	α_v	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin, collagen	RGD	+[a]
β4	α6	Laminin		+[c]
β5	α_v	Vitronectin	RGD	–
β6	α_v	Vitronectin	RGD	–
β7	α4	VCAM-1, fibronectin	EILDV	–
	αIEL			–
β8	α_v	Vitronectin		–

Note: Unless marked otherwise, the presented data on integrin expression summarize the results published by Belkin *et al.* (1990), Clyman *et al.* (1992, 1994), Glukhova *et al.* (1993), Skinner *et al.* (1994), and Mechtersheimer *et al.* (1994).

[a]Expressed only in cultured smooth muscle cells (Skinner *et al.*, 1994).

[b]Transiently expressed in developing smooth muscle (Sheppard *et al.*, 1994).

[c]Expressed only in the smooth muscle cells of the small vessels (Cremona *et al.*, 1994).

are given in earlier reviews by Hynes, 1987, 1992; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991). The β subunits are structurally related to each other and they define different subfamilies of integrins. Most of the α subunits can form heterodimers with only a single β subunit. However, several α subunits (α4, α6, αv) can associate with more than one β subunit. Another level of integrin complexity is obtained by the alternative splicing of cytoplasmic (in β1, β3, β4, α3, α6, α7) and some parts of extracellular (αIIb) domains (reviewed by Hynes, 1992).

Ligand specificity of integrins is largely determined by heterodimer composition and particularly by the α subunit, although the same integrin in different tissues can exhibit different specificities. Individual integrins can often interact with more than one extracellular matrix component, and individual ligands are often recognized by several integrins. There are at least nine receptors for fibronectin (α3β1, α4β1, α5β1, α8β1, αvβ1, αIIbβ3, αvβ3, αvβ6, α4β7) and six receptors for laminin (α1β1, α2β1, α3β1, α6β1, α7β1, α6β4). For several integrins the recognition site in the ligands has been very precisely defined: Arg–Gly–Asp in fibronectin and vitronectin, Lys–Gln–Ala–Gly–Asp–Val in fibrinogen, and Asp–Gly–Glu–Ala in type I collagen (Table V). The various laminin receptors recognize specific regions of the laminin molecule: α3β1, α6β1, and α7β1 interact with the E8 fragment of the long arm of laminin whereas α1β1 and α2β1 recognize the E1 fragment of the core region of laminin (reviewed by Mercurio, 1990; Mercurio and Shaw, 1991).

The number of integrins expressed by different cell types varies significantly. Cultured cells are able to express from 2 to 10 different integrins. Many integrins are cell type specific and their expression appears to be regulated during development (reviewed by Glukhova and Thiery, 1993). In the embryo, the proper temporal and spatial expression of particular integrins makes it possible for cells to interact with appropriate adhesive molecules and to regulate their navigation during cell movements. *In vitro*, interactions of extracellular matrix components with integrins play an important role in the differentiation of skeletal myoblasts (Menko and Boettinger, 1987) and in the phenotypic modulation of aortic smooth muscle cells (reviewed by Thyberg *et al.*, 1990).

Analysis of the integrin repertoire in human vascular smooth muscle cells *in vivo* or immediately after isolation from the tissue reveals mainly α1β1 and small amounts of α3β1, α5β1, and αvβ1 integrins (Belkin *et al.*, 1990; Glukhova *et al.*, 1993; Mechtersheimer *et al.*, 1994; Skinner *et al.*, 1994). In addition, α4 was reported to be expressed in developing mouse arteries and is absent from adult human

arteries (Sheppard *et al.*, 1994; Mechtersheimer *et al.*, 1994). The α6β4 integrin was found in the smooth muscle cells of the small vessels (Cremona *et al.*, 1994), and α8β1 was identified in visceral and vascular smooth muscles (Bossy *et al.*, 1991; Schnapp *et al.*, 1995).

A high level of α1β1 integrin expression is an exceptional feature of smooth muscle cells. Despite a limited number of integrins, smooth muscle cells potentially are able to interact with basic components of the extracellular matrix, laminin, collagens (via α1β1, α3β1 integrins), and fibronectin (via α3β1, α5β1, α8β1, avβ1). In cultured ductus arteriosus smooth muscle cells, seven integrins have been identified (α1β1, α2β1, α3β1, α5β1, α7β1, αvβ1, αvβ3) (Clyman *et al.*, 1990, 1992). *In vitro*, the adhesion of smooth muscle cells on fibronectin, laminin, and collagens depends exclusively on functioning β1 integrins, whereas cell migration over these substrates depends on the αvβ3 receptor (Clyman *et al.*, 1992). Among the integrins expressed on smooth muscle cells *in vivo*, α3β1 is considered to be more involved in cell–cell rather than in cell–substrate interactions (Kaufmann *et al.*, 1989; Carter *et al.*, 1990; Larjava *et al.*, 1990). There is a bit of evidence that cell–cell interactions may be important for maintenance of the differentiated smooth muscle cell phenotype (reviewed by Schwartz *et al.*, 1990; Thyberg *et al.*, 1990). When seeded in culture with high density, smooth muscle cells are able to maintain a differentiated phenotype or even to reexpress once lost features of it.

The expression of integrins which are present in smooth muscles (α1β1 and α5β1) is dynamically regulated during development (Belkin *et al.*, 1990; Muschler and Horwitz, 1991; Duband *et al.*, 1992). During skeletal muscle development, the α1β1 integrin is expressed by myogenic precursors, during myoblast migration, and in differentiating myotubes (Duband *et al.*, 1992). The α1β1 integrin disappears from skeletal muscles as they become contractile. In visceral and vascular smooth muscle cells, the α1β1 integrin appears during early smooth muscle differentiation and is later permanently expressed after cell maturation (Duband *et al.*, 1992). In the chicken embryo, the α5β1 integrin is expressed in the majority of cell types, including visceral and vascular smooth muscle cells (Muschler and Horwitz, 1991). In adult tissues, the expression of the α5β1 integrin is greatly diminished, only capillary endothelial cells and some connective tissue fibroblasts express high amounts of this receptor (Muschler and Horwitz, 1991).

Expression of α1β1 and α3β1 integrins during development of human aorta has been shown to be developmentally regulated, but differently for these two integrins (Table IV) (Belkin *et al.*, 1990; Glukhova *et al.*, 1993). In 10-week-old fetal aorta the only abundant

laminin- and collagen-binding integrin in media is α1β1, and thus it might interact with the α1-β1-γ1 laminin form, which is also already present in aorta. The smooth muscle cell–fibronectin interaction at this developmental stage may be mediated by the α5β1 integrin. By the 24th week of gestation, the amount of the α1β1 integrin is significantly reduced in the aortic media (fourfold for the α1 subunit) compared with that in the 10-week-old aortic smooth muscle cells (Belkin *et al.*, 1990), while at the same time the α3β1 integrin appears. After birth, the expression of α1β1 and α3β1 integrins increases. Smooth muscle cells from intimal thickenings of adult aorta express fivefold less of the α1 integrin subunit than smooth muscle cells from adult aortic media. The functional relevance of the dynamic pattern of α1β1 integrin expression in developing and adult smooth muscle might be consistent with the changes in expression of α1β1 integrin ligands, especially laminin chains. As mentioned before, arrangement of the basement membrane of smooth muscle cells often correlates with the state of cell differentiation. There is good reason to suggest that a specific interaction between collagen–laminin receptors, α1β1 and α3β1 integrins, and corresponding components of basement membrane (particularly, α1-β2-γ1 laminin) promotes the maturation of smooth muscle cells and maintenance of differentiated state.

To summarize, the major integrins expressed in adult smooth muscle cells of large arteries *in vivo* are α1β1, α3β1, α5β1, and αvβ1; such a repertoire provides medial smooth muscle cells the ability to recognize collagens, laminin, and fibronectin. Expression of the integrins in human aortic smooth muscle cells is developmentally regulated so that final smooth muscle cell maturation is accompanied by an increasing expression of α1β1 and α3β1 integrins and downregulation of α5β1; integrin expression in smooth muscle cells from the intimal thickening of adult aorta is reduced compared to medial smooth muscle cells.

VI. Different Stages of Smooth Muscle Development: Phenotypic Transitions and Heterogeneity of the Smooth Muscle Cells from Human Aorta

Studies of the expression of smooth muscle-specific cytoskeletal and contractile proteins performed in different species including human, rat, rabbit, bovine, swine, and chicken have permitted the identification of three stages of vascular smooth muscle cell development; fetal, postnatal, and adult. The earliest steps of vascular smooth muscle cell development when few, if any, contractile and cytoskeletal markers are expressed have practically not been studied. By 8–10 weeks of

gestation in humans, vascular smooth muscle cells express at least α-smooth muscle actin and smooth muscle myosin heavy chain SM-1, significant amounts of ED-A- and ED-B-containing fibronectin, α1β1 integrin, and two important components of basement membrane, collagen type IV and laminin (Tables II and IV). At this particular step of development, cytokeratin 8 is transiently expressed in aortic smooth muscle cells (Fig. 2) and in other smooth muscles. Between 10 and 25 weeks of gestation, human aortic smooth muscle cells undergo significant phenotypic changes manifested by an alteration in the spectrum of cytoskeletal, contractile, and extracellular matrix proteins expressed. The proportion of α-smooth muscle actin, smooth muscle myosin heavy chain SM-2, and h-caldesmon increases, whereas in the extracellular matrix, the β2 subunit of laminin appears and the secreted fibronectin molecules no longer contain ED-A and ED-B. The α1β1 integrin content transiently decreases, whereas α3β1 starts to be expressed.

The second step of smooth muscle cell development, postnatal, is characterized by a further increase in the expression of cytodifferentiation-related contractile and cytoskeletal proteins, particularly of *meta*-vinculin, h-caldesmon, and smooth muscle myosin heavy chain SM-2. In humans, this step is rather long and takes several months.

In the adult organism, the majority of medial smooth muscle cells keep the differentiated phenotype (Fig. 3). However, the smooth muscle cells that are involved in the formation of diffuse intimal thickening and atherosclerotic lesions lack many of the properties of mature medial cells (Tables II and IV, Fig. 4).

The heterogeneity of smooth muscle cells in aortic intima and media has been studied mainly using antibodies specific for smooth muscle and nonmuscle variants of cytoskeletal and contractile proteins. The immunomorphological approach allows to follow the expression of smooth muscle markers in individual cells. Numerous cells containing certain smooth muscle markers but lacking other markers or expressing the nonmuscle variant were revealed in experimental intimal thickenings in animals, in normal human subendothelial intima, and in atherosclerotic plaques. These cells include α-smooth muscle actin-positive, smooth muscle myosin heavy chain-negative cells (Babaev *et al.*, 1990); smooth muscle myosin heavy chain-positive, smooth muscle calponin- or h-caldesmon-negative cells; and smooth muscle calponin-positive, h-caldesmon-negative cells (Fig. 4, Frid *et al.*, 1992). Such cells are especially abundant in the subendothelial part of the intima. This particular layer of the arterial wall contains cells with low levels of *meta*-vinculin and h-caldesmon; numerous cells depleted of the α1β1 integrin; and, finally, cells with an altered

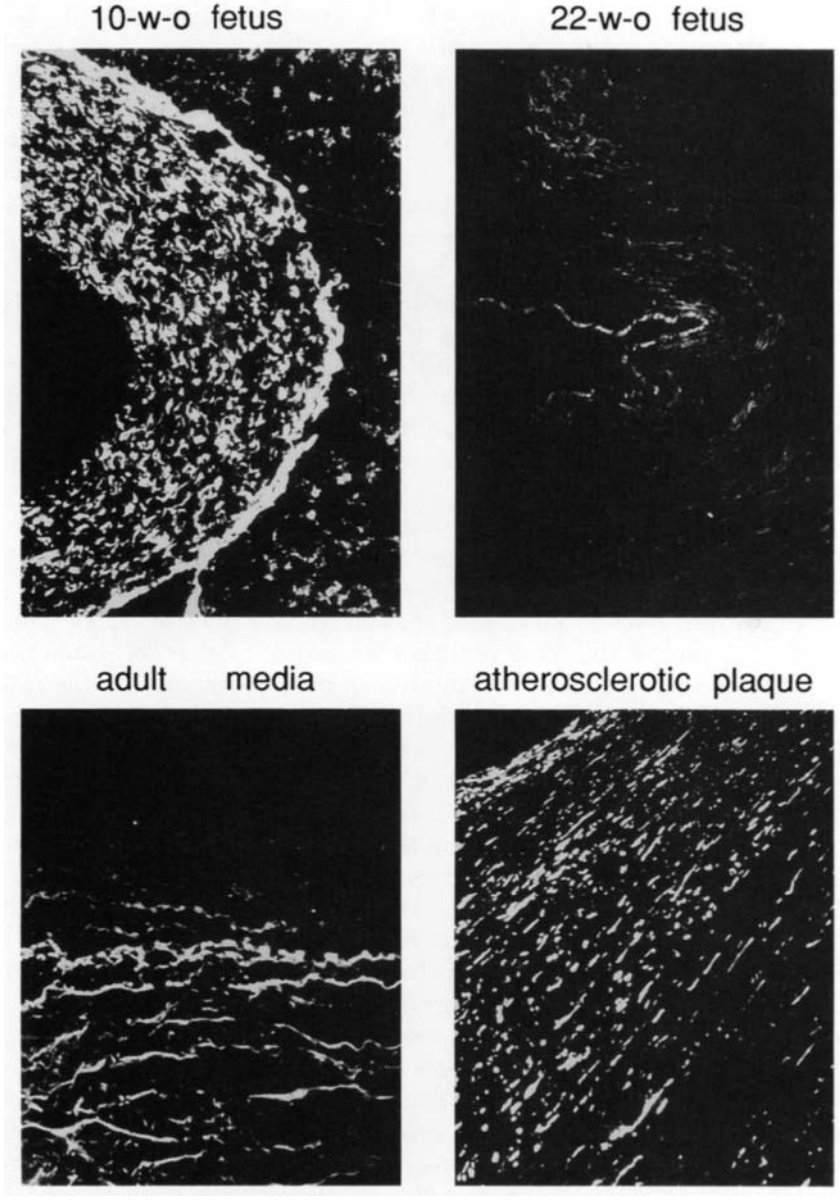

FIG. 2. Indirect immunofluorescence labeling of sections through fetal and adult human aorta with anti-cytokeratin 8. Aortic smooth muscle cells from a 10-week-old fetus contain cytokeratin 8, whereas in the smooth muscle cells from a 22-week-old fetus and adult normal aortic media, cytokeratin 8 is not detected. Clusters of cytokeratin 8-positive smooth muscle cells are found in the intimal thickenings and appear to be more abundant in the atherosclerotic fibrous plaques.

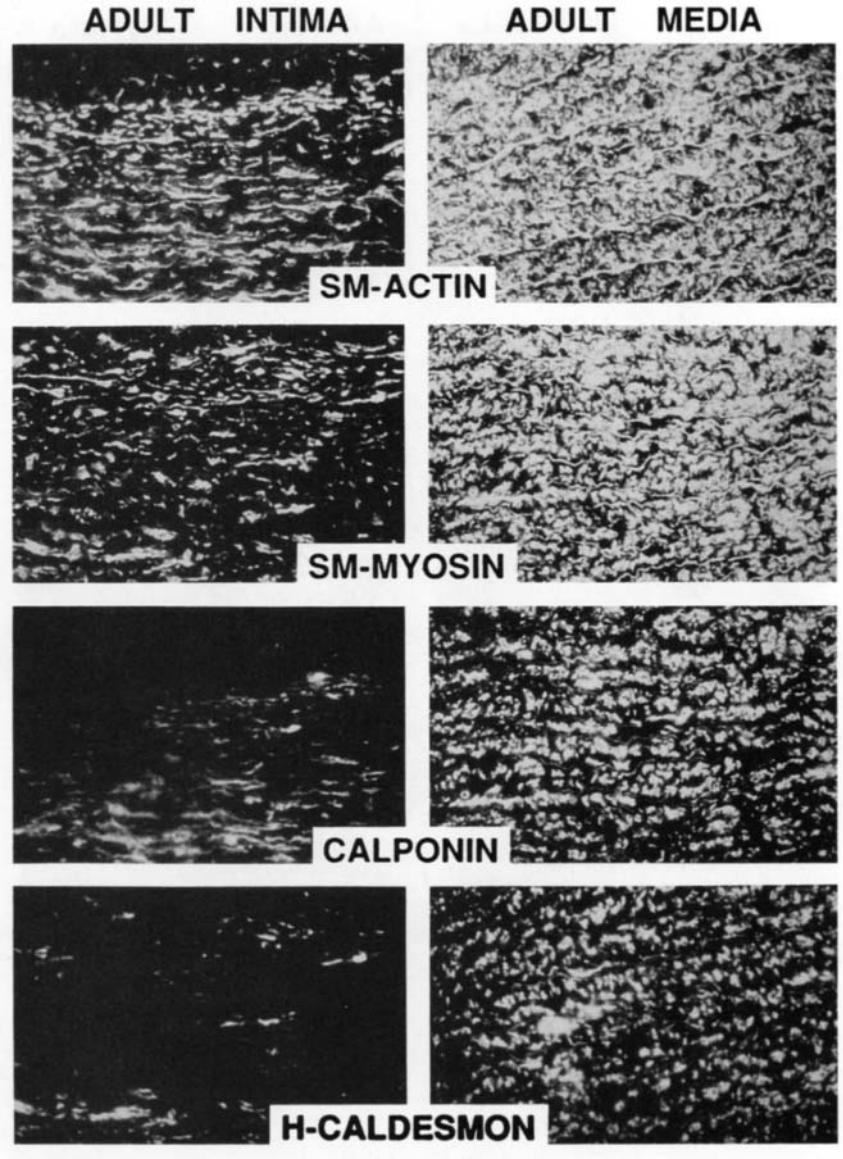

FIG. 3. Indirect immunofluorescence staining of the serial sections of 38-year-old human aorta with antibodies to different smooth muscle markers. In media, the majority of smooth muscle cells are labeled with antibodies to α-smooth muscle actin (SM-actin), to smooth muscle myosin heavy chains (SM-myosin), calponin, and h-caldesmon. In intima, the majority of smooth muscle cells are labeled with anti-α-SM-actin and anti-SM-myosin, and much less smooth muscle cells are labeled with antibodies to h-caldesmon and calponin.

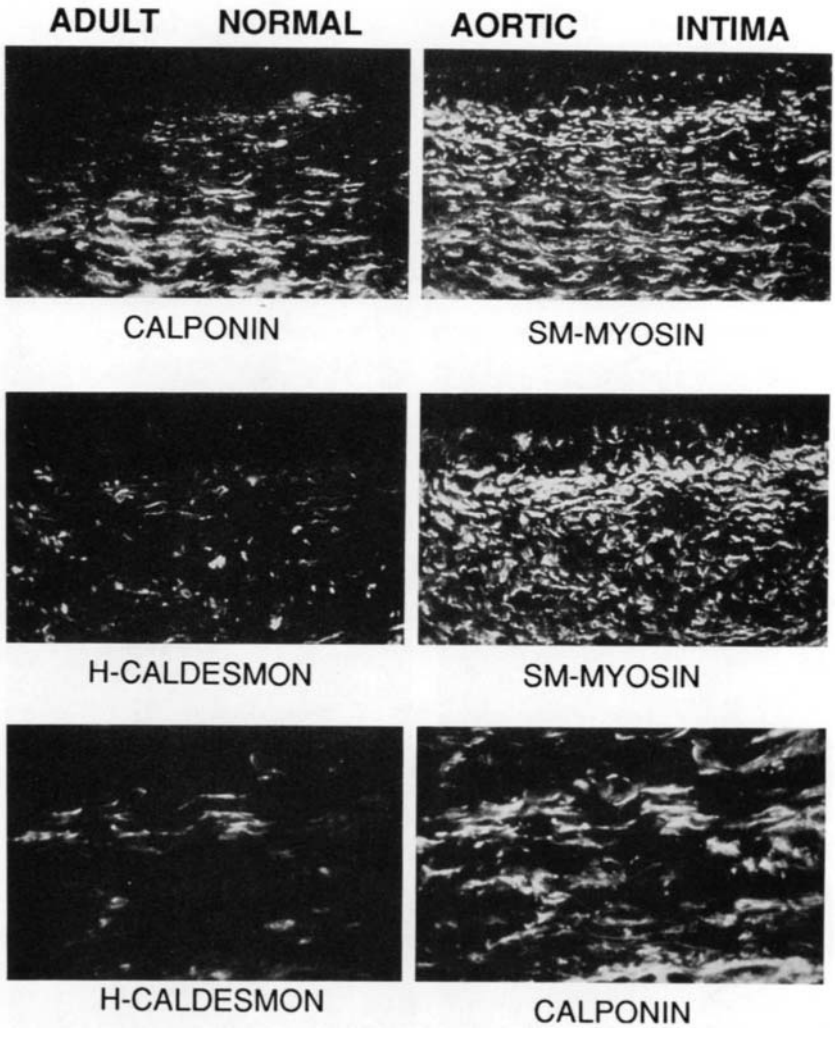

FIG. 4. Indirect immunofluroescence double labeling of the sections of the intimal thickening from 38-year-old human aorta. Only a portion of smooth muscle myosin heavy chain (SM-myosin)-positive cells contain calponin and h-caldesmon; only a portion of calponin-positive cells contain h-caldesmon.

composition of basement membrane, i.e., a decreased amount of β2 and α1 laminin chains and rich in β1 chain. A similar heterogeneity in regard to the expression of the smooth muscle markers (smooth muscle myosin, calponin, desmin, and *meta*-vinculin) has been described in bovine pulmonary artery media (Frid *et al.*, 1994).

Another group of data includes the discovery of intimal smooth muscle cells expressing fetal markers, namely cytokeratins 8 and 18, nonmuscle myosin heavy chains, tenascin, and ICAM-1 (Jahn and Franke, 1989; Printseva *et al.*, 1989, 1992; Zanellato *et al.*, 1990a; Glukhova *et al.*, 1991b; Hedin *et al.*, 1991; Okamoto *et al.*, 1992). The cells that express such markers usually form clusters of different sizes.

Two conclusions can be made on the basis of these data. Heterogeneity of the intimal smooth muscle cell population demonstrates a lack of coordinate expression of muscle-specific proteins. However, the proteins acquired later during the maturation process seem to be lost first. At least in human atherosclerotic plaques, the population of smooth muscle calponin-, h-caldesmon, and *meta*-vinculin-negative cells is much more important than that of smooth muscle myosin heavy chain- and α-smooth muscle actin-negative cells. The phenotypic changes that intimal smooth muscle cells undergo appear to be asynchronous, i.e., altered cells may be either singular or forming clusters. Surprisingly, during development, the acquisition of the differentiated phenotype by the aortic smooth muscle cells is also not a synchronized process, as (1) nonmuscle myosin heavy chains disappear from the aortic wall during the postnatal period, leaving some clusters of positive cells rather than from all the cells simultaneously (Frid *et al.*, 1993); and (2) in the late fetal period (27 weeks of gestation), smooth muscle calponin and h-caldesmon are present only in singular smooth muscle cells distributed unevenly in the aortic media (M. A. Glukhova, unpublished data).

The similarity between cells of atherosclerotic lesions and fetal smooth muscle cells has been extensively discussed (reviewed by Schwartz *et al.*, 1990; Thyberg *et al.*, 1990; Glukhova *et al.*, 1991a). The concept of fetal phenotype expression in the intimal thickenings is, on the one hand, based on a decreased expression of smooth muscle-specific proteins and a lack of certain morphological features of differentiated smooth muscle cells and, on the other hand, on the expression of positive markers of the fetal phenotype, cytokeratins 8 and 18, ICAM-1, and the β1 chain of laminin. The regulation of expression of these positive markers is poorly understood. An analysis of cytokeratins 8 and 18 gene expression in teratocarcinoma cells has shown that the rate of expression depends on the extent of DNA methylation. A high level of expression requires coexpression of c-*fos* and c-*jun* oncogenes encoding for factors involved in transcriptional control (Oshima *et al.*, 1988, 1990). It was also suggested that the intrinsic instability of the inactive state of cytokeratin 8 and 18 genes is responsible for the occurrence of cytokeratins 8 and 18 in nonepithelial tissues and tumors (Knapp and Franke, 1989).

The findings of spontaneous changes in cytoskeletal protein expression in a subpopulation of smooth muscle cells in atherosclerotic plaque and more rarely in normal intima or media may be relevant to the problem of intrinsic smooth muscle cell heterogeneity. The appearance of small smooth muscle cell subpopulations expressing different marker proteins might represent an example of generation of cell diversity within the arterial wall, particularly in atherosclerotic plaques. The environmental conditions, organization and composition of extracellular matrix, and the presence of blood-borne cells, may regulate the frequency of smooth muscle cell diversity generation and favor the proliferation of a subtype of smooth muscle cells with an altered differentiation state.

VII. The Dense Plaque as a Smooth Muscle-Specific Structure Involved in Linkages Between Extracellular Matrix and Cytoskeleton

Smooth muscle cells possess a unique anatomical structure: surface-associated dense bodies or dense plaques. These are adherens junctions of the cell–extracellular matrix type (see chapter by Small and North). Smooth muscle cells are characterized by a high level of expression of major protein constituents of adherens junctions; vinculin, talin, paxillin, α-actinin, and integrins. Some of these molecules have variants expressed mainly in smooth muscle (*meta-* and γ-vinculin, α1 integrin subunit, smooth muscle α-actinin). The colocalization of major adherens junctions proteins in dense plaques suggests that all of these proteins are able to form higher order structures (Fig. 5) (Schollmeyer *et al.*, 1976; Geiger *et al.*, 1981; Volberg *et al.*, 1986; Burridge and Connel, 1983; Small, 1985; Belkin *et al.*, 1988b; Draeger *et al.*, 1989; Turner *et al.*, 1991).

Adherens junctions are relatively dynamic structures which may undergo reorganization (disassembly and reassembly, exchange of their constituents with extra-junctional pool) in response to different stimuli such as growth factors, tumor promoters, changes in extracellular $Ca2^{+}$ concentration, and local proteolysis (reviewed by Geiger *et al.*, 1990). For example, vinculin is lost from focal adhesions of smooth muscle cells in response to platelet-derived growth factor (Herman *et al.*, 1987). A similar disruption of stress fibers was observed in response to tumor promoters (Kellie *et al.*, 1985). Several components of adherens junctions, vinculin, talin, paxillin, and the β1 subunit of integrin, have been shown to be substrates for protein kinase C and tyrosine kinases (reviewed by Burridge *et al.*, 1988; Geiger *et al.*, 1990; Turner and Burridge, 1991). Development and ini-

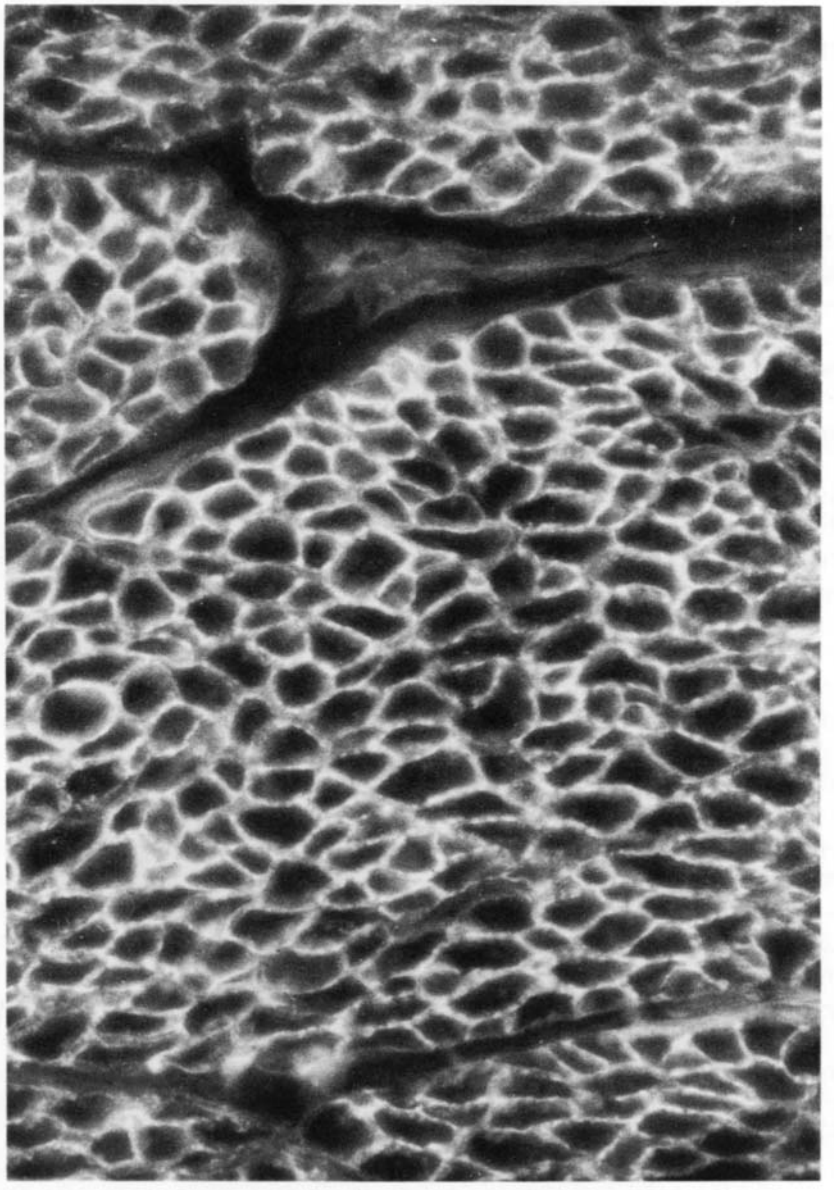

FIG. 5. Indirect immunofluroescence labeling of a transverse section of human colon smooth muscle with antibody against the laminin β2 chain. The laminin β2 chain was found in the basement membrane of adult visceral smooth muscle cells and was concentrated in dense plaque regions. Integrin subunits, vinculin, paxillin, talin, and vinculin were also localized in dense plaques (not shown).

tial steps of cell maturation may be accompanied by changes in the environment, i.e., extracellular matrix composition, presence, and local concentration of various growth factors. Embryonic tissues, including smooth muscle, contain much more phosphotyrosines than adult tissues (Maher and Pasquale, 1988).

One of the most characteristic features of differentiated smooth muscle cells is their spindle-like shape that results from its unique cytoarchitecture and specific interrelations with basement membrane. *In vitro*, under tissue culture conditions, the components of adherens junctions (focal contacts) are involved in the regulation of cell shape (reviewed by Ben-Ze'ev, 1991). Occupation of integrin by extracellular matrix ligands leads to clustering of the receptors, and their intracellular domains may serve as foci for organization of the cytoskeleton, i.e, formation of focal contacts results in an increase in the amount of microfilament bundles and increased anchoring in the plasma membrane. Similarly, smooth muscle cell shape changes from fibroblast-like in the early fetus to spindle-like in the adult occur in parallel with increases in the microfilament system organization as well as in the number of dense bodies and dense plaques. In this context, it is important to mention that stellate or mesenchyme-like cells of the intimal thickenings are somewhat similar to fibroblasts treated by cytochalasin in culture (reviewed by Bershadsky and Vasiliev, 1988). Such a treatment leads to shortening of actin filaments, loss of focal contacts with substrate, and a subsequent change in cell shape, arborization. The coordinate reorganization of smooth muscle cell adherens junctions and cell shape may occur when smooth muscle cells migrate from media through the extracellular matrix into the subendothelial region during vessel remodeling (intima formation). Importantly, this process is accompanied by alterations of dense plaque components, as was demonstrated for β1-associated α integrin subunits and vinculin variants for the cellular part and laminin and fibronectin variants for the extracellular domain (Tables II and IV). On the contrary, in normal adult tissue a certain stability of structure and composition of dense plaques in accordance with their established environment may be important for the maintenance of smooth muscle cells in the differentiated state.

It is commonly accepted that adherens junctions are involved in transduction of signals and mechanical forces mediated by extracellular matrix and accompanied by changes in cell shape. In this respect, integrins play a crucial role because they physically link the intracellular cytoskeleton with the extracellular matrix. Mechanical force transmission through integrins may be a very important factor regulating morphogenesis and tissue remodeling (Ingber, 1991). Mechani-

cal forces produce a biochemical response through adherens junctions, and integrins are particularly involved. One of the possible responses is the force-dependent release of secondary messengers. Cell surface interaction with immobilized extracellular matrix components has been shown to alter phosphatidylinositol metabolism, activate the Na^+/H^+ antiporter, and release intracellular calcium (reviewed by Ingber, 1991). The biological activity of at least one important component of adherens junctions, α-actinin, which may anchor actin filaments to the plasma membrane through interactions with β1 subunit of integrin. F-actin, and vinculin, is regulated by phosphatidylinositol-4,5-biphosphate (Fukami *et al.*, 1992). Therefore, complexes of integrin with corresponding ligands in the dense plaque of smooth muscle cells may induce different chemical signals which can dramatically alter the organization of the smooth muscle cell cytoskeleton and, as a result, change cell shape.

VIII. Smooth Muscle Development and Alternative Pre-mRNA Splicing

The RNA transcripts of many smooth muscle cell genes can be spliced in different ways according to the cellular environment. As a result, several variants (isoforms) of the extracellular matrix, the contractile apparatus, and cytoskeletal components can be produced. At the present time the function of these splice forms in most of the cases is not clear. During aortic morphogenesis the patterns of alternative splicing of several genes change (Glukhova *et al.*, 1988, 1989, 1991a,b). Table VI summarizes data concerning the changes in patterns of alternative splicing of the genes encoding four proteins: fibronectin, vinculin/*meta*-vinculin, caldesmon, and smooth muscle myosin heavy chains (SM-1 and SM-2). While vinculin and caldesmon genes are expressed in many cell types, the smooth muscle-specific variants of vinculin and caldesmon, *meta*-vinculin, and h-caldesmon are generated in smooth muscle cells by tissue-specific alternative splicing of vinculin and caldesmon pre-mRNAs (Humphrey *et al.*, 1991; Hayashi *et al.*, 1991, 1992; Koteliansky *et al.*, 1992b). In contrast smooth muscle-specific myosin heavy chains are encoded by a single gene that is expressed only in smooth muscle. However, because of alternative splicing, two variants of the protein are produced in smooth muscle cells (Nagai *et al.*, 1988, 1989; Babij and Periasamy, 1989; Babij *et al.*, 1992). A detailed discussion of alternative splicing of smooth muscle myosins is offered in the chapter by Periasamy and Nagai. No smooth muscle-specific form of fibronectin exists; however, the alternative splicing patterns of the fibronectin pre-mRNAs vary

TABLE VI

HUMAN AORTIC SMOOTH MUSCLE DEVELOPMENT AND CHANGES IN ALTERNATIVE SPLICING REGULATION[a]

Periods of smooth muscle cell development	SM-2[b]	*meta*-Vinculin	h-Caldesmon	Fibronectin ED-A	Fibronectin ED-B
Prenatal					
(8 to 25-week-old fetus)	Yes (+)	No (−)	Yes (+)	Yes (−)	Yes (−)
Postnatal					
(2 to 18-month-old child)	Yes (+)	Yes (+)	Yes (+)	No (−)	No (−)
Subendothelial intima formation	n.d.	Yes	Yes	Yes	No
(5 to 10-year-old child)		(+)	(+)	(+)	(−)

Note: (−), exon was skipped from pre-mRNA; (+), exon was not skipped from pre-mRNA; yes, the pattern of alternative splicing was changed; no, the pattern of alternative splicing was not changed; n.d., not determined.

[a]The table summarizes data published by Glukhova *et al.* (1988, 1990 a,b), Belkin *et al.* (1988a,b), and Frid *et al.* (1993).

[b]Smooth muscle myosin heavy chain SM-2.

depending on cell type and functional state, and also change during development (reviewed by Hynes, 1990; Schwarzbauer, 1991). The differentiated smooth muscle cells express fibronectin which lacks the ED-A and ED-B segments (Glukhova *et al.*, 1989, 1990b). Comparison of the splicing patterns of vinculin, caldesmon, fibronectin, and smooth muscle myosin heavy chain genes in developing smooth muscle cells allow us to make two main conclusions: (1) the alternative splicing patterns for vinculin, caldesmon, fibronectin, and smooth muscle myosin heavy chain pre-mRNAs change during smooth muscle development; and (2) at certain developmental stages, there is a coordinate regulation of alternative splicing of smooth muscle-specific exons. It is important to mention that in aortic smooth muscle cells, changes of alternative splicing patterns of vinculin, caldesmon, and smooth muscle myosin heavy chain are asynchronous with changes in expression of smooth muscle-specific proteins regulated on the transcriptional level (α-smooth muscle actin, smooth muscle myosin heavy chains). The system of regulation of alternative splicing in smooth muscle cells may be more sensitive to external factors than the transcriptional machinery.

On the basis of current information, two different mechanisms of

cell-specific alternative splicing regulation have been suggested: either cell-specific differences in the concentrations or activities of general splicing factors could regulate alternative splicing or cell-specific patterns of alternative splicing can be mediated by specialized tissue-specific factors that control alternative splicing of specific pre-mRNAs (reviewed by Latchman, 1990; Maniatis, 1991). The regulation of alternative splicing in smooth muscle is poorly understood. Developmentally regulated alternative splicing of the vinculin gene makes it an attractive model. Compared to vinculin mRNA, the muscle-specific splice form of *meta*-vinculin mRNA contain a 204 nucleotide segment encoded by single exon 19 flanked by two introns of 2.8 and 2.2 kb (Fig. 6). In most tissues and cultured cells, during splicing the *meta*-vinculin-specific exon is excised from the vinculin pre-mRNA (Glukhova *et al.*, 1986; Gimona *et al.*, 1987; Belkin *et al.*, 1988a). In muscles, especially in smooth muscle, this exon remains. The levels of *meta*-vinculin vary during different stages of smooth muscle differentiation (Glukhova *et al.*, 1988, 1990a). Interestingly, the alternative splicing pattern of the vinculin gene in cultured aortic smooth muscle cells is dependent on culture conditions (Shirinsky *et al.*, 1991). In sparse cultures, in which smooth muscle cells dedifferentiate very rapidly, the level of *meta*-vinculin also drops, whereas in dense cultures, the level of *meta*-vinculin does not change. Therefore, cell–cell interactions may be involved in the regulation of vinculin pre-mRNA alternative splicing. What mechanism is responsible for smooth muscle-specific and developmentally regulated processing of vinculin pre-mRNA? It was shown that one of the causes of exon skipping is the presence of weak 5′ and 3′ splice sites in the skipped exon, which affects the splicing of both flanking introns (Nasim *et al.*, 1990). Both splice sites of the *meta*-vinculin exon (E19) deviate substantially from the consensus and thus would be expected to act as weak sites (Koteliansky *et al.*, 1992b). At the same time, the 5′ splice site of the exon MV-1 (E18) and the 3′ splice site of exon MV+1 (E20) correspond to the classical consensus sequences. Therefore, in nonmuscle cells, a negative regulator *cis*-acting sequence within vinculin pre-mRNA may play a critical role in skipping of the *meta*-vinculin-specific exon. In smooth muscle cells, the regulation of alternative splicing of vinculin pre-mRNA can be mediated by specialized *trans*-acting factors that control alternative splicing in a tissue-specific manner (Fig. 6). These factors must act recognizing *cis*-acting sequences within the vinculin RNA transcript itself. Interaction of these smooth muscle cell-specific factors with such sequences could promote splicing at the site of the *cis*-acting sequence at the expense of the alternative splice site, although these regula-

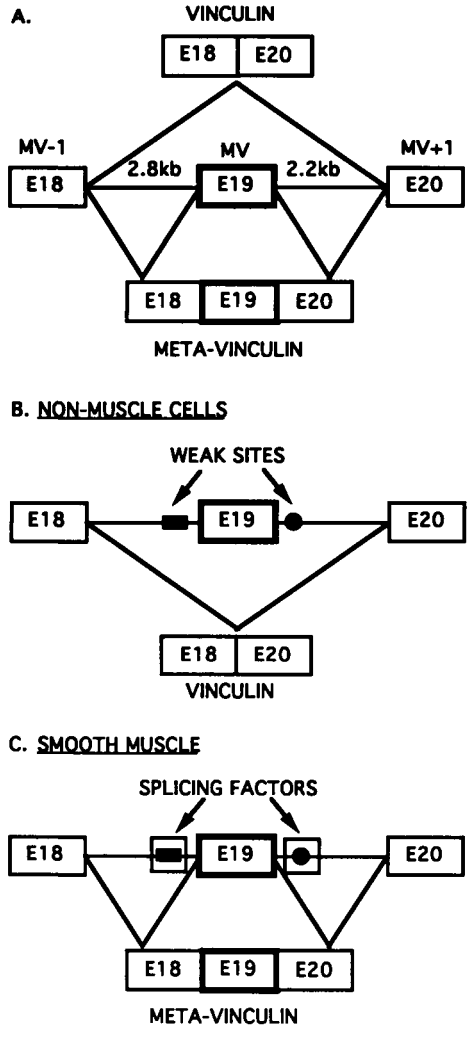

FIG. 6. Alternative splicing of vinculin pre-mRNA. (A) The origin of vinculin and *meta*-vinculin mRNAs by alternative splicing. (B and C) Possible models for positive control of vinculin pre-mRNA splicing by the smooth muscle-specific alternative splicing factor(s). In (B), an exon E19 cannot be joined to exons E18 and E20 because the adjacent introns contain two weak potential splicing sites. In (C), the factors are binding to a *cis*-acting sequence (the weak splicing sites) promoting its use. E19 (MV), *meta*-vinculin-specific exon; E18 (MV-1) and E20 (MV + 1), adjacent 5′ and 3′ exons.

tory factors may act by modifying the activities of general splicing factors.

Alternative splicing of fibronectin pre-mRNA is another example of alternative splicing, which is regulated in smooth muscle cells. The patterns of alternative splicing of fibronectin pre-mRNA in smooth muscle cells are developmentally regulated and phenotype dependent (Glukhova *et al.*, 1989, 1990b). The ED-B-containing fibronectin variant is expressed only in early fetal aorta, whereas ED-A-containing fibronectin is found mostly in modulated smooth muscle cells. It was

shown that alternative splicing of fibronectin pre-mRNA is altered during aging and in response to growth factors (Magnuson *et al.*, 1991). In nonmuscle cells, transforming growth factor β, retinoic acid, and 1,25-dihydroxyvitamin D_3 are able to change the alternative splicing pattern of fibronectin pre-mRNA. The mechanisms of alternative splicing of ED-A- and ED-B-containing exons are not completely understood. Transfection studies demonstrated that the splicing of both exons are independent events and that their cell type-specific alternative splicing may require *trans*-acting regulator factors (Paolella *et al.*, 1988; Barone *et al.*, 1989). The centrally located 81 nucleotide exon sequence is absolutely necessary for inclusion of the ED-A exon in fibronectin mRNA (Mardon *et al.*, 1987). The stimulating sequence contains a purine-rich stretch of nine nucleotides (GAAGAAGAC) (Lavigueur *et al.*, 1994). This sequence plays an important role in splice site recognition through interactions with factors binding to the 3′ splice site (Lavigueur *et al.*, 1994). Cell type-specific splicing of the ED-B exon from rat fibronectin pre-mRNA is dependent on sequences in the intron immediately downstream of ED-B (Hug and Hynes, 1993). The short repeated TGCATG motif from this intron can activate an alternatively spliced exon (Hug and Hynes, 1994). The development of smooth muscle-specific splicing extracts and transfection of *meta*-vinculin-, ED-A- and ED-B-containing minigenes in smooth muscle cells and nonmuscle cells may permit the purification of smooth muscle-specific alternative splicing regulatory factors, characterization of their properties and their interactions with vinculin and fibronectin pre-mRNAs, and identification of *cis*-acting sequences involved in the regulation of pre-mRNA alternative splicing in different cell types. An *in vitro* alternative splicing assay showed that the smooth muscle cell-specific regulation of α-tropomyosin pre-mRNA alternative splicing was dependent on smooth muscle-specific regulatory factors (Gooding *et al.*, 1994).

IX. Conclusions

This chapter has tried to analyze the expression patterns of several cytoskeletal, contractile, extracellular matrix proteins, and integrins in smooth muscle cells of developing, adult, and atherosclerotic human aorta. The chapter's intentions were (1) to describe the phenotypic properties of smooth muscle cells during development and in disease states; (2) to characterize the diversity of human vascular smooth muscle cells; and (3) to analyze possible strategies for the regulation of individual gene expression used during smooth muscle development and phenotypic transitions of smooth muscle cells in adult,

and the role of genetic (intrinsic) and environmental (extrinsic) factors in smooth muscle cell phenotypic expression. Whereas intrinsic control plays a major role in the early events of smooth muscle cell differentiation, extrinsic factors probably serve as signals for morphogenetic events and determine the phenotypic transitions of mature smooth muscle cells. This chapter has focused on analyzing the coordinate changes in the expression of extracellular matrix components, laminin, fibronectin, extracellular matrix receptors, integrins, and cytoskeletal differentiation markers of smooth muscle cells. We are especially interested in the mechanisms by which adhesion-mediating proteins are involved in the control of smooth muscle cell phenotypic transitions. The main conclusions from this analysis are:

1. Several cytoskeletal and contractile proteins, extracellular matrix components, and integrins can serve as markers of smooth muscle cell phenotypic transitions. Their expression is changed during smooth muscle cell development, formation of intimal thickening, and in atherosclerotic plaques. Smooth muscle cells can roughly be characterized as belonging to three phenotypic classes: (a) a fetal class characterized only by the expression of smooth muscle α-actin. This phenotype weakly defines smooth muscle since other cells can also express α-smooth muscle actin; (b) an intermediate class characterized by the expression of some proteins that are restricted to smooth muscle cells; and (c) a fully differentiated class characterized by a high level of the expression of the entire set of smooth muscle markers.

2. A remarkable heterogeneity of smooth muscle cells was found in different aortic layers. Such a heterogeneity was manifested more in the subendothelial intima of normal vessels, especially in atherosclerotic plaques. The origin of these different smooth muscle cell subpopulations is not clear. One possibility is that a portion of mature smooth muscle cells migrate into intima and asynchronously lose some features of the differentiated phenotype. Another option is that different smooth muscle cell subpopulations originate from distinct cell lineages that may or may not have a common precursor with a major population of medial smooth muscle cells expressing the stable contractile phenotype.

3. Adherens junctions proteins (vinculin/*meta*-vinculin, talin, paxillin, integrins, laminin, fibronectin, etc.) play an important role as mediators of signaling systems that are involved in the regulation of the processes of smooth muscle cell differentiation, maturation, and phenotypic transitions from differentiated to less differentiated states. In smooth muscles, all these proteins are associated with specific smooth muscle cell adherens junctions, dense plaques. The expression patterns of all major structural components of dense plaques

are developmentally regulated, are specific for different smooth muscle cell phenotypes, and can be correlated with smooth muscle cell phenotypic transitions. The specific repertoire of adherens junction proteins at particular stages of development and in particular subpopulations of smooth muscle cells can provide the structural basis for temporal and spatial regulation of smooth muscle cell phenotypic expression. The variation in dense plaque composition can also generate the diversity in signal transduction system controlling smooth muscle differentiation.

4. The analysis of smooth muscle differentiation marker expression demonstrates the existence of certain groups of genes whose expression might be regulated by different strategies. For example, integrins, actin, myosin heavy chains, and laminin variants are encoded by multigenic families, and the specific smooth muscle expression patterns of these genes are regulated at the transcriptional level. Another strategy for controlling smooth muscle differentiation is regulating gene expression via alternative splicing of pre-mRNAs encoding smooth muscle proteins involved in signaling mechanisms of smooth muscle differentiation. Smooth muscle-specific *trans*-acting splicing factors may regulate the selection of specific exon expression according to the cellular environment. This strategy is used for regulation the expression of several smooth muscle-specific proteins (*meta*-vinculin, h-caldesmon, smooth muscle myosin heavy chain SM-2, and fibronectin variants).

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Relevance of Smooth Muscle Replication and Development to Vascular Disease

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I. History

The modern history of vascular biology began with attempts to grow cells in culture and therefore to understand cell replication. In the early 1970s methods were discovered for *in vitro* culture of endothelial cells and smooth muscle cells(1).

For the endothelium, two quite different sets of growth requirements evolved. Human cells showed a very short replicative life span until Levine and co-workers found that the life span of human umbili-

cal vein endothelial cells could be greatly expanded by use of heparin plus fibroblast growth factor (FGF)(2,3). The reasons why primary cells lack an FGF requirement remain unexplored. In contrast to human endothelium, bovine endothelium was easily grown for many passages without exogenous FGF(4), although earlier studies suggested that FGF was needed at least for clonal expansion of primary bovine cultures(5).

Our topic here, however, is smooth muscle proliferation. The early cell culture studies have had a dramatic effect on our concepts of smooth muscle biology and pathology. Ross(1), in particular, was disturbed by the appearance of factors in serum that were able to stimulate smooth muscle replication; today we would call these factors "growth factors." We have compiled a long list of growth factors and growth inhibitors for smooth muscle cells in the chapter by Jackson. Aside from such traditional polypeptide growth factors as platelet-derived growth factor (PDGF), this chapter reviews the growth-stimulating effects of several neuropeptides and angiotensin as well as growth inhibition by other molecules, including heparin and somatostatin. The questions addressed in the present chapter include whether smooth replication occurs in vascular lesions and where antiproliferative approaches may have therapeutic value.

The appropriate place to start may be with the origins of the smooth muscle cells themselves. Endothelial formation of the vascular primordia is beginning to be well understood(6). In contrast, surprisingly little is known about how vessels acquire a coat of smooth muscle cells. In the rat, the precursors of the carotid arteries lack a smooth muscle layer as late as day 12 of gestation (Meyers, Thayer, and Schwartz, unpublished data). The first smooth muscle cells appear as a condensation around these endothelial tubes over a period of about 24 hr. As the mesenchymal cells invest the endothelial tube, the cells begin to express smooth muscle α-actin(7). This, however, is not a definitive marker of smooth muscle lineage since smooth muscle α-actin can also be found in many nonsmooth muscle cell types(8). More definitive smooth muscle-specific proteins are expressed later in development. These include desmin, calponin, smooth muscle γ-actin, and the smooth muscle myosins(9–12). These genes characterize the bulk of the cells that comprise the arterial media. Expression of most "smooth muscle" genes, however, is typically lost in cultured cells or in cells that form the neointima.

The nature of the intima is itself an important question. In the late 1960s, French offered a seminal review describing the unique properties of the arterial intima(13). His major points, given the technology of the day, were that the smooth muscle cells of the intima had a

unique morphology as seen by either light or electron microscopy (Fig. 1). Moreover, intimal formation appeared to be the characteristic response of arteries to almost any imaginable injury, as well as occurring during normal development and aging. Finally, French emphasized the importance of the intima as the unique soil for the development of atherosclerosis. As we will review, two different, but not mutually exclusive, hypotheses have been proposed as to why the intima is such a unique soil. The Campbells and others(14,15) have published extensively on the loss of differentiation when smooth muscle cells grow in culture and have suggested that similar adaptive changes explain the lack of smooth muscle markers in cells of the normal or atherosclerotic intima. In contrast, this laboratory has suggested that intimal cells may belong to a distinct smooth muscle subset characterized by expression of its own unique set of genes. Of course, both ideas may be correct, as will be discussed later in this chapter.

Returning to the subject of proliferation, not much is known about the time course of intimal formation. French(13) suggests that normal arterial intima formation is a function of animal size, as arteries of smaller animals do not form an intima unless the vessel first undergoes trauma. The absence of an intima in some arteries has been essential to studies identifying molecules critical to neointimal formation after injury (see later). In those animals and vessels in which an intima is present, the intima, however, grows rapidly after birth—at least at certain sites(16–19). The most interesting of these in humans is the origin of the left anterior descending coronary artery, a site with a high probability of developing atherosclerosis in later life. This intima develops spontaneously after birth and increases rapidly until about 6 months of age(16,19). Presumably, this is one time of life in which we should expect to see quite high levels of smooth muscle replication, although replication has never actually been studied in this artery at this early age. The only data we have are in older individuals in whom the intima is already well formed and may already be atherosclerotic. As discussed later, there is no evidence of elevated replication in either normal intima or in plaque, despite reports that these tissues often contain growth factors.

Another site where spontaneous intimal formation has been studied is the ductus arteriosus(20,21). As described in the chapter on smooth muscle embryology and contrary to conventional wisdom, the ductus forms an intima spontaneously before birth. This event may be essential for ductus closure rather than as a result of injury occurring during closure. Indeed, the characteristic stenosis seen in the ductus suggests that the function or malfunction of the intima could

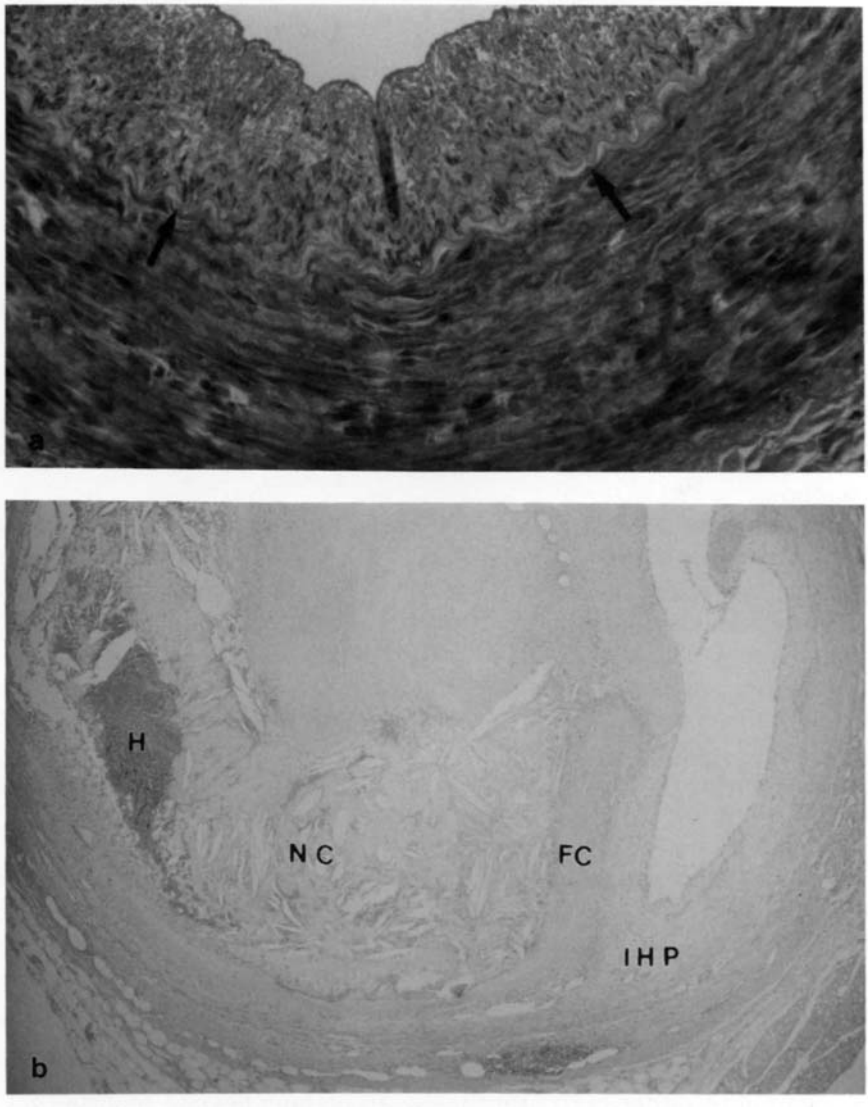

FIG. 1. (a) Histology of intima vs media. Coronary artery from adult patient with a idiopathic dilated cardiomyopathy. In the absence of atherosclerosis, diffuse intimal hyperplasia remains. The intima is several cell layers thick, is composed of smooth muscle cells, and is separated from the distinct media by the internal elastic lamina (arrows) (hematoxylin and eosin, ×200). (b) Advanced atherosclerosis of the left circumflex coronary artery. This lesion was treated by percutaneous balloon angioplasty 23 days prior to the patient's death. Note the complex nature of the lesion with a distinct fibrous cap (FC), intimal hyperplasia (IHP), intramural hemorrhage (H), and a necrotic core (NC) composed of inflammatory cells and accumulated lipid (hematoxylin and eosin, ×100).

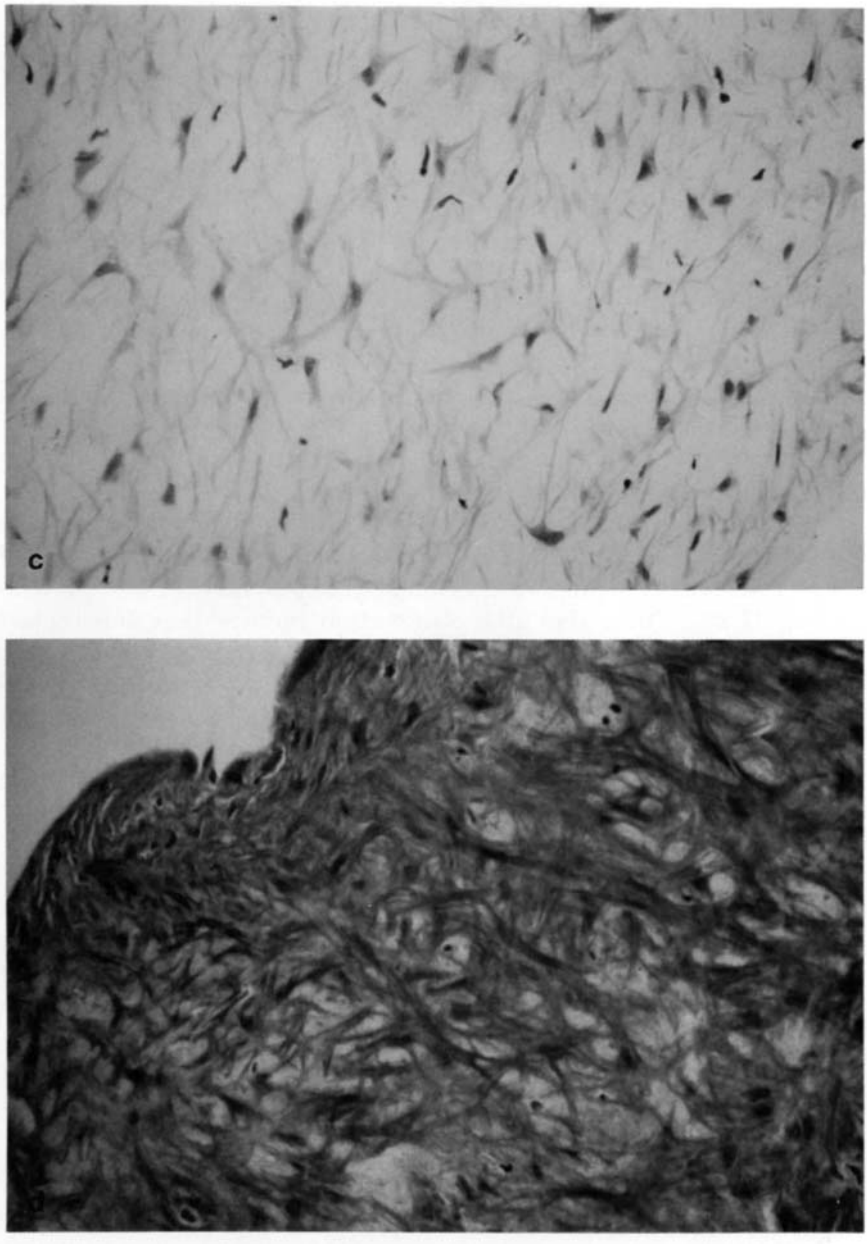

FIG. 1. (*Continued*) (c) Myxomatous tissue. Directional coronary atherectomy specimen from a restenotic lesion showing stellate-shaped smooth muscle cells. Smooth muscle cells with this appearance are often regarded as evidence of proliferation. Note the homogeneity of these cells and the absence of inflammatory and endothelial cells in this region (hematoxylin and eosin, ×200). (d) Directional coronary atherectomy specimen from a primary lesion. Again, note the presence of stellate-shaped smooth muscle cells, except in a denser connective tissue matrix (hematoxylin and eosin, ×200).

play a key role in the control of vascular lumen size—a key issue in atherosclerosis, restenosis, and hypertension. Again, we lack direct measures of cell replication at this site.

Before leaving this historical view of smooth muscle replication, we need to consider a critical paper on the pathology of the vessel wall that was written in the early 1970s. In 1973, Benditt reported that atherosclerotic plaques were monoclonal. He used glucose-6-phosphate 1-dehydrogenase (G6PD) as an allotypic marker in plaques of black females(22). The original observation has been reproduced by two other groups(23–25). Because G6PD isoforms can only be identified in blocks of tissue, the original studies did not provide direct evidence that the cell type being studied was the smooth muscle cell type. The remaining cells in these lesions are lymphocytes, macrophages, and endothelial cells. Hansson *et al.*(26) later showed that lymphocytes in lesions are polyclonal. While plaque macrophage and endothelial cells do replicate, there are no known examples of these cell types forming monoclonal growths other than in neoplasms(27,28). Thus, Benditt's observation implies that substantial smooth muscle replication must have occurred at some time during the origin of the lesions. Given the high rate of intimal growth in infants before frank lesion formation, it is tempting to ask whether the bulk of smooth muscle replication in a monoclonal lesion may not have occurred in the first weeks of life, perhaps before the lesion has developed a fatty core(17).

II. Identity of the Intimal Cell

The question of smooth muscle replication may have become especially confused by claims that proliferative smooth muscle cells have a characteristic "smooth muscle phenotype." It is important to realize that this is not the case. Despite the use by pathologists of the term "proliferative," there is no distinctive morphology other than mitosis itself that allows us to identify smooth muscle cells as being in the cell cycle.

The confusion between morphologic change and replication began with the already cited observation by the Campbells. They noted that cultured smooth muscle cells lost much of their characteristic contractile apparatus and at the same time acquired a characteristic exaggerated endoplasmic reticulum(15,29). Later, these morphological studies were augmented by immunocytochemical studies showing that smooth muscle cells lost characteristic proteins, such as desmin or smooth muscle α-actin. Similar changes were found in fetal vessels and in the neointima formed after injury. Finally, studies *in vitro* re-

lated factors required to maintain differentiation to the time course of initiation of replication in the culture dish, and other studies showed an increase of smooth muscle α-actin or other smooth muscle-specific proteins as cells became quiescent(30–32). This issue is addressed more fully in other chapters; however, one vital point needs to be emphasized here: to our knowledge, there are no experimental data showing that smooth muscle cells must dedifferentiate before replication. In our own work we have seen smooth muscle cells *in vivo* replicate within 24 hr of injury, before any loss of smooth muscle α-actin at an immunocytochemical level. Similarly, newborn smooth muscle cells are actually more stable in their expression of smooth muscle-specific proteins than are typical adult cells(33–35) and, at least for the PDGF isoforms, there is a confusing dissociation between loss of phenotype and ability to stimulate smooth muscle replication. Again, the important point is that loss of phenotype, "modulation," in the terminology used by the Campbells, Nilsson, and others, is not necessary for, nor does it imply, cell replication.

An even more fundamental question is whether there is such a thing as a smooth muscle cell at all. Put another way, what do we mean when we identify a cell as belonging to a specific cell type? Do we mean, as we mean when we discuss species of animals, that cells of one type cannot give rise to cells of another type? Like many common-sense ideas, however, this one is overly simplistic. For example, few would argue that primitive endoderm and the liver are of the same cell type. In this case, what we mean by the distinction in cell type is developmental; that is, one cell type can evolve into the other. In general, however, when we refer to cell types, we are implying adult cell types and the idea that these cell types, while perhaps able to dedifferentiate, are not able to give rise to each other. On the other hand, studies of endodermally derived cells show a surprising plasticity; that is, cells of one type can undergo a complete phenotypic conversion when plated on matrices derived from other cells types(36).

This concept of cell type is particularly important when we think about the artery wall. The artery wall is the simplest organ in the entire body, made up of only two cell types. A thin epithelium, the endothelium, lines the lumen. This epithelium is surrounded by multiple layers believed to be of a single cell type, the smooth muscle cell. The available evidence suggests that smooth muscle cells are locally derived. A particularly intriguing example is the origin of smooth muscle cells of the uterus. At birth, at least in the rat, the uterine epithelium is surrounded by an undifferentiated, fibroblast-like stroma. The ability of this stroma to differentiate into smooth muscle cells appears to depend on interactions with the uterine lining or with some

other endoderm. This suggests that endoderm can act as an inducer of smooth muscle cells from more primitive mesenchyme. It is intriguing to imagine that similar local phenomena may give rise to the initial smooth muscle coat surrounding blood vessels, although no direct evidence for this possibility exists. At this point, then, one might imagine structures consisting of an endothelial tube surrounded by locally recruited smooth muscle cells. It is important to realize that local recruitment may mean that smooth muscle cells surrounding different vessel beds may have quite different properties. For example, a report has suggested that the endothelial primordium that forms the left anterior descending coronary artery is separate from the aorta, and, in fact, after forming must invade through the smooth muscle coats that form the aortic wall(37). This suggests that at a junction particularly known for the development of atherosclerotic lesions, we may well have smooth muscle cells that have been derived by quite different processes. Particularly relevant to this hypothesis are the observations by Rosenquist and his colleagues that smooth muscle cells in the head and neck have a neural crest origin, whereas smooth muscle cells in the rest of the animal come from more conventional mesoderm(38). In summary "smooth muscle cell" may be an oversimplified term for cells with diverse origins.

It is the intimal layer, the final layer of the vessel wall to form, which is the subject of most of this chapter. Our central thesis remains much like the ideas offered by French. We suggest that the arterial intima is a distinct tissue. The list of smooth muscle cell genes that are either uniquely expressed or overexpressed in the intima relative to the underlying media is long and rapidly increasing (Table I). Even if we exclude genes whose intimal overexpression can be attributed to the chronic inflammatory process of atherosclerosis(27,39), a large number of molecules distinguish intimal smooth muscle cells from underlying normal medial smooth muscle cells. Furthermore, it is likely that this unique pattern of intimal gene expression is responsible for the unique pathology of the intima, including the origins of atherosclerosis.

Perhaps the most debated issue regarding the intima is the clonality of the cells that give rise to atherosclerotic plaques(23–25). As already noted, we have known since the early 1970s that plaque smooth muscle cells are monoclonal(22). It is intriguing to consider which special properties of the intima could result in the monoclonal expansion of the plaque and to wonder how clonal plaque cells may differ *in vitro* from polyclonal medial cells. A final reason for focusing attention on the intima is its role in restenosis. As will be discussed later, the process of luminal renarrowing following angioplasty of atheroscle-

rotic lesions is widely believed to be the result of intimal hyperplasia. Thus, control of intimal formation is one of the major goals of contemporary research in vascular biology.

III. Neointimal Formation: A Generic Response of Vessels to Injury?

If intima is the natural layer that forms between the endothelium and the internal elastic lamina, a similar structure, the "neointima," is the intima that forms in response to injury. We recognize neointima because many arteries, especially smaller ones, either do not form an intima at all or do so only slowly as the animal ages(13,40). Neointimal formation, however, occurs in all arteries as a response to a wide variety of injuries, including irradiation, application of turpentine to the adventitia, wrapping the vessel, or electrical stimulation, as well as mechanical injuries, including placement of a suture, scratching with a probe, or dilatation of the common carotid artery with an embolectomy balloon catheter(13,41–50). These changes are seen in large arteries, small arteries, and even in transplanted veins that undergo arterialization.

The important point is that the normal vessel wall is composed almost entirely of smooth muscle cells; thus, the typical response to injury will be somewhat different from the classical response seen in a skin wound. The brain, too, has a unique cellular composition and responds to injury by gliosis. Similarly, we may consider neointimal formation as the peculiar response of vessels to injury. The most obvious questions about this process are:

1. To what extent does the neointima form by migration of cells from the media versus replication of cells in the intima?
2. Does neointimal formation require medial smooth muscle replication?
3. How does neointimal formation relate to spontaneous development of an intima?
4. Are intimal or neointimal cells, like glia, a distinct cell type?
5. What molecules control neointimal formation?

IV. Pharmacology of Neointimal Formation

While the identification of a large number of molecules that stimulate smooth muscle replication *in vitro* has been seen since the mid-1970s (see chapter by Jackson), the central theme of this chapter is the relevance of smooth muscle replication to pathology *in vivo*. We

TABLE I

INTIMAL UNIQUE GENE EXPRESSION

		Humans								
		In vitro				In vivo				
Function	Gene	Fetal	Intimal	Adult	Plaque	Fetal	Intimal	Adult	Plaque	Restenosis
Adherence	Integrin α_1			+		+++	+	+++	+	
Adherence	Integrin $\alpha_3\beta_1$			+		+	+	+++	+	
Adherence	Integrin α_5			+		++	+	++	+	
Adherence	Integrin $\alpha_v\beta_1$			+				++		
Adherence	Vitronectin									
Adherence	*meta*-Vinculin			+/−		−	+	+++	+	
Adherence	Phosphoglucomutase			+/−		+/−	+	+++	+	
Adherence	15 lipoxygenase								+++M?	
Angiotensin	ACE									
Angiotensin	AT-1									
Angiotensin	AT-2									
Coagulation	PAI-1							+	++	
Coagulation	Tissue factor							−	++	
Coagulation	Thrombomodulin									
Cytochrome P450	CYPIA1									
Cytokine	Interferon			Inducible					Assumed present	
Cytoskeleton	Vinculin									
Cytoskeleton	SM actin			+++++	+++	++	+/−	++++	++	+/−
Cytoskeleton	Calponin					−	+	+++	+	
Cytoskeleton	Cytokeratins 8 and 18	++		+/−		+++	−	−	+ focal	
Cytoskeleton	Desmin						+	++	++/−	
Cytoskeleton	H caldesmon			+/−		+/−	+	+++	+	
Cytoskeleton	NMM					++		+/−	++	++++

Cytoskeleton	Gelsolin														
Cytoskeleton	SM 1 MHC							++		+++		+++			
Cytoskeleton	SM 2 MHC							+/−		+		+++			
Cytoskeleton	SM22														
Development	HOX B7	+++++		−											
Development	Gax														
Differentiation marker	Ferritin														
Growth factor	bFGF											+++		−	
Growth factor	IGF-1														
Growth factor	IGF-2														
Growth factor	PDGF-A					++						−		+++	
Growth factor	PDGF R α														
Growth factor	PDGF-B	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Immune response	MHC II			Inducible										++++	
Inflammation	ICAM			++		+++						−		++	
Inflammation	IL-1			Inducible										++	
Inflammation	IL-2			−	+										
Inflammation	IL-6			Inducible								+		+	
Inflammation	IL-8			Inducible											
Inflammation	MCSF-1			Inducible								+		+++ in rabbit and human lesions in SMC	
Inflammation	TNFα														
Inflammation	VCAM									−		−		+++	
Inflammation	c-*fms*			Inducible										++M?	
Lipids	aLRL-R			Inducible											
Lipids	APO-E														
Lipids	LPL													++M?	
Macrophage	MCP-1													+	

continues

TABLE I *continued*

INTIMAL UNIQUE GENE EXPRESSION

		Humans								
		In vitro				In vivo				
Function	Gene	Fetal	Intimal	Adult	Plaque	Fetal	Intimal	Adult	Plaque	Restenosis
Matrix	Col I(a1)[a]									
Matrix	Elastin									
Matrix	Elastin									
Matrix	Fibronectin ED-A	++++	++++	++++			++++	–	++++	
Matrix	Fibronectin ED-B	+	+	+	+	+++	–	–	–	
Matrix	GLA[b]									
Matrix	GLA[b]							?	++	
Matrix	Laminin A B1 B2					+++++	+++++	–	+++++	
Matrix	Laminin A S B2						+++++	+++++	+++++	
Matrix	OP	++	?	++	++	–	–	–	+++	+++
Matrix	Tenascin	++								
Matrix	Versican						==	++	+++	+++
Matrix	CHIP28									
Membrane channel	Phospholamban									
Protease	Stromelysin				+++					
Receptor	Thrombin receptor							–	+++	
RNA regulatory protein?	F31									
SR protein	1RA1									
TGF-β family	BMP 2a							–	+++	
Unidentified sequences	2E10									

		Rats/rabbits						
		In vitro			In vivo			
Function	Genes	Pup	Contractile adult	Synthetic adult	Fetal	Media	Intima	Refs.
Adherence	Integrin α_1				++	+		+++ 165
Adherence	Integrin $\alpha_3\beta_1$							165
Adherence	Integrin α_5							165
Adherence	Integrin $\alpha_v\beta_1$					+	++++	165
Adherence	Vitronectin					−	++	252
Adherence	*meta*-Vinculin			+/−		+++	+	253
Adherence	Phosphoglucomutase		+/−			+++		Koteliansky, personal communication
Adherence	15 lipoxygenase							254
Angiotensin	ACE					+	++++[a]	85,255[a]
Angiotensin	AT-1			++++		++	+++++	85
Angiotensin	AT-2				+	+	+	85
Coagulation	PAI-1					−	++	252,256–258
Coagulation	Tissue factor						Transient	176,177,259
Coagulation	Thrombomodulin					−	+++	260
Cytochrome P450	CYPIA1	++++	−	−	−	−	Inducible	172
Cytokine	Interferon						Assumed present	261
Cytoskeleton	Vinculin			++	+	+++		253,262
Cytoskeleton	SM actin		++++	++++		+++++		34,159,253,262–265
Cytoskeleton	Calponin		+	−		+++		263,266
Cytoskeleton	Cytokeratins 8 and 18							253,267

continues

TABLE I *continued*

INTIMAL UNIQUE GENE EXPRESSION

Function	Genes	Rats/rabbits						Refs.
		In vitro			In vivo			
		Pup	Contractile adult	Synthetic adult	Fetal	Media	Intima	
Cytoskeleton	Desmin							263
Cytoskeleton	H caldesmon							266,269
Cytoskeleton	NMM		+	+++	++	+++	++	270–273
Cytoskeleton	Gelsolin							274
Cytoskeleton	SM 1 MHC		++	+		+++++		32,263,275–279
Cytoskeleton	SM 2 MHC		++	+		+++++		32,265,266,279
Cytoskeleton	SM22		++++	+++		+++++		263
Development	HOX B7							Miano, unpublished data
Development	Gax		++	++				280
Differentiation marker	Ferritin		++++	+				281
Growth factor	bFGF					++++	++	282,283
Growth factor	IGF-1			++		+++	+	284–286
Growth factor	IGF-2				+++			287
Growth factor	PDGF-A	+++	+++			+	++	27,83,101,102,182
Growth factor	PDGF R α		++++	++++		+++	+++	83
Growth factor	PDGF-B	+++	−!!	−		−	+++!!	83,182
Immune response	MHC II						+++	39,288,289
Inflammation	ICAM					−	++	254,290–292
Inflammation	IL-1			Inducible[a]				293–295
Inflammation	IL-2							206
Inflammation	IL-6							295
Inflammation	IL-8							296
Inflammation	MCSF-1							296–298

Inflammation	TNFα							299,300
Inflammation	VCAM					–	++	292,301,302
Inflammation	c-*fms*							297,303
Lipids	aLRL-R			+++ rabbit				303,304
Lipids	APO-E							305
Lipids	LPL							254,306
Macrophage	MCP-1							307,308
Matrix	Col I(a1)[a]	—+++++		+++++	+++++	++	++++?	169,175
Matrix	Elastin			+++	+	++++		263
Matrix	Elastin	+++++	+	+	+++++	+		169,253,309,310
Matrix	Fibronectin ED-A				++++	–	++++	253
Matrix	Fibronectin ED-B							253
Matrix	GLA[b]		+++	+		+++++		263
Matrix	GLA[b]							311
Matrix	Laminin A B1 B2							253
Matrix	Laminin A S B2			+/–				165
Matrix	OP	++++	?	++	+	+	+++	185,263,316
Matrix	Tenascin	+++++	++	++?		++	++++	167,168,312
Matrix	Versican	–	+++++	+++++	?	?	?	313, Lemire, unpublished data
Matrix	CHIP28		+++	+		+++		263
Membrane channel	Phospholamban		++	–		++		263
Protease	Stromelysin							205
Receptor	Thrombin receptor				+++	0	+++	115,116,119,314,315
RNA regulatory protein?	F31	–	?	–	+++	–	+++	287
SR protein	1RA1		–	–		++		263
TGF-β family	BMP 2a							186
Unidentified sequences	2E10		~	–		+++		263

Note: Classes: PI, PI gene in rat; MU, adult specific in rat; CF, contractile phenotype; NI, neointimal: S, smooth muscle cell lineage marker; I, human intimal marker; R, restenosis in man; F, chronic inflammatory response; Ca, calcium related; Pl, plaque specific.

[a]Contradictory data exist.

[b]Different GLA proteins.

will begin by discussing the best studied model for *in vivo* replication: neointimal formation in the response of the rat carotid artery to balloon angioplasty(43,46,51). It is important to realize that this model is much simpler than the same response in larger, more complex human arteries which already are diseased. First, as previously discussed, the rat carotid artery has only rare intimal cells(52). Even normal arteries in larger animals have a preexisting intima(53,54). Second, unlike the response of arteries in larger animals, including rabbits, swine, and nonhuman primates, the response of rat arteries to most injuries involves platelets, but there is no deposition of fibrillar fibrin or adherence of leukocytes(55–64). The simplicity of the rat model has made it possible to develop methods for detailed kinetic analyses of the processes leading to neointimal formation and to define four waves of response to injury with the molecules responsible for each of these waves(41,65–69).

The balloon injury model begins with complete destruction of the endothelium as well as extensive death of medial smooth muscle cells(51). The first response to balloon injury, called "first wave," consists of medial smooth muscle cell proliferation and begins about 24 hr after the injury. In elegant studies, Reidy and colleagues(70–71) have shown that this wave of replication can be completely accounted for by release of basic FGF (bFGF) from dying smooth muscle cells. Among other candidate molecules, studies with infused PDGF, as well as studies with anti-PDGF antibodies, have shown that this molecule does not play a significant role as a mitogen(72,73). In addition, other molecules may be active, as more limited data suggest that α-adrenergic antagonists and angiotensin II (AII) antagonists can block medial replication(74–76).

The migration of smooth muscle cells across the internal elastic lamina to form the intima constitutes the "second-wave." Smooth muscle cells are readily observed on the luminal side of the internal elastic lamina 4 days after injury(51). The duration of the second wave is not known. Several molecules can contribute to smooth muscle migration, including PDGF, transforming growth factor β (TGF-β), bFGF, and AII. The relative contributions of these different molecules are not known, nor do we know whether other molecules are involved(71,76,77). Interestingly, the effects of angiotensin-converting enzyme (ACE) inhibitors may result from the ability of these drugs to elevate bradykinin levels by preventing its degradation rather than the expected effect of these agents on lowering levels of AII. ACE inhibitors are not specific for the conversion of AI to AII(78–81). Among other effects, these drugs inhibit the degradation of bradykinin, and there is evidence from studies involving bradykinin antagonists that

elevation of bradykinin can explain part of the effect of ACE inhibitors on neointimal formation(78). The effect of PDGF is noteworthy, given the emphasis from *in vitro* studies that this molecule is a mitogen, despite the apparent lack of mitogenicity *in vivo*(72).

Once smooth muscle cells arrive in the intima, they may replicate for weeks to months(82). This replication is called the "third wave." As of the time of writing this chapter, no specific molecular antagonist has been shown to inhibit this replication. Even antibodies to bFGF, which are so effective in inhibiting the first wave, are impotent against third-wave replication(71). Therefore, although we cannot say that any specific molecule has definitively been identified as a third-wave mitogen, a few potential candidates appear to be present in the intima. For example, the PDGF-A chain is overexpressed in the intima, but will not stimulate replication if infused, nor do antibodies to PDGF suppress third-wave replication(72,83,84). Other growth-control molecules that appear to be overexpressed in the rat neointima include the AII receptor and TGF-β(77,85). Insulin-like growth factor-1 (IGF-1) is also overexpressed following injury; however, it is overexpressed in the media(86). Although we cannot identify the critical molecules that sustain elevated replication in the third wave, we do know that the neointima can be stimulated to show a further increase of replication by infusion of other molecules. This increased responsiveness to mitogens can be called a "fourth wave," and involves at least TGF-β, bFGF, or AII as agonists(70,74,77). Again, PDGF does not appear to be mitogenic(72).

Table II summarizes the molecules that control the three waves.

V. Specific Molecules in the Rat Model

The role of the angiotensin system in neointimal formation is of particular relevance because of the failure of extensive clinical trials using ACE inhibitors to prevent restenosis(87). Exploring possible explanations for this failure requires a consideration of the pharmacology of the renin–angiotensin system.

The renin–angiotensin system is not restricted to the kidney. Components of the pathway may be found together in the intima or neointima(80,88). The principal AII receptor, AT1, is elevated in the neointima, although both AT1 and AT2 receptors are also present in the normal wall(85). The second angiotensin receptor, AT2, is probably not important in these processes, although it can be found at modest levels in the vessel wall. The one report of inhibition by an AT2 receptor antagonist required that the drug be given locally at very high doses(89). The source of AII in the wall may be local activation of an-

TABLE II

LIKELY MEDIATORS OF NEOINTIMAL FORMATION

	Description	Mediators	Inhibition		Stimulation
			Antibody	Antagonist	Agonist
First wave (0–3 days)	Replication of SMC within the media	FGF	+	NA	+
		PDGF	±	NA	−
		TGF-β	NA	ND	±
		AII	ND	+	NA[a]
Second wave (3–14 days)	Migration of SMC from the media into the intima	PDGF	+	NA	+
		AII	ND	+	ND
		FGF	+	NA	+
Third wave (7 days to a month)	Proliferation of SMC within the neointima	FGF	−	NA	±
		PDGF	−	NA	−
		TGF-β	NA	NA	+[b]
		AII	ND	−	+[b]

Note: AII, angiotensin II; +, supporting evidence; PDGF, platelet-derived growth factor; −, evidence against hypothesis; FGF, basic fibroblast growth factor; ±, weak response; TGF-β, transforming growth factor β; ND, not done; NA, not available.

[a]AII does stimulate medical smooth muscle.

[b]"Restimulation" experiments (71–74,77,98).

giotensin I. Angiotensinogen is available from the circulation. Although Rakugi *et al.*(90–92) state that they were able to use quantitative *in situ* hybridization to demonstrate an increase in angiotensin gene expression in both the media and the intima during the first 2 weeks after balloon injury to the rat carotid, this is difficult to evaluate since methods for quantitative analyses of *in situ* hybridization are not yet well established. Kininase II (ACE) has been found in the normal vessel wall, even in the absence of its highest source, the endothelium(93). Whether vascular ACE is upregulated following injury is still a matter of controversy. Rakugi *et al.*(94) reported that, 2 weeks after injury, the rat carotid artery expresses increased levels of ACE activity, as well as immunoreactivity using a polyclonal human and monoclonal rat ACE antibody. Viswanathan and colleagues(85), however, used quantitative autoradiography and reported that binding to ACE in the aortic neointima 15 days after injury was not different from binding in the underlying media or the media of sham-operated animals. Taken together, these observations suggest that ACE activity per unit of wall mass is not increased in the vascular wall 2 weeks after injury. Finally, the possibility that the vessel wall contains angiotensinogenases with renin-like activity has been raised(95). In summary, there is sufficient evidence for components of the angiotensin system in the injured vessel wall.

Moreover, AII itself, like FGF, is able to stimulate the first wave and it is also able to restimulate replication in the neointima. First-wave replication, migration into the intima, and intimal thickening can be prevented by angiotensin receptor antagonists as well as converting enzyme inhibitors(96,97). Furthermore, the persistence of replication after injury and the elevated replicative response of the neointima to infused angiotensin, compared to uninjured media, could be due to increased intimal expression of angiotensin I receptors(74). Thus, it is quite reasonable to interpret the effect of ACE inhibitors in blocking neointimal formation as being the result of blocking formation of AII(98).

Despite all of this data, why did the clinical studies fail? One explanation may lie in a misunderstanding of the pharmacology of converting enzyme inhibitors. Existing studies with ACE inhibitors are inconclusive in part because higher drug doses are needed to achieve significant suppression of intimal thickening (3–30 mg/kg/day) than to obtain a maximal (20%) reduction of blood pressure in the same strain of normotensive rats (3 mg/kg/day)(99). This difference in dosage requirements may reflect the higher doses necessary to achieve inhibition of vascular tissue ACE or to effectively block degradation of bradykinin at the site of injury(100). The latter is of special

interest because of evidence that bradykinin elevation as a result of inhibition of the normal catabolism by converting enzyme may act as an endogenous inhibitor of neointimal formation(78).

A second issue surrounds the mechanism of action of angiotensin itself in the vessel wall. The studies done in humans assume that AII acts directly via angiotensin receptors. In the rat, however, the mitogenic effect of angiotensin on smooth muscle cells in the media of blood vessels is mediated indirectly via α-adrenergic receptors, yet the effect on intima is via AT1 receptors(75). The relative role of these two pathways in human restenotic vessels is, of course, unknown.

Finally, it is important to consider the possibility that the endpoint in the animal studies, intimal thickening, is not the critical process in the renarrowing of human vessels; this is discussed later.

The next molecule of special interest is PDGF. Infused PDGF is at best a minor mitogen *in vivo*(72). Instead, PDGF seems to function primarily in promoting migration(27,72). The absence of a direct mitogenic effect does not rule out an indirect role for vessel wall PDGF in replication *in vivo*. The PDGF-A chain is chronically overexpressed in neointima(83) and atherosclerotic plaque(101,102). The mitogenic effect of a number of mild mitogens, including bFGF, TGF-β, and AII, has been shown to be substantially blocked *in vitro* by antibodies to PDGF-A(73). This is true despite the observation that PDGF-A is itself a nonmitogen or a weak mitogen *in vitro*(72). Thus, we need to consider the possibility that the localized overexpression of PDGF-A seen in the neointima may act as a cofactor that increases the fourth-wave responsiveness to the factors just discussed and perhaps elevates neointima replication in response to the baseline levels of these same molecules.

TGF-β may also play an important role in third- and fourth-wave replication. As already noted, we found that infused TGF-β was a mitogen and there is an accumulation of TGF-β in the neointima(77). These *in vivo* data are at odds with studies *in vitro* in which TGF-β is sometimes seen as a growth stimulant and at other times as an inhibitor. Some of the variability may reflect the strain of smooth muscle cells studied and their state of confluence(103–105). A particularly intriguing hypothesis for a role for TGF-β as a growth inhibitor comes from a report by Grainger *et al.*(106). These authors propose a link between TGF-β as an endogenous growth inhibitor and elevated atherosclerosis risk due to elevated Lp(a). It has been suggested that Apo(a) levels correlate with an increased incidence of restenosis(107,108). The mechanism of action of Apo(a) is believed to be due to its homology with plasminogen and its ability to inhibit the formation of plasmin(109,110). Atherosclerosis may then depend

on inadequate clot lysis(109,110). Plasmin, in addition to its role in fibrinolysis, is essential to the activation of TGF-β(111). Grainger and colleagues(106) found that Apo(a) is mitogenic for smooth muscle cells in culture. They were also able to attribute the mitogenic effect to inhibition of an autocrine growth inhibitor, TGF-β. They propose that Apo(a) blocks the formation of plasmin and the subsequent ability of the cells to activate TGF-β(106,112,113). In this view, the predominant role of TGF-β would be as an endogenous inhibitor of lesion formation at sites of active coagulation and Apo(a) would enhance lesion formation by diminishing the production of activated TGF-β. Perhaps in contradiction to Grainger's hypothesis, a study of atherectomy specimens found elevated levels of TGF-β in restenotic lesions(114).

Thrombin is another neointimal molecule that is attracting renewed interest because of the recent cloning of a thrombin receptor and the recognition that thrombin can have a direct mitogenic effect on cells independent of the coagulation cascade(115,116). Particularly intriguing is the evidence that thrombin activity is chronically elevated in the neointima and that the neointima as well as the human atherosclerotic plaque overexpresses the thrombin receptor(117–119). In addition, studies involving animal model systems more complex than the rat, especially the pig coronary artery stent model, suggest that thrombosis may well play a key role in the early events leading to restenosis(120).

The last two molecules for this discussion are somatostatin and heparin. These molecules are of special interest because of the extensive studies of their ability to inhibit neointimal formation. Somatostatin analogs, in particular, angiopeptin, have been found to be effective in a range of animals species and different models of arterial injury, including fat-feeding, balloon angioplasty, neointimal formation in vein grafts, and transplant atherosclerosis(121–125). Clinical trials are underway; however, preliminary results appear to be equivocal. The pharmacological rationale for this approach is unclear; however, it is important to note that somatostatin receptors are widespread in the body and may act as vasodilators or vasoconstrictors in blood vessels(126–129). Failure or success of angiopeptin trials to prevent restenosis will be especially important because the dosages of the drug that were used in humans were comparable to those used in animals. Failure could suggest that these animal models fail to accurately mimic the clinical problem of restenosis.

Similarly, heparin has been widely studied as an inhibitor of intimal formation in animal models(130). As with somatostatin, the mechanism of action of heparin as an inhibitor of intimal hyperplasia

is poorly understood. While much attention has been focused on the potential role of heparin on c-*myb*, this molecule is expressed in late G1 of the cell cycle and likely represents only one of several defects when growth is inhibited(131). Equally intriguing are the role of heparin in inhibiting migration and suggestions from Lindner and Reidy(70) that the major action of heparin may be to wash bFGF out of the injured vessel wall after the mitogen is released from dying cells. Unfortunately, clinical trials using heparin to prevent restenosis have been disappointing(132,133). Ellis and colleagues(132) administered intravenous heparin to patients over the first 18–24 hr postangioplasty and found no difference in the restenosis rates of patients treated with heparin compared to those given a dextrose infusion (41% vs 37%, respectively, P = NS). Similarly, a subsequent attempt to limit restenosis with a single daily subcutaneous injection of 10,000 IU of heparin was halted prematurely due to higher rates of restenosis and clinical events in the heparin treatment group compared to the usual care group(133). This lack of clinical benefit is difficult to explain; however, Edelman and Karnovsky(134) suggest that differences in heparin dose scheduling may be critical. For example, the antiproliferative effect of heparin requires that the drug be administered for at least 4–7 days after injury in the rat carotid balloon injury model(51). Furthermore, cell proliferation and the intima:media area ratio are made worse when rats are treated with heparin dosages and administration schedules similar to those used clinically(134). Low molecular weight heparins also appear to be ineffective for the prevention of restenosis(135).

Finally, attention has been focused on other anticoagulant strategies as a means of treating restenosis, including clinical trials using monoclonal antibodies or antagonists to the platelet glycoprotein IIb/IIIa receptor(136–138). For example, as part of the EPIC study, the chimeric monoclonal antibody Fab fragment (c7E3), which is directed against the platelet glycoprotein IIb/IIIa receptor, was administered to patients undergoing angioplasty or atherectomy who were at high risk for ischemic complications(138). This therapy resulted in a reduction in acute ischemic complications (e.g., nonfatal myocardial infarction, emergency revascularization procedures), although at the risk of increased bleeding complications. While it is easy to imagine that these trials are affecting only platelets, these antagonists to Gp IIb/IIIa, a platelet adhesive protein, may also affect a closely related integrin, $\alpha_v\beta_3$. Antagonists to $\alpha_v\beta_3$ have been shown to block smooth muscle migration *in vitro* and intimal formation *in vivo*(139). Moreover, we have found that this same receptor is required for movement of smooth muscle cells in response to osteopontin, an abundant and

specific marker of intimal smooth muscle cells(140). In summary, there is an increasing body of circumstantial data suggesting that the second wave, i.e., migration, is a critical step in neointimal formation and possibly in restenosis.

VI. The Role of Intimal Replication in the Formation of an Atherosclerotic Lesion

The role of intimal replication in atherosclerosis is confusing. Is intimal thickening a result of atherosclerosis or a cause? Does it precede or follow formation of the atherosclerotic lesion? Before answering these questions, it is important to point out a major, practical difference between studies of replication in animals, where it is practical to use labeled analogs of thymidine, either BUdR or [^{3}H]TdR, and studies in humans where this is generally not possible *in vivo*. Figure 2 illustrates the ways replication can be measured in human tissues. [^{3}H]TdR or BUdR can be used *in vitro*. Villaschi used this method with plaque specimens and found very low levels of replica-

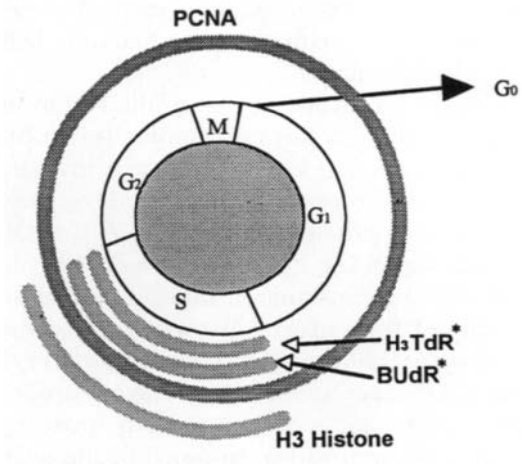

FIG. 2. Cell cycle markers. Replication can be detected either directly, by labeling DNA synthesis with thymidine analogs, or indirectly, by antibodies of in situ hybridization for RNA or proteins that are upregulated during the cell cycle. PCNA is useful because it is a general marker for the cell cycle. Because PCNA is not linked to a specific phase of the cell cycle, this method may provide overestimates of the frequency of replicating cells. In contrast, H3 histone mRNA, detected by in situ hybridization, is only known to be elevated during DNA synthesis. Therefore, H3 may be a marker comparable to pulse labeling with [^{3}H]TdR or BUdR.

tion, less than a percent. This method has one major source of error: the tissue *in vitro* is likely to be deteriorating during the incubation and the labeled material may not penetrate to a cell of interest until that cell has died. Thus one might expect *in vitro* incubation studies to underestimate the levels of replication(141). The first alternative procedure used in the vessel wall was staining for "proliferating cell nuclear antigen" (PCNA). This is a bit confusing since PCNA will stain cells that are generally replicating instead of identifying cells in a specific part of the cell cycle. Thus, PCNA tends to overestimate levels of replication. Nonetheless, studies of plaque show low levels of PCNA. The one confusing exception is a report(142) of high levels of PCNA in primary lesions sampled by atherectomy. The reasons for these high values are unclear, but the simplest possibility is that PCNA is easily overstained. The best control is a piece of gut or tonsil since these tissues have been independently studied with labeled analogs and we know what to expect. When this was done, two laboratories found low levels of replication, under about 1%(28,143). Finally, a new and appealing cell cycle marker is histone H3. The RNA for this protein is present at extremely high levels during S and only during S phase. Thus *in situ* hybridization for H3 may be a very good substitute for use of labeled analogs in human tissue. We have confirmed the PCNA studies of primary atherosclerotic lesions using H3 (Shoval *et al.*, unpublished data).

Of course, one might not expect to see proliferation in advanced lesions. Fatty streaks, however, form at many sites in humans as well as in other fat-fed animals(144–146). Many investigators regard these as early lesions and one can see higher levels of replication in fat-fed animals(147). Nonetheless, when PCNA was used to look at human fatty streaks, again the PCNA levels were very low(143).

The absence of evidence for smooth muscle replication needs to be considered in terms of the natural history of the atherosclerotic lesion. We have already noted that the monoclonality of lesions implies that proliferation must occur at some time during lesion development. The obvious question is not whether smooth muscle cells proliferate—proliferation must occur since so much of the lesion is made up of smooth muscle cells. A better question would be when smooth muscle replication occurs. Stary *et al.*(146) state that intimal thickening is not the early lesion of atherosclerosis because intimal thickening is not lipid rich and is a normal developmental change at specific vascular sites in our species. These authors do note, however, that lipid accumulation can occur deep in the wall, within the preexisting intimal mass. This sequence of events, with a localization and initiation of atherosclerosis, is reminiscent of experiments in balloon-injured ani-

mals in which lesions develop selectively at sites of previous injury and intimal formation(52). Thus, the initiation of atherosclerosis, or at least the form of atherosclerosis that leads to classical lesions in humans, may begin in the "soil" of the intima. The peculiar properties of the intima that lead to such a phenomenon have not been identified. However, there have been suggestions that endothelial regrowth and specific glycosaminoglycans may promote local lipid accumulation(148,149).

More important to our present discussion, Stary's description raises the possibility that most of the replication required to form the plaque may occur in the soil, i.e., the intima, that exists prior to lipid accumulation. The time course of human intimal smooth muscle proliferation is not known. Ross has suggested that proliferation of smooth muscle cells is important in advanced lesions(27,150). As just discussed, this idea is not supported by cell kinetic studies measuring either the frequency of cells able to incorporate labeled thymidine *ex vivo* or the number of cells identified as replicative on the basis of staining for a cell cycle-specific marker(28,151). Thus, monoclonality implies that smooth muscle proliferation must occur during the formation of the lesion and that the initial group of cells giving rise to the lesion must be very small. Such an early expansion of intimal smooth muscle mass has been described to occur in the proximal anterior descending coronary artery (LAD), a common site for occlusive coronary artery lesions in adults(16,19). If the increase in mass described in the newborn LAD is correlated with smooth muscle replication, then very few further doublings may be required to account for the mass of smooth muscle seen in an adult lesion. The low replicative rates seen in advanced lesions correlate with studies of cells cultured from lesions. Moss and Benditt(152) were the first to culture these cells. They found that plaque smooth muscle cells have a greatly shortened life span relative to normal medial cells. This observation has been reproduced by others(153,154). Indeed, we have shown that plaque smooth muscle cells have an accelerated rate of programmed cell death *in vitro*. If this happens *in vivo* as well, then even higher rates of replication would be required to explain increases in plaque mass in late events (Bennett and Schwartz, unpublished data).

It is important, however, to put these measures of replication into perspective. First, very few doublings would be required to convert the mass of the normal intima into the mass of an occlusive lesion. Such doublings might occur at rare intervals, intervals too rare to detect by the random samplings we now have. These doublings might even occur in bursts, consistent with angiographic studies that

demonstrate rapid growth, or at least loss of lumen, at sites that are initially relatively normal(155). Finally, we also need to consider the proliferation of cells other than smooth muscle cells. A study from this laboratory shows focal levels of endothelial replication as high as 10%(156). This may be evidence that angiogenesis is a very active and chronic process adding to plaque mass. Finally, while most studies have focused attention on the smooth muscle cell, Gordon *et al.*(28) found more impressive evidence for macrophage replication than for smooth muscle replication.

In summary, the available direct evidence suggests that smooth muscle replication occurs very early in the formation of atherosclerotic lesions(16,19). It is also likely that monoclonality precedes or occurs early in lesion formation. Unfortunately, testing this hypothesis would require novel methods for serial study of clonality at a histological level in human tissue. We also cannot rule out the possibility that smooth muscle replication occurs at a very low rate over several years or that replication occurs at a high rate in an episodic fashion.

VII. How Does the Intima Contribute to Atherosclerosis?

Regardless of when the intima is formed, special properties of the atherosclerotic intima are essential for atherogenesis since this process either only occurs in the presence of an intima or results in the formation of an intima as an intrinsic part of atherogenesis. Atherosclerotic lesions are progressive, and it seems obvious that progression must depend on unique properties either of the intima or of the plaque itself(29). Table I represents an attempt to collate those properties with special regard to genes that are underexpressed or overexpressed by the intimal or plaque smooth muscle cell relative to medial smooth muscle cells. The table emphasizes rat, rabbit, and human data, the species about which the most is known. In addition, the table includes data from cultured smooth muscle cells since the preservation of properties *in vitro* identifies intimal smooth muscle cells that may be differently differentiated from medial smooth muscle cells. The table points out one critical issue: intima and media are quite different tissues. Thus, the pharmacology of vessels with an intima, especially an atherosclerotic intima, is likely to be very different from the pharmacology of the normal vessel wall.

The differences between intima and medial smooth muscle cells could represent two different kinds of phenomena: intimal cells might, as already suggested, be a distinct subtype of smooth muscle cell or the phenotype of the intimal cells could represent an adaptation of

these cells to the conditions in the intima, especially to the inflammatory milieu. The latter hypothesis is a variation on the concept of modulation already discussed. In this view, plaque smooth muscle cells overexpress or repress certain molecules as a result of mediators present in the plaque environment. Particularly important among such mediators are oxidation products or more traditional inflammatory mediators(39,157). Collins(158) has suggested that a common factor linking inflammation and oxidation is the role of NF-κB as a *trans*-acting factor induced by oxidized radicals and by many cytokines. Table I includes plaque-specific genes whose overexpression may be explained as an inflammatory response. In some cases, however, this may be misleading since overexpression in the plaque is permanent, whereas overexpression of cytokines is often only transient.

As already discussed, smooth muscle cells do not have to lose their phenotype to replicate. Nonetheless, it is certainly true that the phenotype of smooth muscle cells in the intima is very different from the phenotype of medial smooth muscle cells. As already discussed, loss of expression of genes associated with the fully differentiated, "contractile" phenotype was first described by the Campbells and has been confirmed by many investigators(15,159–161). The Campbells have given the change from a "contractile" to a "synthetic" phenotype the name "modulation." The term "synthetic" is used because cultured cells lose much of their microfilaments and, as seen by transmission electron microscopy, acquire an extensive rough endoplasmic reticulum. Molecules lost in modulated cells include smooth muscle myosin, desmin, and caldesmon(10,162). Unfortunately, the term synthetic has been loosely applied to this lack of smooth muscle phenotype because intimal cells as well as many cells in the media may lack the contractile phenotype without showing evidence of an active synthetic apparatus(159; Gordon and Schwartz, unpublished data). Thus, the equation of synthetic phenotype with proliferative cells seen in cell culture may not apply very well to the quiescent but modulated cells seen *in vivo* inside plaques. Similarly, the media of avian aortae contain a second cell type which, while apparently quiescent, lacks morphological features of the contractile smooth muscle cell(38,163,164).

VIII. Intimal Cells Overexpress Certain Genes *in Vitro* as well as *in Vivo*

The second possible explanation for intimal unique gene expression is that the intimal smooth muscle cell is actually a different cell type. This hypothesis is supported by observations that, in addition to be-

ing "modulated," i.e., lacking characteristics of other smooth muscle cells, intimal cells have properties of their own and overexpress certain genes even when there is no activation by injury or atherosclerosis. In humans, the intima overexpresses the B1 chain of laminin and the ED-A splice form of fibronectin(165,166). In the rat, a large number of genes are overexpressed after injury, but so far only tenascin has been shown to be overexpressed in the few intimal cells seen in the normal rat artery(167,168).

Most importantly, at least in rat smooth muscle cells, many of these properties are retained *in vitro*, implying that expression is due to some property of the cells themselves rather than the environment of the injured vessel wall. Smooth muscle cell lines cultured from 2-week-old rat pups have several unique properties. These include an epitheloid morphology, the ability to grow without platelet-released growth factors, and secretion of PDGF(169,170). These same properties appear when neointimal cells, isolated 2 weeks after arterial injury, are placed in culture(171). When the same cells were studied for gene expression, a group of genes was found that showed overexpression or even unique expression in pup and intimal cells as compared with normal medial cells cultured from adult arteries(169,172,173). This collection of properties and genes led us to suggest that the vessel wall, at least in the rat, contains two types of cells: π (pup-intimal) vs μ (medial-unmanipulated) cells. Genes overexpressed by π cells include the PDGF-B chain, CYPIA1 (cytochrome p450a), elastin, tenascin, and osteopontin(169,172,173; Sharifi, unpublished data).

Unlike the distinction between contractile and synthetic phenotypes, which is lost when all cells put in culture become synthetic, the π phenotype seems to be maintained in passage, and cells with π or μ properties can be isolated by cloning cells from mixed cultures(174,175).

Moreover, some of these genes, although first identified *in vitro*, are also overexpressed or uniquely expressed in the neointima *in vivo*. At least for the rat, then, we suggest that smooth muscle cells contain two distinct "cell types"—one of which takes a special part in the formation of the neointima. Comparable evidence has not yet been discovered for subsets in human smooth muscle cells. However, as already noted, human intima preserves certain genes that are also seen in the fetal vessel(10). Moreover, if atherosclerotic lesions are monoclonal, the origin of lesions in the intima suggests that lesions are derived from a unique subset of vessel wall smooth muscle cells localized to this layer(22).

IX. Plaque-Specific Gene Expression by Smooth Muscle Cells

Whether or not intimal smooth muscle cells belong to a uniquely differentiated subset, it is clear that smooth muscle cells in human plaques overexpress a large number of genes that are generally not seen in the normal wall and that these properties likely account for many features of plaque progression. Some of the more interesting genes shown to be overexpressed in plaques are discussed next.

A. Tissue Factor

The normal vessel wall contains little or no tissue factor at a level demonstrable by *in situ* hybridization or immunocytochemistry(176). High levels of tissue factor seen in plaque may promote coagulation, a factor that is likely important to both plaque progression and the final morbid outcome(176,177). The time course for the appearance of tissue factor in plaques is not known; however, Davies and others(178–180) have suggested that thrombosis is a critical event in plaque progression, and it is likely that accumulation of this molecule is critical to the morbidity of lesions.

B. PDGF Chains

The PDGF-A chain is found in the plaque and, as already discussed, it is not a potent smooth muscle mitogen *in vitro*. Nonetheless, antibodies to this mitogen will inhibit replication induced by other molecules, including TGF-β and bFGF(102–104,181). Interestingly, PDGF-A chain synthesis is induced in cultured cells by a number of molecules likely to be present in advanced plaques, including thrombin, angiotensin, and phenylephrine(182–184). *In vivo*, PDGF mRNA levels may be lowered by antagonists to thrombin(182) or phenylephrine(83).

The PDGF-B chain is also seen in plaque macrophage and endothelial cells, although from *in situ* hybridization studies the latter cells are probably the major source of synthesis(27,102). The failure to detect the PDGF-B chain in plaque smooth muscle, however, may be misleading. Studies of rat neointima show that PDGF-B chain mRNA is confined to about 10% of the most superficial cells in the neointima (Lindner, Giachelli, Reidy, and Schwartz, unpublished data). Such a low percentage would be difficult to detect if a similar low frequency were confined to a portion of primary or restenotic human lesions.

C. MCSF/GMCSF

The presence of these leukocyte growth factors, as well as receptors for leukocyte factors (see Table I), is of special importance because of growing evidence that plaque macrophages, rather than plaque smooth muscle cells, may comprise the only unique proliferative element in the plaque(28,151). The unique properties of the proliferative plaque macrophage have yet to be explored.

D. Osteopontin and Bone Morphogenic Protein

Osteopontin and bone morphogenetic protein 2a have both been found in smooth muscle cells of atherosclerotic plaques(185,186). The presence of these molecules is supportive of the hypothesis that plaques are derived from unique subsets of smooth muscle cells because osteopontin was first found in neointimal cells of the rat pup artery as part of the π phenotype(173). Bone morphogenetic protein 2a, on the other hand, is also seen in plaques. Intriguingly, cells found to express bone morphogenetic protein 2a also express immunocytochemical markers associated with a special form of smooth muscle cell, the pericytes seen around small vessels, again suggesting a unique lineage for plaque smooth muscle as compared with medial smooth muscle(186). The association of osteopontin and bone morphogenetic protein 2a with areas of calcification in the plaque also suggests that the mechanisms of bone mineralization may also play a role in vessel wall calcification.

E. Constitutive Nitric Oxide (NO) Synthase

An excellent example of the difference between a vessel with only a media and one with an atherosclerotic intima is the issue of vascular contractility. Normally the vessel wall exists in a relaxed state due to the endogenous production of NO by endothelium. NO-dependent relaxation, however, is greatly impaired in atherosclerotic vessels(187). This loss of NO function has been attributed to endothelial injury or to the inactivation of NO by free radicals produced in the plaque(188). In contrast, Joly and colleagues(189) described the appearance of the inducible form of NO synthase following balloon injury. Similarly, as described earlier, the neointima overexpresses angiotensin receptors and the plaque has been shown to overexpress endothelin(190). While to this point we have not distinguished effects of atherosclerosis from those of the more general appearance of the neointima, our point is simply that we might expect the vasomotor activity of a diseased artery with an intima to be very different from vasomotor regulation in a normal artery.

The contribution of this altered pharmacology to the ability of the vessel wall to maintain a normal lumen caliber is largely unexplored.

X. Mechanism of Lumen Occlusion in Atherosclerosis

Until this point, this chapter has focused on the formation and properties of the arterial intima. We have assumed that increased intimal mass occludes the lumen.

The experimental data supporting the assumption that intimal mass obstructs the lumen of large vessels is unclear. For example, Glagov *et al.*(191) noted that human vessels can undergo massive accumulations of atherosclerotic mass without lumen narrowing. The vessel wall compensates for the new mass by dilating to permit a normal level of blood flow until an adaptational limit is exceeded. This limit appears to occur when approximately 40% or more of the area bounded by the internal elastic lamina is occupied by intimal mass. The compensatory dilation is a structural change in the vessel wall and is usually called "remodeling." Decreased lumen size probably does not occur until Glagov's limit is exceeded and may depend on pharmacological mechanisms associated with the neointima or the plaque that cause a failure of normal remodeling events.

Remodeling is a normal response that allows the vessel to maintain normal levels of blood flow and wall stress, and can be seen in small muscular arteries as well as in large elastic arteries(192,193). Branching patterns of conduit vessels seem to be genetically determined(194). Thus, the only way arteries can respond to a demand for an increase or a decrease in blood flow is by changing vessel caliber. These changes rely on rearrangement of existing vessel wall components rather than on synthesis of new mass or cell replication(195). Until recently, the concept of vessel wall remodeling as a part of the response to injury has been largely ignored in animal models of arterial injury and repair. The extent of remodeling versus loss of lumen due to intimal formation is illustrated by an important experiment by Jamal and coworkers(193). When Jamal and colleagues studied the balloon-injured rabbit carotid artery, they found no narrowing of the lumen despite a 100% increase in wall thickness. The same study, however, showed a significant (14%) narrowing in response to an experimental restriction of flow in the vicinity of the thyroid artery where endothelium had regenerated. This effect of the endothelium in an injured vessel is consistent with observations that structural adaptation to changes in flow requires an endothelium(193). Since reendothelialization generally correlates with a diminution of intimal thickening, these data may

even imply a negative correlation of intimal mass with luminal narrowing(196). The role of the endothelium in controlling lumen size may be especially important given the often neglected fact that plaques have a prominent microcirculation composed of capillaries arising from the adventitia(197–199). The role of these small vessels in regulating structural change or, for that matter, contractile properties of the atherosclerotic vessel remains unexplored.

If the correlation of intimal (or plaque) mass with lumen caliber is not a simple one, how do we account for angiographic changes seen after aggressive lipid-lowering therapy(200–202)? The actual degrees of improvement of stenosis diameter in these studies is small (e.g., 0.7–5.3%, or an increase of 0.003 to 0.117 mm in minimum absolute diameter). To put these results in perspective, one should note that 6 months after percutaneous coronary angioplasty, there is an average improvement of 16% diameter stenosis units and a 0.47-mm increase in minimum absolute diameter(203). It is critical to realize that angiographic studies provide no insight into the mechanisms that may account for loss of lumen caliber. Thus, it is possible that apparent changes in mass reflect changes in adherent thrombotic material or the state of vasospasm rather than in the extent of lumen narrowing due to the accumulation of lipid and necrotic material in the atherosclerotic intima(204).

These angiographic regression studies showed only a modest change in lumen caliber compared to the beneficial change in clinical events(200,202). This raises the intriguing possibility that lipid-lowering therapies may improve clinical outcome by stabilizing the lesion rather than by altering the lumen. Davies and others(178–180) have suggested that the formation of fissures in plaques is the critical step leading to vascular occlusion. They propose that fissuring results from a combination of biochemical events that weaken the fibrous cap over lesions and may include the expression of certain proteases(205). Another group has added to this concept by proposing that the accumulation of tumor necrosis factor-α may lead to necrotic changes that result in plaque rupture(206). While Arbustini and colleagues(206) were unable to find a clear correlation of fissuring with acute ischemic heart disease, and Davies *et al.*(179,207) found a high incidence of fissuring in atherosclerotic "control" arteries of patients dying of noncoronary events, the control patients may have had undetected myocardial infarctions and these fissures may simply represent the sequelae of advanced atherosclerosis. Davies' hypothesis would imply that we may be able to develop diagnostic tests, based on plaque composition as assessed by magnetic resonance imaging (MRI), intravascular ultrasound (IVUS), or even gene expression pat-

terns in atherectomy specimens, that might indicate lesion prognosis or the effectiveness of drugs targeted at stabilizing the lesion.

It is important to emphasize the potential value of new modalities for imaging human coronary artery lesions (e.g., MRI or IVUS) in testing the impact that plaque fissuring and subsequent thrombosis may have on eventual lumen diameter. Animal models with rare and spontaneous occlusive atherosclerotic arterial diseases, even with fat feeding, are poorly characterized. Human data, as of now, however, are confusing. As just discussed, it is difficult to define a control population if one depends on autopsy material. Serial angiographic studies in humans, moreover, suggest that the majority of myocardial infarctions may occur due to thrombotic occlusion of arteries that previously did not contain significant stenoses (e.g., < 50%)(208,209). However, postmortem studies do not bear this out. For example, Qiao and colleagues(210,211) have reported that in both native coronary arteries and saphenous venous bypass grafts, atherosclerotic plaque rupture with thrombosis most commonly occurred at sites with severe narrowing (e.g., >90% area stenosis). Possibly, this is just another example of the inability of angiography to estimate the extent of atherosclerotic disease.

XI. The Nature of Restenosis Following Angioplasty

This section discusses the loss of arterial caliber seen following angioplasty, i.e., "restenosis." Figure 1 shows the "proliferative" tissue that characterizes coronary artery restenosis as seen in atherectomy specimens or at autopsy(212). The first step is to compare the course of events in the animal model with the course of events following angioplasty in humans. It is important to emphasize that the first three waves of replication in the rat model represent an acute response of a normal vessel forming a neointima. It may be entirely incorrect to assume that a comparable pattern of cell kinetics occurs following angioplasty of human lesions. As discussed earlier, we have used immunocytochemical labeling for PCNA to determine the proliferative profile of atherosclerotic lesions and found only very low levels of smooth muscle replication. We have used the same method to study 100 restenotic coronary atherectomy specimens(213). To our surprise, the vast majority of the restenotic specimens (74%) had no evidence of PCNA labeling. Moreover, in those specimens with proliferation, only a modest number of PCNA-positive cells were present per slide (typically <50 cells per slide). PCNA labeling was detected over a wide time interval after the initial procedure (e.g., 1 day to 390 days), with no obvious proliferative peak. There were no differences in the proliferative profiles of restenotic specimens collected in the first 3 months,

4–6 months, 7–9 months, or >9 months after the initial interventional procedure (Spearman rank correlation coefficient = 0.081, $P = 0.43$). Furthermore, only 12 of 30 specimens obtained within 60 days of the initial coronary interventional procedure had one or more PCNA-positive nucleus per slide (including 9 specimens collected within 6 days of the initial procedure, only 3 of which had immunolabeling of 1, 7, and 20 cells per slide). In support of these findings, a preliminary study using *in vitro* bromodeoxyuridine labeling also found low levels of proliferation in restenotic atherectomy specimens(214). Similarly, Strauss *et al.*(215) found no PCNA-positive cells in atherectomy specimens from seven restenotic stented coronary artery lesions.

As already discussed, these results and those of Strauss *et al.*(215) contrast with those of Pickering *et al.*(142). In their study, both primary and restenotic coronary and peripheral arterial specimens had surprisingly high percentages of cells that were considered PCNA positive (e.g., mean ± SD: 15.2 ± 13.6% and 20.6 ± 18.2% by immunoctychemistry and *in situ* hybridization, respectively). However, only 4 of the 19 restenotic atherectomy specimens were obtained from coronary artery lesions and none were obtained within 1 month of the initial interventional procedure (e.g., 1.6, 5.2, 6.1, and 7.9 months). Overall, the labeling indices reported by Pickering *et al.*(142) seem exceptionally high (e.g., as high as 59% of cells being PCNA positive) and resemble those of malignant neoplasms(216). It is important, however, to realize that PCNA is not a direct measure of replication (Fig. 2). Thus, the detection of differences in the frequency of PCNA-positive cells in the Pickering study should raise concerns that differences between primary and restenotic tissue in Pickering's study could reflect changes specific to peripheral arterial lesions, a tissue not included in our study.

These data on replication force us to reconsider the relevance of animal models to the human problem. This issue is illustrated by Fig. 3. Almost all animal studies, particularly those performed in the rat, measure a decrease in lumen size from the initially normal situation. This can rightly be called stenosis. In contrast, the clinical problem is defined by the extent of dilatation that is lost after an atherosclerotic vessel has been dilated to achieve what the interventional cardiologist believes is an optimal diameter. The loss of this optimal diameter, rather than the preexisting vessel caliber, is rightly called restenosis (e.g., a popular clinical definition of restenosis is >50% loss of initial luminal diameter gain). By current angiographic criteria, even a return of the human vessel to its predilation diameter would be defined as restenosis, despite no change in intimal mass. Thus, stenosis, as seen in most animal models of arterial injury, is very different from

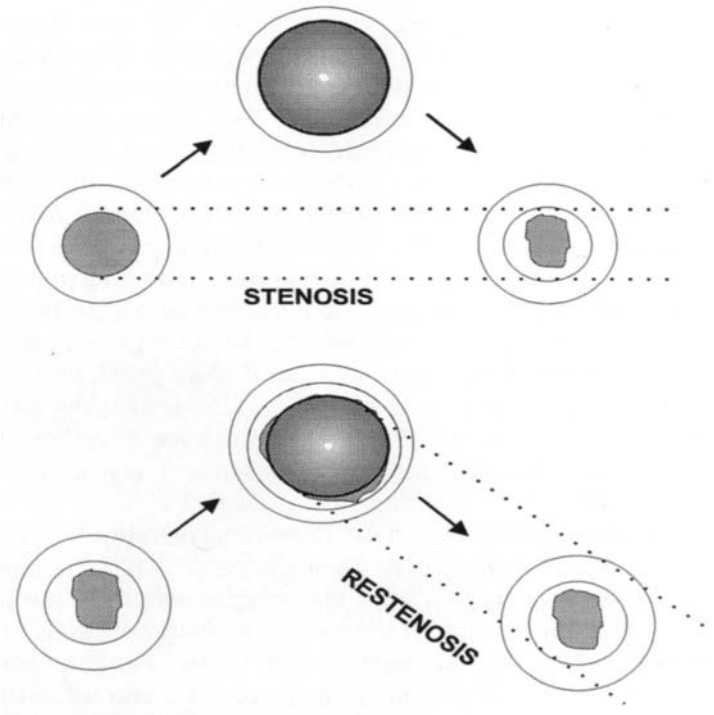

FIG. 3. (Top) Most animal models describe the formation of a neointima in a normal artery, i.e., the creation of a stenosis. (Bottom) Human coronary arteries that are treated by angioplasty already have severe atherosclerotic disease and a preexisting stenosis. Clinical angioplasty produces a larger lumen; however, in approximately 30–50% of these lesions, restenosis develops as the arterial lumen renarrows.

the clinical process called restenosis and may not be useful in predicting the result of restenosis therapies. This statement is especially true of studies that equate intimal hyperplasia with restenosis. The relationship of intimal mass to loss of lumen caliber is unclear even within animal models. For example, luminal narrowing after injury to the rat carotid artery is more pronounced after 2 weeks (75%) than after 12 weeks (35%), implying that early stenosis may be due to smooth muscle contraction of the vessel. Loss of lumen caliber could depend more on the extent of remodeling of the vessel wall to compensate for a change in mass than on the intimal mass itself(191). Therefore, it may be more appropriate to define stenosis as a decrease in lumen size from the caliber existing prior to angioplasty. Unfortunately, such an incremental change in diameter is difficult to measure with existing quantitative angiography.

Using these definitions, we would consider the response of the rat carotid artery as stenosis. To the best of our knowledge, angiographic studies in humans do not show that angioplasty produces stenoses similar to those seen in animal models. This, of course, would be difficult to demonstrate angiographically as the initial lesion usually already has a critical stenosis. Furthermore, animal models, perhaps by design, require that all manipulated arteries show narrowing, i.e., stenosis, as shown in Fig. 3. There is no animal model in which 50% (or any significant percentage) of manipulated arteries remain dilated beyond their initial, unmanipulated caliber; yet this 50% rate (or higher) is the success rate seen when atherosclerotic human arteries are dilated with an angioplasty balloon. Perhaps, with more than 50% of human arteries remaining free of restenosis months after angioplasty, we may want to ask whether we have a sufficient animal model that allows us to explain why angioplasty is ever successful in the first place.

Only recently have larger animal models been developed that take remodeling into account after angioplasty(217–220). Remodeling of blood vessels, however, has been discussed at length in the hypertensive microvasculature and in the response of larger vessels to changes in blood flow(195,221). Of particular interest, Langille and O'Donnell(221) showed that the initial response of a carotid artery to reduced flow is active vasospasm. After 2 weeks, however, this active process is replaced by a fixed remodeling that cannot be reversed by vasorelaxants. Finally, as already discussed earlier, Langille and O'Donnell(221) found no loss of lumen despite a 100% increase in wall mass due to intimal thickening.

Another way of thinking about this problem is to ask whether restenosis is the result of an increase in wall mass due to intimal hyperplasia, remodeling of the vessel wall to reestablish its preangioplasty caliber, or a combination of these processes. Our ability to evaluate human vessels is changing because of new technologies; in particular, the use of IVUS to image the affected wall and atherectomy to biopsy the same tissue. Previous data, based on histologic and imaging studies, suggest that plaque compression, disruption with fracture and dissection of the intima and media, and stretching of the more normal portions of the media are involved in creating a bigger lumen(222–229). However, newer concepts are emerging. For example, Losordo *et al.*(230) used IVUS to study 40 patients immediately before and after iliac artery angioplasty. The areas of the arterial wall, plaque, lumen, and neolumen resulting from the procedure were examined. Over 70% of the increase in luminal area immediately postangioplasty was contained within the plaque fracture (the

so-called neolumen). The plaque cross-sectional area decreased by approximately one-third, but the total artery cross-sectional area increased only minimally (approximately 5%) with the dilatation. Thus, the major effects of angioplasty may be to redistribute the components of the wall. Conversely, loss of lumen, i.e., restenosis, might be due to healing of the fissure rather than formation of new mass.

The emphasis on neointimal formation as the cause of restenosis is not simply by analogy to animal models. The original studies on the pathology of human restenosis emphasized a histologic change called intimal hyperplasia. For example, Nobuyoshi *et al.*(212) examined 39 dilated lesions from the postmortem coronary arteries of 20 patients who had undergone angioplasty. The extent of "intimal proliferation" was defined by the histologic appearance of stellate, fibroblast-like cells with a myxomatous appearance (Fig. 1). It is essential to note that the term "proliferative" as used here is a morphological term, not a measure of replication such as thymidine index or mitotic frequency. Cells having this proliferative morphology are not unique to restenosis. Similar cells are commonly seen beneath the endothelium in areas of nonatherosclerotic intimal thickening as well as in primary atherosclerotic coronary artery lesions that have never been exposed to an interventional device(231,232). While the myxomatous tissue seen in atherosclerotic or restenotic lesions does not appear to be actively proliferative, most observers agree that there is an increase in the amount of this tissue in restenotic vs primary lesions(28,213,231–234).

We do not know whether the increase in the amount of proliferative tissue represents a redistribution of the components of lesions due to compression by the catheter or some reaction such as formation of extracellular matrix, migration, or cell proliferation at a low level not measured by current methods. Postmortem examination of stented coronary arteries supports the idea that this is not simply a redistribution of preexisting plaque components, yet to date has failed to show evidence of replication(215). Perhaps the wires push into the wall or wall components migrate around the wires.

Preliminary intracoronary ultrasound studies suggest that increases in plaque area with restenosis following angioplasty are actually small (e.g., 5–7%)(235). The clinical significance of this small increase in plaque mass in an artery that is already severely diseased is unknown. The authors speculate that intimal hyperplasia may not be a dominant factor in the restenotic lesion, and that instead, "chronic recoil" or remodeling may account for approximately 60% of late lumen loss(236). This concept of chronic recoil is similar to the phenomenon of vascular remodeling or to the wound-healing model discussed earlier.

In summary, the available data do not demonstrate that cell proliferation is a major component of coronary restenosis. If this is true, antiproliferative approaches to therapy with the elegant use of molecular biology to inhibit growth may be irrelevant(131). It is intriguing to note that a study in the swine stent model actually showed an increase in restenosis when an injured wall was irradiated to prevent cell proliferation(237). As ultrasound technology improves, it will be possible to test these hypotheses based on serial measurements of changes in vessel wall mass. The kinetics of those changes may be useful in estimating the expected rate of cell replication in tissues undergoing restenosis.

XII. Future Direction

Before ending this chapter, it is important to state clearly that smooth muscle proliferation does occur, although perhaps not as dramatically as had been suggested by data based on studies *in vitro*. Moreover, while we have concentrated on the proliferation associated with atherosclerosis and restenosis, there is much better evidence for proliferation in hypertensive vascular injury(142,143,238–243), transplant atherosclerosis(244), AV shunt grafts(245) and, of course, in animal models of angioplasty(246,247). While the human data on angioplasty seem inconsistent, it is important to realize that the human tissue is complex, the lesions are much more advanced than those we usually see in animals, and the atherectomy is a relatively inefficient biopsy tool for such a complex lesion.

Whatever the case for angioplasty and replication, these other forms of smooth muscle replication are important in their own right, but only angioplasty has been explored at a mechanistic, molecular level. Even here, most of the mechanistic data are in the rat, a much simpler vessel than the human coronary artery. The rat lesion, for example, typically does not involve macrophage or fibrin formation. These processes might result in very different proliferative mechanisms in angioplasty responses in larger animals including pigs, monkeys, and humans. Similarly, it is much too early to conclude that the molecules now identified with the various waves of the angioblast response are the same molecules responsible for the proliferative response seen in hypertension or transplantation atherosclerosis.

Hypertensive replication is a special issue. There is clear evidence that large vessels of hypertensive animals have an elevated DNA content. It is tempting to think that this increase in DNA could serve as an enlarged template for protein synthesis, accounting for the structural changes described by Folkow and others(192,248). This hypothe-

sis is supported by embryologic studies in genetically hypertensive rats(249), showing that an increase in vascular mass is present even *in utero*. On the other hand, stereologic studies of resistance arteries, the microvessels responsible for maintaining pressure, have failed to find evidence that the microvessels are hyperplastic(248). If this is true, then the relevance of smooth muscle replication to the etiology of critical structural changes in hypertension would be unclear. At least in one form of hypertension, angiotensin infusion, we see clear evidence of elevated PCNA frequencies, suggesting that replication does occur, but the increase in cell number is either compensated for by cell death or is too small to be measured by the stereologic approach (Wiener *et al.*, unpublished data).

Finally, this chapter has attempted to distinguish between proliferation and phenotypic changes. While we have been pessimistic about simplistic equations of intimal proliferation with loss of lumen in atherosclerosis or restenosis, this should not in any way diminish the obvious fact that the pathology of both of these clinical problems depends on the special properties of the intima. In atherosclerosis it is the intima that accumulates fat, becomes calcified, expresses tissue factor, and ultimately breaks down, leading to occlusive vascular disease. Restenosis, of course, would not be a problem if we did not need to treat atherosclerosis. Moreover, any mechanism likely to account for restenosis is probably going to depend on new tissue formed after injury. This new tissue is important and may be the result of replication, migration, or extracellular matrix accumulation. Furthermore, this new tissue may contribute to lumen narrowing by occupying space, causing tissue contraction, or geometric remodeling of the vessel wall.

At a basic science level, we need to know three things. First, we need to know much more about the generality of the molecular mechanisms underlying proliferation and migration in forming a neointima. Do these mechanisms apply to other forms of smooth muscle response to injury? Second, even in the rat angioplasty model, we do not know why the intima remains hyperproliferative for so many months after injury.

Third, the question of the hyperproliferative property of the neointima is presumably part of the larger question of why the intima is different from the media. As already discussed earlier, two possibilities exist. Intimal smooth muscle cells may belong to different lineages, just as different skeletal muscle cell phenotypes appear during differentiation of that cell type. Definitions of "lineage" are rapidly changing because of the identification of genes and 5′ sequences that determine the expression of cell type-specific proteins. Such determi-

nation elements have not yet been identified for smooth muscle cells. Presumably, the identification of such elements will lead to a much better definition of smooth muscle at a molecular level and, therefore, to an understanding of why intimal cells lose the usual patterns of gene expression seen by classical medial smooth muscle cells. Based on our data showing two distinct lineages in rat smooth muscle, we would like to imagine that similar mechanisms may eventually distinguish human intimal cells from medial smooth muscle cells.

Alternatively, we have discussed the role of the inflammatory process in maintaining distinct phenotypes in smooth muscle cells within the atherosclerotic intima. Like any chronic inflammatory tissue, the intima of an atherosclerotic plaque is extremely confusing. Diagrams purporting to explain behavior of the intima based on these mediators are complex and unappealing because it is difficult to identify individual critical processes or molecules. It would be intriguing, however, to see if this complexity might not be simplified by identifying a critical inflammatory agent or eliciting antigen among the products of oxidation, lipid accumulation, necrosis, and coagulation. Such a critical molecule would offer an ideal therapeutic target to inhibit lesion progression. Oxidation products are especially interesting given evidence that antioxidants, independent of lipid levels, can cause lesion regression or failure of progression(250). Apo(a) and related components of coagulation are also intriguing candidates, especially given the surprising observation that human Apo(a) transgenic mice develop lipid deposition and atherosclerotic lesions despite lack of evidence that this apoprotein interacts with lipid or alters serum lipids(251).

Finally, theories of both atherosclerosis and restenosis place smooth muscle replication in a central role. These hypotheses simplistically assume that an increase in mass of the intima is the cause of loss of lumen caliber. We know that this is not simply true, but we lack a more comprehensive hypothesis. Terms like "late loss," "chronic recoil," or "remodeling" simply put names on poorly understood processes. Concepts of plaque enlargement by mural thrombosis or breakdown of plaque with critical fissuring offer a tantalizing prospect of molecular targets for therapy that again depend on our knowing why these events occur in the progressing plaque. One can guess where advances are likely to occur. Better, preferably noninvasive, imaging methods are needed to give us more precise definitions of the clinical problems. This, in turn, will hopefully lead to the development of better animal models. In turn, tissue from humans and from better animal models should help us learn how to control the expression of the molecules that lead to plaque narrowing and ultimately death.

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Smooth Muscle Gene Expression during Developmental Maturation

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I. Introduction

The basic process of blood vessel development is not well understood. It is known that vessel formation is initiated by the aggregation of endothelial precursors (angioblasts) as well as by the invasion of endothelial cells into existing tissue (reviewed in Dieterien-Lievre and Pardanaud, 1993). Subsequently, there is recruitment of cells from the surrounding mesenchyme and likely from the cardiac neural crest that give rise to smooth muscle cells (SMCs) (Manasek, 1971; LeLievre and Ledouarin, 1975). In medium and large vessels, there is continued proliferation of SMCs, organization of these cells into distinct layers separated by elastic laminae, and eventual cessation of growth (Karrer, 1960; Nakamura, 1988). This last phase of blood vessel development, which we have termed "developmental maturation," defines the transition of SMCs from an immature (high proliferative, high biosynthetic, and noncontractile) to a mature (low proliferative, low biosynthetic, and contractile) phenotype. This phase of blood vessel development spans the interval from late prenatal to early postnatal. Research interests in the developmental maturation of SMCs arise from the morphological and biochemical similarities that have

been noted between developmentally immature SMCs and lesional SMCs in the adult animal (Gabbiani *et al.*, 1984; Kocher *et al.*, 1985; Glukhova *et al.*, 1990; Kuro-o *et al.*, 1989, 1991; Majesky *et al.*, 1992; Aikawa *et al.*, 1993; Sartore *et al.*, 1994). These similarities have led to the suggestion that SMCs in atherosclerosis and in hypertension may recapitulate certain aspects of earlier developmental events (Kocher *et al.*, 1985; Schwartz *et al.*, 1986). To permit a critical analysis of this possibility, it is crucial that we first obtain a better understanding of the events associated with the transition of fetal SMCs to a developmentally mature phenotype. Additionally, this complex and poorly understood developmental process is critical for the establishment of a functional blood vessel and is of interest in its own right. This chapter describes studies that utilize molecular screening to identify genes that are expressed in the immature prenatal smooth muscle but not in the adult smooth muscle. The regulation and possible function of these developmentally regulated genes are examined and the utility of cultured SMCs to study developmental maturation is discussed.

II. Mammalian Prenatal Blood Vessel Development

The most comprehensive, quantitative histological analysis of mammalian prenatal smooth muscle development has been performed by Nakamura (1988). He examined blood vessel development of the rat thoracic aorta from gestational days 12 to 21. At phase I (gestational day 12), the dorsal aorta consists of a single layer of endothelial cells loosely surrounded by mesenchymal cells. At phase II (gestational days 13–16), multiple layers of compact SMCs are observed surrounding the endothelium and some of these cells begin to acquire small clusters of myofilaments with dense bodies, rough endoplasmic reticulum, and a discontinuous basal lamina. SMCs adjacent to the endothelium are generally the first to acquire myofilament bundles and initiate elastogenesis. At phase III (gestational days 17–19), there are five to eight layers of SMCs and the three major divisions of the vessel wall are clearly distinguishable: intima, media, and adventitia. At phase IV (gestational day 20 until parturition), elastic laminae are present in several inner layers of the media, and SMCs appear as contractile cells with massive bundles of myofilaments anchored in dense bodies.

We have been studying prenatal blood vessel development in the rabbit which has a gestational period of 30 days and the duration from days 19 to 30 is roughly equivalent to gestational days 13–21 in the rat. Proliferating cell nuclear antigen (PCNA) staining of aorta

from gestational days 20 to 29 and neonatal animals was performed to determine a rough index of SMC replication in this tissue. At gestational day 20, there were approximately three to four layers of SMCs with 65% of the cells being PCNA positive and the percentage of PCNA-positive cells remained similar up to day 25. However, the percentage of PCNA-positive cells was reduced to 35% by gestational day 29 and approximately 10% of the cells were positive in newborn animals. The reduction in SMC proliferation was associated with an increase in vessel wall elastic fiber content and organization (Fig. 1). At gestational day 20, only weakly stained, diffuse elastic fibers were present in the aorta. However, by gestational day 29, the elastic fiber content of the vessel wall was greatly increased and became more organized. These results clearly illustrate that concurrent with a high rate of SMC proliferation there is a dramatic reorganization of the blood vessel wall between gestational day 20 and birth in the rabbit.

Overall, the pattern of prenatal blood vessel development in the rabbit is very similar to that previously described for the rat and the mouse (Nakamura, 1988; Davis, 1993). From these and other studies, several generalizations regarding mammalian prenatal blood vessel maturation can be made. First, smooth muscle maturation as defined by the formation of myofilament bundles, dense bodies, and a basal lamina as well as elastogenesis is clearly evident prenatally. This implies that the signal(s) necessary to initiate and propagate the process that will ultimately result in a fully mature SMC is present early during fetal blood vessel development. Second, there does not appear to be a simple inverse relationship between fetal SMC proliferation and maturation; these events appear to occur independently. This is supported by the observation that the process of smooth muscle maturation occurs throughout the entire period of blood vessel wall formation, including the early phases where an extremely high rate of cell division is observed (Nakamura, 1988; Cook *et al.*, 1994; D. K. M. Han and G. Liau, unpublished observations). Moreover, in the rat, the mouse, and the rabbit, smooth muscle maturation apparently initiates in the medial layers adjacent to the lumen of the blood vessel (Nakamura, 1988; Davis, 1993; Giuriato *et al.*, 1992). If there is a inverse relationship between cellular proliferation and maturation, one would expect to see a greater number of proliferating SMCs in the outer layers of the blood vessel as prenatal development progresses. Yet such a localization was not observed in the rat aorta (Cook *et al.*, 1994) or in the rabbit aorta (D. K. M. Han and G. Liau, unpublished observations). These results support the possibility that independent signals may be involved in the regulation of SMC proliferation versus SMC maturation. For example, cell proliferation in the fetal blood

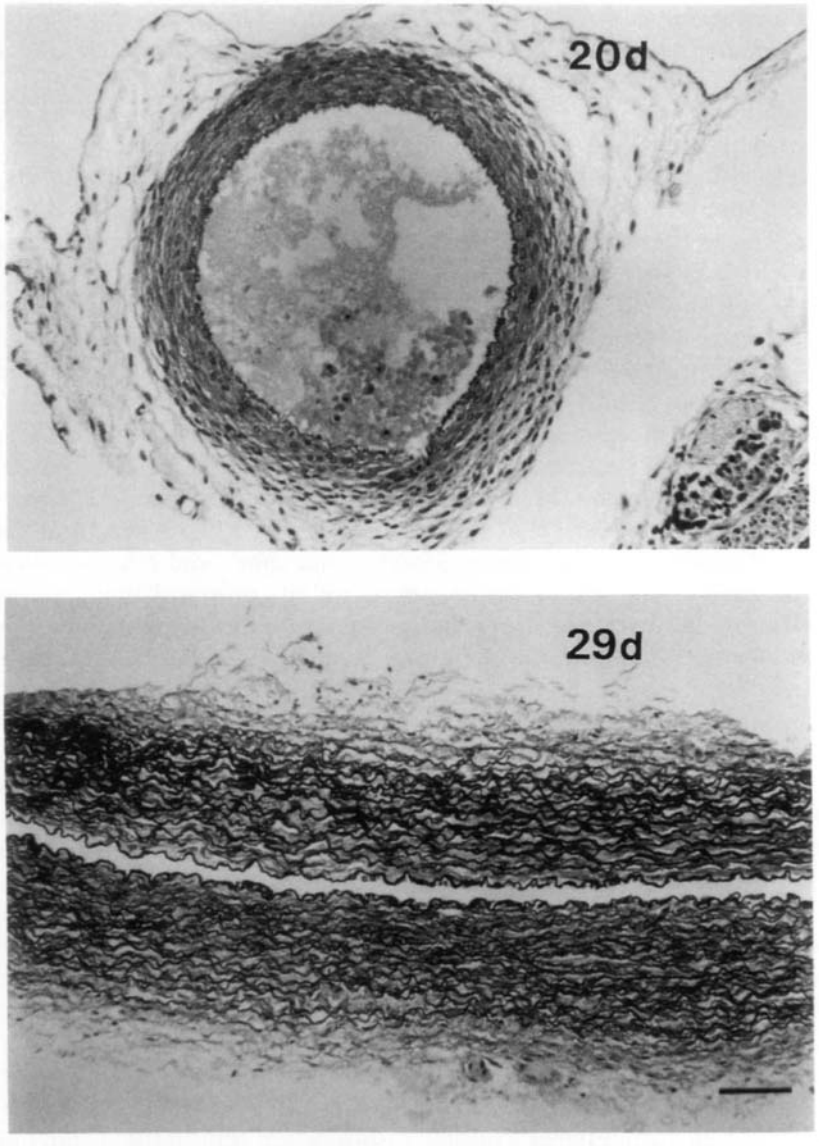

FIG. 1. Verhoeff's elastic fiber staining of the developing aorta. Paraffin-embedded aortic rings from gestational day 20 to 29 animals were stained by Verhoeff's technique which stains elastin dark-blue to black. Bar = 15 μm.

vessel may be dependent on the concentration of a local trophic factor(s) that becomes insufficient to support additional rounds of cell division or is regulated by endogenously produced inhibitors such as heparan sulfate proteoglycans that limit further cell division. In contrast, maturation signals may be initiated by mechanical stresses such as an increase in blood pressure or flow that is somehow transduced directly to the inner SMCs or via the endothelium.

III. Isolation and Characterization of Developmentally Regulated Genes

The isolation and characterization of genes specifically downregulated during the developmental maturation of vascular smooth muscle may provide additional insights into this complex process. We used differential cDNA screening to attempt to identify such genes. A schematic representation of this strategy is shown in Fig. 2. Differential screening involves the construction of cDNA libraries from two distinct sources and the subsequent identification of cDNAs encoding mRNAs that are in higher abundance in one source. This is accom-

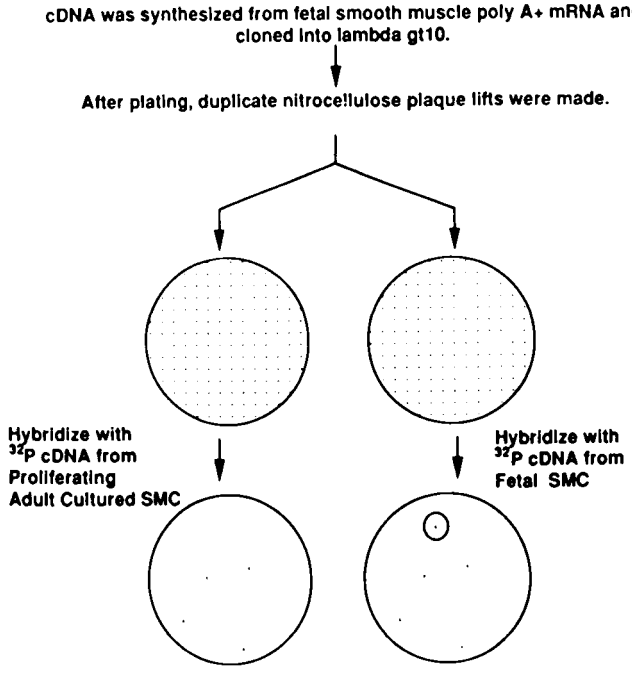

FIG. 2. Schematic representation of the differential library screening protocol to identify cDNAs expressed higher in fetal vs adult smooth muscle.

plished by hybridization of the cDNA library of interest with ^{32}P-labeled cDNA probes generated from the two sources. However, one potential problem with such an approach is that since a major difference between these two SMC populations is their proliferative state, it is likely that many of the known SMC proliferation-inducible genes such as c-*fos*, c-*myc*, and thrombospondin will be identified by this technique (Janat and Liau, 1992). We, therefore, chose a strategy that involved initial comparison of fetal aortic smooth muscle with proliferating cultured adult SMCs and subsequent confirmation that the identified clones were not expressed *in vivo* in adult smooth muscle (Han and Liau, 1992). By this method we isolated a 405-bp cDNA (F-31) that hybridized to a 2.3-kb mRNA present in fetal smooth muscle but not in the adult (Han and Liau, 1992). In addition, we also identified the insulin-like growth factor-II (IGF-II) gene as being preferentially expressed in fetal smooth muscle (Han and Liau, 1992). The mRNA expression of F-31, IGF-II, and actin in fetal, newborn, and adult aortic smooth muscle is illustrated in Fig. 3. Expression of F-31 was high in gestational day 25 fetal smooth muscle, diminished in newborn tissue, and not detectable in the smooth muscle of 4-week-old animals. In contrast, the expression of IGF-II mRNA was detectable only in fetal smooth muscle whereas the total actin mRNA level was unchanged. These results demonstrated that IGF-II and F-31 are both developmentally regulated genes in rabbit aortic smooth muscle.

The initial 405-bp cDNA fragment of F-31 was used to isolate two larger, overlapping F-31 cDNA fragments of 1.2 and 1.8 kb and these were sequenced (Han and Liau, 1992). Comparison with the GenBank database revealed that there was approximately a 70% identity with

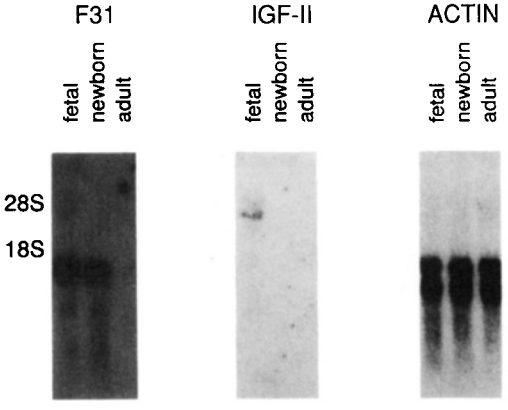

FIG. 3. Analysis of *in vivo* mRNA levels of F-31, IGF-II, and actin in rabbit vascular smooth muscle. Rabbit aortic RNA isolated from fetal (gestational day 25), newborn, and adult (4-week-old) animals was analyzed by Northern blotting.

the sequence of a developmentally regulated gene called H19 (Pachnis *et al.*, 1988; Brannan *et al.*, 1990). F-31 may be the rabbit homolog of the mouse and human H19 gene or it is possible that F-31 may encode a related gene. To distinguish between these possibilities, we first compared the mRNA expression of F-31 in the rabbit with that reported for H19 in the mouse (Han and Liau, 1992; Poirier *et al.*, 1991; Pachnis *et al.*, 1984). We found that the mRNA size, developmental expression pattern, and general tissue distribution of F-31 and H19 are similar. However, some discrepancies exist in the expression of F-31 and H19 mRNA in the adult. In particular, expression of F-31 mRNA was fourfold more prominent in the adult rabbit lung than in the skeletal muscle and was detectable in the brain but not in the heart (Han and Liau, 1992). This was in contrast to that reported for H19 where expression was detected in the adult mouse heart but not in the brain and the H19 mRNA level was considerably higher in the skeletal muscle than in the lung (Poirier *et al.*, 1991). Interestingly, there is an additional discrepancy between two studies on the mouse with one report indicating that the expression of H19 in the adult liver was equal to or greater than that in the heart (Pachnis *et al.*, 1984), whereas a second report was unable to detect H19 expression in the adult mouse liver (Poirier *et al.*, 1991). We were also unable to detect H19 expression in the rabbit adult liver. This comparative analysis was inconclusive and left open the possibility that two related genes may exist.

To attempt to resolve the relationship between F-31 and H19, we used a 3-kb genomic DNA fragment that encompassed the entire mouse H19 gene (kindly provided by Dr. S. Tilghman, Princeton University, NJ) to analyze RNA expression in rabbit tissues. This DNA probe was unable to identify the H19 RNA in rabbit tissues under high stringency Northern hybridization conditions. However, under less stringent hybridization (40% formamide, 37°C) and wash conditions (0.2× SSC, 50°C), it hybridized to a RNA transcript of 2.3 kb in rabbit tissues that is similar in size to F-31 (Fig. 4). Analysis of the respective RNAs in rabbit placenta and in a variety of fetal tissues including skeletal muscle, esophagus, lung, liver, and heart revealed that the relative content of the F-31 RNA and the presumed H19 RNA in these tissues were quite similar (Fig. 4 and results not shown). Additional analysis of RNA samples obtained from mouse, human, and rat placenta indicated that both the rabbit and the mouse DNA probes were able to detect a 2.3-kb transcript in RNA samples derived from mouse and rat placenta (Fig. 4). Interestingly, under these conditions, the F-31 cDNA probe also detected RNA transcripts of 4 and 7 kb in mouse, human, and rat placenta RNA whereas

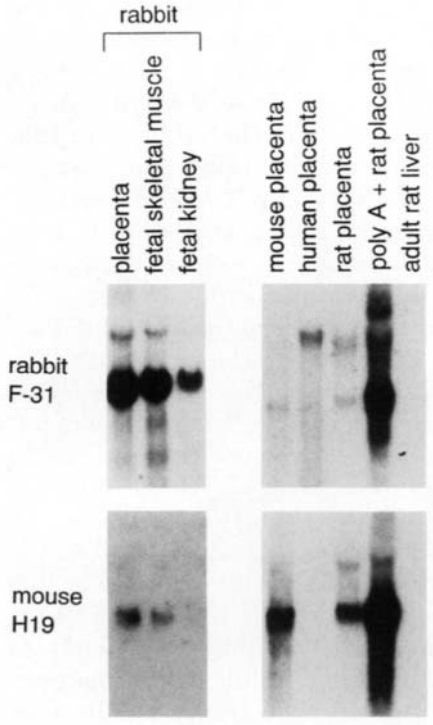

FIG. 4. Comparative Northern blotting analysis using rabbit F-31 and mouse H19 DNA probes under low stringency hybridization conditions.

the mouse H19 DNA probe was much less sensitive in the detection of these larger transcripts (Fig. 4). Cross-species Southern blotting experiments to determine if F-31 and H19 recognized the same genomic DNA fragments in the rat were unsuccessful. However, Southern blotting analysis of rabbit genomic DNA revealed that *Eco*R1-digested genomic DNA contained a single 11-kb DNA fragment that hybridized to the 1.8-kb F-31 cDNA probe. These studies indicate that F-31 is most likely the rabbit homolog of the mouse and human H19 gene.

Because of the poor sensitivity in cross-hybridization experiments, we were unable to directly address the discrepancy in the expression of F-31 in the adult rabbit tissues versus H19 expression in the adult mouse tissues. It is possible that differential expression of F-31/H19 in adult tissues may be due to species difference or difficulties in accurately assessing the low level of this RNA. However, it is our hypothesis that F-31/H19 expression in adult tissue is likely due to the presence of undifferentiated blast cells and that the most likely explanation for the observed discrepancy is a result of limited sampling. This last possibility can reasonably account for the difference in H19

expression between the two mouse studies just described (Pachnis *et al.*, 1984; Poirier *et al.*, 1991). Thus, a variable number of blast cells in the analyzed tissue or an altered activity state of these blast cells within the tissue can explain the observed difference in the level of F-31/H19 RNA.

IV. F-31/H19 and Blood Vessel Developmental Maturation

The H19 gene was first isolated as a gene expressed in fetal but not in adult mouse liver (Pachnis *et al.*, 1984). H19 expression is also induced during embryonic stem cell differentiation and C3H10T1/2 conversion to myoblasts (Pachnis *et al.*, 1988; Poirier *et al.*, 1991; Davis *et al.*, 1987). Although the function of H19 is currently unknown, two studies provide direct evidence that H19 has an important role during development and in cell proliferation. First, it was found that the introduction of excess copies of the H19 gene in mice resulted in a very low frequency of transgenic progeny and that these animals did not express the introduced H19 gene (Brunkow and Tilghman, 1991). Analysis of embryos during various gestational times revealed that animals expressing the transgene were present only up to gestational day 14 and subsequently were resorbed. This dominant lethality was due to overexpression of intact H19 since deletion of a 900-bp internal fragment of the H19 gene allowed expression of the transgene at high levels in neonates (Brunkow and Tilghman, 1991). These results strongly suggest that the H19 gene has a crucial role during development. Second, based on genetic evidence that a human tumor-suppressor gene is present on chromosome 11p15.5, several tumor cell lines were transfected with an H19 expression vector (Hao *et al.*, 1993). It was found that overexpression of H19 resulted in growth retardation and morphological changes in two independent embryonal tumor cell lines. Furthermore, in-depth analysis of one cell line revealed that H19 overexpression abrogated the ability of these cells to exhibit anchorage-independent growth and form tumors *in vivo* (Hao *et al.*, 1993). Based on its critical function during development, induction during *in vitro* differentiation, tumor-suppressor activity in embryonal tumor cell lines, and developmentally regulated expression in aortic smooth muscle, we postulate that F-31/H19 may have an important role in the developmental maturation of SMCs.

We have examined the developmental expression of F-31/H19 in the rabbit aorta by *in situ* hybridization. Preliminary evidence indicated that during prenatal blood vessel development, F-31/H19 was expressed by all cells of the aortic wall (D. K. M. Han and G. Liau, unpublished observations). However, with increased gestational age, the

decrease in F-31/H19 expression was not uniform; SMCs proximal to the lumen tend to be the first cells to exhibit a decreased F-31/H19 mRNA level (D. K. M. Han and G. Liau, unpublished observations). This spatial gradient of F-31/H19 expression during development is interesting since as discussed earlier, by morphological criteria, smooth muscle maturation is initiated in the layers proximal to the lumen in both the rat and the mouse (Nakamura, 1988; Davis, 1993). Although a careful morphological analysis of prenatal blood vessel development in the rabbit has not been reported, there is evidence to support a similar lumenal to ablumenal progression of smooth muscle maturation. Giuriato *et al.* (1992) analyzed myosin isoforms in the developing rabbit vessel and found that in gestational day 19 rabbit fetuses, smooth muscle myosin heavy chain 1 (SM-MHC-1) was particularly evident in the region proximal to the lumen of the aortic wall whereas the entire wall had become SM-MHC-1 positive in gestational day 29 animals. These results suggest that there is likely a similar lumenal to ablumenal progression of smooth muscle developmental maturation in all three mammalian developmental models. Interestingly, developmental maturation in the chick is apparently initiated in the deeper layers of the media and gradually proceeds toward the lumen (Takagi, 1969; Kadar *et al.*, 1971). Whether this represents a distinct difference in smooth muscle development in the chick is presently unclear.

Our data indicate that loss of F-31/H19 expression during rabbit aortic wall development is temporally and may be spatially related to SMC maturation. Taken together with the critical role this gene has in development and in the growth of some tumor cell lines (Brunkow and Tilghman, 1991; Hao *et al.*, 1993), a better understanding of the function of this gene could provide new insight into the process of SMC maturation. The function and, indeed, the nature of the H19 gene product is somewhat obscure. Although the mouse H19 gene is transcribed by polymerase II and exhibits classical properties of a translated mRNA such as RNA splicing and polyadenylation, it is not associated with polyribosomes in fetal and neonatal mouse liver or in C3H10T1/2 cells (Pachnis *et al.*, 1988; Brannan *et al.*, 1990). We analyzed the distribution of the rabbit F-31/H19 RNA in poly(A)$^+$ and poly(A)$^-$ RNA fractions derived from the rabbit placenta and found that it was likewise enriched in the poly(A)$^+$ fraction (Fig. 5). In addition, we have examined the theoretical translational open reading frames of the rabbit F-31/H19 sequence and compared it with that present in the human and mouse H19 sequences (Han and Liau, 1992). Four putative AUG start sites found in rabbit F-31/H19 were also present in human and mouse H19 and the largest of these could

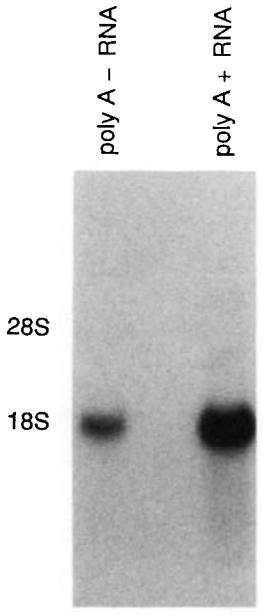

FIG. 5. Analysis of the distribution of F-31 RNA in poly(A)$^+$ and poly(A)$^-$ fractions of total placenta RNA.

encode a 121 amino acid protein in the rabbit. However, interspecies comparison of the putative translated products indicated that except for the region immediately adjacent to the four AUGs, the protein sequences were disparate (Han and Liau, 1992). Consistent with the lack of a conserved open reading frame, *in vitro*-transcribed rabbit F-31/H19 RNA was unable to support the synthesis of translation products in rabbit reticulocyte lysate or in wheat germ translation systems. Pachnis *et al.* (1988) used a similar strategy to generate a 14-kDa polypeptide from a truncated mouse H19 cDNA. However, antibodies directed against this theoretical peptide were unable to identify a protein product in cells expressing a high level of the H19 RNA directed from the endogenous gene or the transfected gene (Brannan *et al.*, 1990). The poor level of interspecies conservation is rather convincingly illustrated when a multiple alignment is performed at the nucleotide level among rabbit, mouse, and human H19 (Fig. 6). Although there is approximately a 70% sequence identity between any two of the three H19 sequences, when all three sequences are compared, the level of sequence identity decreased to 47%. Generally, small stretches of highly homologous sequences of approximately 50–100 nucleotides are dispersed within a background of very poorly conserved sequences. One explanation for this type of homology is

FIG. 6. Comparison of the DNA sequence of human and mouse H19 and rabbit F-31/H19. The consensus sequence is also indicated and reflects identity in all three positions.

```
            1121                                                                1200
rabbit {h19} gggagctgtg cccgcc.gcc acgggaggcc cggacgcca. ....ggtgca ggcgagccga gggacatgac atggtccggt
 human {h19} ggtggacgtg cccaccagct gccgaaggcc aagacgccag gtccggtgga cgtgacaagc aggacatgac atggtccggt
 mouse {h19} ggtggatgtg cctgccagtc actgaaggcg aggatgacag gtgtggtcaa tgtga.caga aagacatgac atggtccggt
   Consensus GG--G--GTG CC--CC-G-- -C-G-AGGC- --GA-G-CA- ----GGT--A -G-GA---G- --GACATGAC ATGGTCCGGT

            1201                                                                1280
rabbit {h19} gtgacggtga ggacagaggg gctggcgtgc ggccttccag aacatcctcg gggggcaccg cgggggagct gccatcagga
 human {h19} gtgacggcga ggacagagga g.gcgcgtcc ggccttcctg aacaccttag g........c tggtggggct gcggcaagaa
 mouse {h19} gtgatggaga ggacagaagg gcagtcatcc agccttcttg aacaccat.. .......... ..gtggcgcc ttgtcgtaga
   Consensus GTGA-GG-GA GGACAGA-G- G----C-T-C -GCCTTC--G AACA-C-T-- ---------- --G-GG-GC- ---------A

            1281                                                                1360
rabbit {h19} gcgagtctct gtccctccg tcgcccaagg agctgacaca cacacacata cccactactg ctgctctctg gacc......
 human {h19} gcgggtctgt .ttctttact tcctccacgg agtcggca.. .......... ..cactatgg ctgccctctg ggct......
 mouse {h19} agccgtctgt .tctttcact tttcccaaag agctaaca.. .......... ..cttcactg ctgctctctg gatcctcctc
   Consensus ----GTCT-T -T---T--C- T---CCA--G AG----CA-- ---------- --C---A--G CTGC-CTCTG G---------

            1361                                                                1440
rabbit {h19} ......cttg gaactctca. .gagaaagaa atggtgctgc ccagctcaag tctgggccct cgaacccggg gact..cgtg
 human {h19} ......ccca gaacccacaa catgaaagaa atggtgctac ccagctcaag cctgggcctt tgaatccgga cac......a
 mouse {h19} cccctacctt gaaccctcaa gatgaaagaa atggtcctac ccagctgatg tctgggcctt tgaatccggg gacttcttta
   Consensus ------C--- GAAC-C-CA- ---GAAAGAA ATGGT-CT-C CCAGCT-A-G -CTGGGCC-T -GAA-CCGG- -AC-------

            1441                                                                1520
rabbit {h19} agtccgtcct gttgtgaatc aagaagactt tgc.ctttgg aacctcgaca cagccttgct caggagtcag ctc.ccaggt
 human {h19} aaaccctcta gcttggaa.. atgaatatgc tgcactttac aaccactgca ctacctgact caggaatcgg ctctggaagt
 mouse {h19} agtccgtctc gttctgaatc aagaagatgc tgca.atcag aaccactaca ctacctgcct ca.gaatctg ctc.caaggt
   Consensus A--CC-TC-- G-T--GAA-- A-GAA-A--- TGC---T--- AACC-C--CA C--CCT--CT CA-GA-TC-G CTC---A-GT

            1521                                                                1600
rabbit {h19} gacgcccaag gaaggagacc tc....gccc a....cactg ccatcagcag cagcagcaac gcccccaccc ctcgccc...
 human {h19} gaagctagag gaaccagacc tcatcagccc aacatcaaag acaccatc.g gaacagcagc gcccgcagca cccaccccgc
 mouse {h19} gaagctgaaa gaacagatgg t......... .........g tcaaattttg aaagagcag. actcatagca cccaccc.ac
   Consensus GA-GC---A- GAA------- T--------- ---------G -CA------G -A--AGCA-- -C-C--A-C- C-C-CCC---

            1601                                                                1680
rabbit {h19} .cctgccact ccacctgc.. gcacgcaccc tgcggcatcc gggtg..... .......... ...ctcccgg agctggg..c
 human {h19} accggcgact ccatcttcat ggccaccccc tgcggtggac ggttgaccac cagccaccac atcatcccag agctgagctc
 mouse {h19} ccctgagaat ccatcttcat ggccaactct gccctgaccc gggagaccac ca...cccac atcatcctgg agccaagcct
   Consensus -CC-G--A-T CCA-CT-C-- G--C----C- --C------C GG--G----- ---------- ----TCC--G AGC---G---

            1681                                                                1760
rabbit {h19} cctccgcggg gtg.....gc ccccaccgcc tccctcc..t ttctttcttc ttc..tctct ctctttctct ctctgcctga
 human {h19} ctccagcggg atgacgccgt ccccaccacc tccctct..t cttctttttc atccttctgt ctctttgttt ctgagctttc
 mouse {h19} ctaccccggg atga...... cttcatcatc tccctcctgt ctttttcttc ttcctccttt cctgtaattc tgtttctttc
   Consensus C--C--CGGG -TG------- C--CA-C--C TCCCTC---T -T--TT-TTC -TC---CT-T C---T--T-- -----C-T--

            1761                                                                1840
rabbit {h19} gtgtttgccc tcccattgcc tgagagactc ggagca.ccc ctgactccg. .cccccatc ccctccacgt ttattttgca
 human {h19} ctgt...ctt tcctttttte tgagagattc aaagcc.tcc acgactctgt ttccccctc ccttctgaat ttaatttgca
 mouse {h19} cttttggttc cttccttgct tgagagactc aaagaagccc gtgactctgt ttccccattt acc.ccctttt tgaatttgca
   Consensus -T-T------ -----TT--- TGAGAGA-TC --AG----CC --GACTC-G- --CCCC--T- -C--C----T T-A-TTTGCA

            1841                                                                1920
rabbit {h19} ctaagtcgtc ggcactggtc tggagt..cg gcggcggccc tgag..tctc .........t c.....gcgc gtgtgtgcga
 human {h19} ctaagtcatt tgcactggt. tggagttgtg gagacggcct tgag..tctc agtacgagtg t.....gcgt gagtgtgagc
 mouse {h19} ctaagtcgat tgcactggtt tggagtcccg gagatagctt tgagtctctc cgtatgaatg tatacagcga gtgtgtaaac
   Consensus CTAAGTC--- -GCACTGGT- TGGAGT---G G-G---GC-- TGAG--TCTC ---------- ------GCG- G-GTGT----

            1921                                                                2000
rabbit {h19} gccctggcc aagcccggcc ccggcacctg cctatccgcc catctctgtc ..cggggtcc tgggcgctgg c.gtgtgtct
 human {h19} caccttggca agtgcctgtg cagggcccgg cc..gccctc catct.gggc ..cgggtgac tgggcgccgg ctgtgtgccc
 mouse {h19} ctctttggca atg..ctgcc ccagtaccca cct.gtcgtc catctccgtc tgagggcaac tgggtg.tgg ccgtgtgctt
   Consensus --C-TTGGC- A----C-G-- C--G--CC-- CC----C--C CATCT--G-C ---GGG---C TGGG-G--GG C-GTGTG---

            2001                                                                2080
rabbit {h19} gaggcctcgc cc.cacctcg cctggcctgg gtgctccggc cca....gcg gagctgcctt ..agaagacc cacaaggccc
 human {h19} gaggcctcac cctgccctcg cctagtctgg aagctccgac cgacatcacg gagcagcctt caagcattcc attacgcccc
 mouse {h19} gaggcctcgc cttcccctcg cctagtctgg aag..... ........ca gttccatcat aaag.tgttc aacatgccct
   Consensus GAGGCCTC-C C----CCTCG CCT-G-CTGG --G------- --------C- G--C---C-T --AG-----C ---A-G-CC-

            2081                                                                2160
rabbit {h19} ctctcgccct gtgcccctcc ccaccagggc cccagcagga gccctgg... cctcatcaat aaacacagtt ccggc.....
 human {h19} atctcgctct gtgcccctcc ccaccagggc ttcagcagga gccctggact catcatcaat aaacactgtt ac........
 mouse {h19} acttcatcct ttgcccctcc tcaccagggc ctcaccagag gtcctggtc. ...catcaat aaatacagtt acagtcattg
   Consensus ---TC---CT -TGCCCCTCC -CACCAGGGC --CA-CAG-- G-CCTGG--- ---CATCAAT AAA-AC-GTT -C--------
```

that they may reflect secondary folding constraints of the H19 RNA molecule.

The results, taken together, suggest that the bioactive H19 molecule is not a translated protein but may be the RNA itself. However, it still remains a formal possibility that H19 RNA does encode a polypeptide product and that this protein is only translated under a very specific set of circumstances. A functional open reading frame can conceivably be generated by frameshift translation (Jacks and Varmus, 1985) or by RNA editing (Powell *et al.*, 1987). It is also possible that translation of one of the small conserved open reading frames is sufficient for the biological activity of H19 (Fini *et al.*, 1989; Han and Liau, 1992). How might the H19 RNA function during development? A number of other RNAs that are spliced and polyadenylated but apparently not translated have been described (Calzone *et al.*, 1988; Brown *et al.*,, 1992; Brockdorff *et al.*, 1992). One of these, the Xist gene, is located in the nucleus and is believed to be involved in maintaining X chromosome inactivation (Brown *et al.*, 1992; Brockdorff *et al.*, 1992). Since the H19 RNA has been localized to a 28S cytoplasmic particle (Brannan *et al.*, 1990), it likely does not function directly in the nucleus. Given that it is expressed at a very high level globally during development, it is difficult to imagine that it acts on a specific target such as that described for naturally occurring antisense RNAs against fibroblast growth factor (Kimelman and Kirschner, 1989), or c-*erb*A (Munroe and Lazar, 1991) unless such a target has a global developmental role. Identification of the other molecules within the 28S H19-associated cytoplasmic particle should provide additional insight into this enigmatic RNA molecule.

V. IGF-II and Blood Vessel Developmental Maturation

IGF-II is expressed in many fetal tissues and is especially prominent in cells of mesenchymal origin (Brown *et al.*, 1986; Han *et al.*, 1987; Beck *et al.*, 1987). Demonstration that IGF-II has an important role in fetal growth is provided by transgenic studies in which mice carrying only one allele of the functional wild-type IGF-II exhibited a 10-fold decrease in IGF-II mRNA expression and a 40% decrease in animal size (DeChiara *et al.*, 1990). The developmental regulation of IGF-II expression in vascular SMCs has not been examined previously. We have shown that *in vivo*, fetal rabbit vascular SMCs expressed an IGF-II mRNA transcript of 4.5 kb and that the level of this mRNA decreased dramatically at birth (Han and Liau, 1992). Since IGF-II is generally believed to act locally, our results are consistent

with the possibility that IGF-II may have some autocrine role in the proliferation of fetal vascular SMCs. Whether IGF-II may also have a role in SMC maturation is currently unclear. However, it is interesting to note that there is evidence to support an autocrine role for IGF-II in skeletal muscle differentiation (Florini *et al.*, 1991).

A curious relationship exists between H19 and IGF-II in the mammalian system. First, we have established that H19 and IGF-II are similarly regulated in the aorta during development (Han and Liau, 1992). Indeed, global coexpression of H19 and IGF-II during embryogenesis has been noted previously (Lee *et al.*, 1990; Poirier *et al.*, 1991). Second, H19 and IGF-II are two of a small number of genes that are known to be imprinted, i.e., expressed only from the paternal or maternal allele. The IGF-II and H19 genes are closely linked on mouse chromosome 7 and on human chromosome 11, but are imprinted in the opposite direction with H19 being expressed exclusively from the maternal allele (Bartolomei *et al.*, 1993; Razin and Cedar, 1994). It has been proposed that the transcription of these two genes may utilize the same *cis* regulatory elements originally identified as enhancers for the H19 gene (Bartolomei *et al.*, 1993). In this scenario, transcription factor interaction with these tissue-specific enhancers provides the basis for the similar pattern of H19 and IGF-II expression observed during development. A second possibility for the similarity in expression pattern of these two genes is that the product of one gene is able to regulate the expression of the other.

VI. Maintenance of the Developmentally Immature Phenotype *in Vitro*

We have previously demonstrated that in high cell density cultures, fetal SMCs did not acquire the multilayerd "hill and valley" morphology typically associated with cultured adult SMCs, but remained monolayered and retained a spread and flat morphology (Fig. 7) (Han and Liau, 1992). A somewhat similar cobblestoned, monolayer morphology has previously been reported for cultured rat neonatal SMCs as well as for rat adult SMCs derived from the intima of injured vessels (Gordon *et al.*, 1986; Walker *et al.*, 1986). Additionally, cultured rabbit fetal SMCs exhibited a weak mitogenic response to normally potent SMC growth factors such as fibroblast growth factor 1 (D. K. M. Han and G. Liau, unpublished observations). This is consistent with what has been reported for the rat neonatal and fetal SMCs (Hultgardh-Nilsson *et al.*, 1991; Cook *et al.*, 1994). These results indicate that *in vitro*, fetal and neonatal SMCs have morphological and biochemical features that are distinct from cultured adult cells. How-

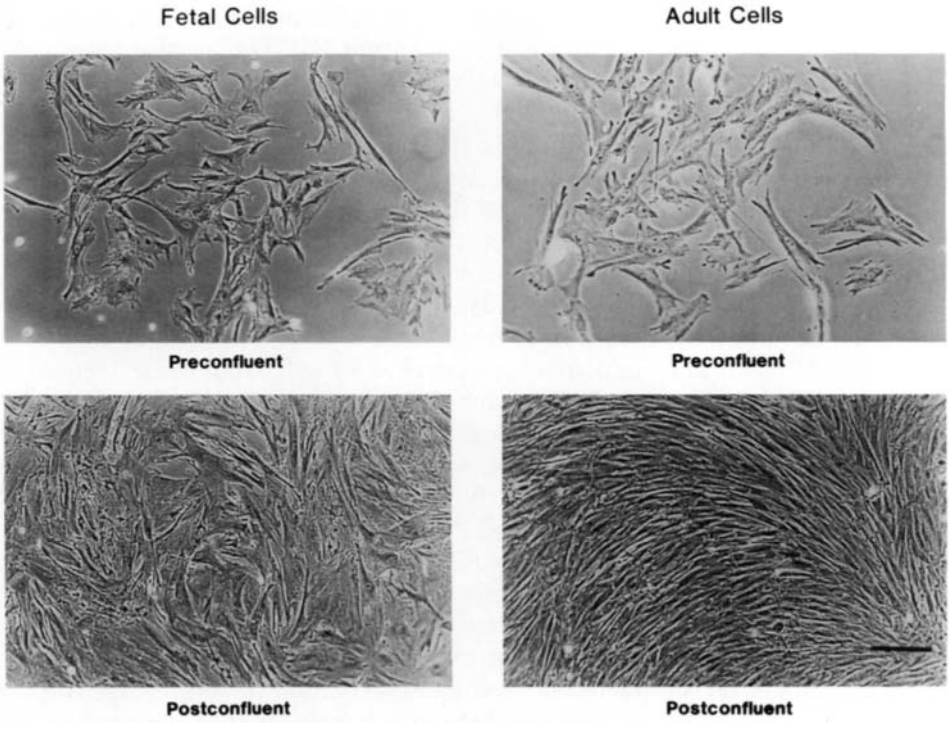

FIG. 7. Morphological comparison of cultured fetal and adult smooth muscle cells.

ever, the relationship between these *in vitro* differences and the *in vivo* developmental process has been unclear.

We have analyzed the expression of F-31/H19 and IGF-II RNA in cultured cells isolated from fetal, newborn, and adult smooth muscle (Han and Liau, 1992). Our analysis revealed that, similar to the *in vivo* situation, F-31/H19 RNA was expressed at an elevated level in both fetal and newborn SMCs but not in the adult cells (Fig. 8). IGF-II mRNA expression in cultured SMCs also faithfully replicated what was observed *in vivo* (Fig. 8). Maintenance of differential expression of these two markers of the developmentally immature phenotype *in vitro* indicates that some of the *in vivo* differences observed during smooth muscle development are maintained in cultured cells. These results strongly suggest that SMCs cultured from different developmental periods can be utilized to gain a better understanding of the process of developmental maturation in the animal.

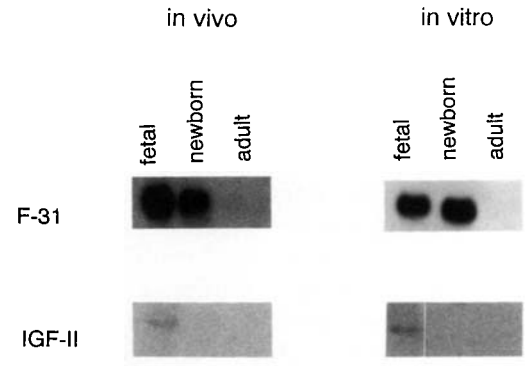

FIG. 8. Comparison of the *in vivo* expression of F-31/H19 and IGF-II RNA in fetal, newborn, and adult aortic smooth muscle with the expression in smooth muscle cells isolated from these tissues.

VII. CONCLUSION AND PERSPECTIVES

The late prenatal phase of mammalian blood vessel development is a complex and poorly understood process that involves dynamic changes in smooth muscle structure and function. We have described this process in terms of cellular maturation events to emphasize two components of this transition. First, that it is a process that occurs over a relatively long period (1–3 weeks in small mammals). Second, that there is not a "synthetic" versus a "contractile" phenotype but rather there is a gradual increase in the amount of morphologically recognizable structures associated with a contractile SMC. During the earlier phases of prenatal development, highly biosynthetic SMCs are observed to also accumulate myofilaments and are elastogenic. A reasonable interpretation of this observation is that these cells are now dedicated toward the synthesis of proteins necessary for the contractile machinery. Therefore, the interesting questions are: What signals initiate this switch in biosynthesis? Is there a relationship between these events and the cessation of cellular proliferation? How is the ongoing biosynthetic process regulated? One approach toward answering some of these questions is by the identification of genes that exhibit dramatic changes in expression during this developmental maturation process. We have identified the F-31/H19 and the IGF-II genes as two that fit this criteria. Although the function of H19 is unknown, it has a critical role in development and has tumor-suppressor activity in embryonal tumor cell lines. Our finding that H19 is developmentally regulated in vascular smooth muscle and that altered H19 expression is likely associated with the developmental maturation of SMCs strongly suggest a functional role for H19 in this process. The IGF-II molecule is likewise an interesting one within the

context of smooth muscle developmental maturation. The availability of IGF-II as well as IGF receptor knockout transgenic mice may provide important clues regarding the possible role of IGFs in blood vessel maturation.

The initial premise that propelled us to identify genes specifically downregulated during the developmental maturation of vascular smooth muscle was that in the injured adult blood vessel, SMCs may recapitulate certain aspects of earlier developmental events. If this scenario is correct, one would expect that F-31/H19 will be reexpressed in the injured adult blood vessel. It has been reported that H19 was reexpressed by intimal SMCs in the injured rat carotid artery (Kim *et al.*, 1994). The data on H19 along with the morphological and biochemical information generated by many other laboratories strongly support the concept that blood vessel development will teach us much about the response of SMCs to blood vessel injury in the adult animal.

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Molecular Identity of Smooth Muscle Cells: Overview

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Cellular differentiation is defined as "the process whereby cells acquire those cell-specific characteristics that distinguish them from other cell types."

The question has been posed elsewhere in this book as to "whether there is such a thing as a smooth muscle cell." The identity of the smooth muscle cell has never been questioned by physiologists or cell biologists. The normal smooth muscle cell is a highly specialized cell, with a well-defined anatomical location, that exhibits unique contractile properties, electrical activities, and agonist responsivity that distinguish it from any other cell type in the body. Consistent with this, the smooth muscle cell has a unique morphology and expresses a repertoire of ion channels, receptors, signal transducing molecules, and contractile proteins that are necessary for its highly specialized form of cell contraction (reviewed in Owens, 1995). This includes smooth muscle isoforms of a variety of contractile proteins that are important for their differentiated functions such as smooth muscle α-actin (Gabbiani *et al.*, 1981; Owens and Thompson, 1986), smooth muscle myosin heavy chains (Rovner *et al.*, 1986a,b), smooth muscle myosin light chains (Hasegawa *et al.*, 1992; Helper *et al.*, 1988), and smooth muscle α-tropomyosin (Bretscher, 1986; Lees-Miller and Helfman, 1991; Majesky and Schwartz, 1990). In addition, differentiated smooth muscle cells also express a number of proteins that are part of the cytoskeleton and/or are purported to be involved in the regulation of contraction, such as calponin (Winder *et al.*, 1991), smooth muscle-22α (Winder *et al.*, 1991), h-caldesmon (Sobue and Sellers, 1991), γ-vinculin (Geiger *et al.*, 1980), (α- and β-) *meta*-vinculin) Geiger *et al.*, 1980; Pardo *et al.*, 1983), and desmin (Gabbiani *et al.*, 1981; Mitchell *et al.*, 1990) which show at least some degree of smooth muscle cell specificity/selectivity (reviewed in Owens, 1995 and elsewhere in this text).

There is thus absolutely no difficulty in identifying the fully differentiated smooth muscle cell and distinguishing it from other cell types *in vivo*. However, it is important to distinguish proteins that are characteristic of a given stage (or state) of smooth muscle cell differentiation/maturation versus proteins that alone can serve as definitive markers for the identification of smooth muscle cell lineages to the exclusion of all other cell types. While it has been the goal of many vascular biologists to identify the latter, at this time no marker strictly meets these criteria for the smooth muscle cell, with the possible exception of smooth muscle myosin heavy chain isoforms, SM-1 and SM-2 (Aikawa *et al.*, 1993; Miano *et al.*, 1995; Rovner *et al.*, 1986a,b) (see the chapter by Periasamy and Nagai). However, as noted in the chapter by Glukhova and Koteliansky, there is evidence that smooth muscle myosin heavy chains can be expressed in non-smooth muscle cells under some pathological conditions (Lazard *et al.*, 1994). As such, clear identification of the smooth muscle cell and assessment of its state of differentiation must rely on multiple criteria, including expression of multiple smooth muscle cell selective proteins, the morphologic and functional characteristics of the cell, and, at least *in vivo*, the anatomical location of the cell.

The ambiguity about smooth muscle cell markers is not unique. Definitive lineage markers have not been identified for many cell types. Moreover, many of the differentiation marker genes that have contributed to our understanding of the molecular basis of the control of skeletal muscle differentiation (see the chapter by Gittenberger-de Groot *et al.*), such as skeletal α-actin, cardiac α-actin, troponins (-T and C), myosin light chain-2, and muscle creatine kinase, are not specific to skeletal muscle (Sartorelli *et al.*, 1993).

The uncertainty regarding the "existence" of the smooth muscle cell derives from two observations. First, abundant evidence is cited in this book and in the literature that the smooth muscle cell can also exist in a multitude of other forms that may bear little resemblance to the highly differentiated cell type found in the medial layer of normal blood vessels (reviewed in Majesky and Schwartz, 1990). Second, as discussed in the chapter by Gittenberger-de Groot *et al.*, it appears that vascular smooth muscle cells have multiple developmental origins, with the ultimate phenotype the cell will manifest being highly dependent on local environmental cues (reviewed in Owens, 1995). These observations have been interpreted by some to mean that the smooth muscle cell is not a distinct lineage in the developmental sense, but rather that a "smooth muscle cell" can evolve from any multipotential mesodermal cell that is exposed to the appropriate local environmental cues necessary for differentiation/maturation of

the smooth muscle cell, whatever those might be. However, several weaknesses undercut the preceding arguments. First, there is not one shred of evidence that differentiated smooth muscle cells can undergo transdifferentiation to other cell types. Second, as discussed in the chapter by Gittenberger-de Groot *et al.*, the embryological origins of vascular smooth muscle cells and, even more importantly, the timing of the inductive event that leads to commitment to the smooth muscle cell lineage have not been determined. As such, we cannot really determine the true developmental origins of smooth muscle cells since we have no way of recognizing the determined, but as yet undifferentiated, smooth muscle cell. An alternative means of addressing this question is to perform careful smooth muscle cell lineage mapping studies. However, such studies have not yet been reported in this important area. Third, the fact that smooth muscle cells within the great vessels of the head and neck are derived from neural crest mesectodermal cells (Hood and Rosenquist, 1992; Lelievre and Ledouarin, 1975), as opposed to ventrolateral plate mesoderm as elsewhere in the body (Lelievre and Ledouarin, 1975), by no means should be construed as evidence that there is not a smooth muscle cell lineage. Note that this exception to the general pattern of origin of the principal cell types in the body is by no means unique to smooth muscle cells, in that in the head, many of the neural crest cells will differentiate into cartilage, bone, and other connective tissues, which elsewhere in the body arise from the mesoderm. Finally, with regard to the issue of local environmental cues playing a key role in the control of smooth muscle cell differentiation as opposed to it being "inherent" to the smooth muscle cell itself, it is a well-established paradigm that cell determination and differentiation of many cell types are highly dependent on local environmental cues and that cell fates (lineages) can be altered to that of the host tissue by transplantation. Thus, the only distinction of the smooth muscle cell is that control of its differentiation/maturation, even in mature organisms, remains highly dependent on environmental cues rather than being relatively fixed and inherent to the cell itself.

Thus, in this author's opinion, it is undeniable that smooth muscle cells can and do exist as a highly unique specialized cell type in normal blood vessels of adult organisms. The key issue is to try to understand the mechanisms underlying the plasticity/multiplicity of smooth muscle cell functons/phenotypes. Why must smooth muscle cells retain a high level of plasticity? What are the key proteins that are required for their differentiated function? What are the key extrinsic signals/local environmental cues that are important for regulating smooth muscle cell differentiation and maturation? What are

the mechanisms that regulate the expression of genes that encode for proteins characteristic of differentiated smooth muscle cells and how is expression of these proteins coordinately regulated? How is expression of these smooth muscle cell differentiation marker genes altered in disease states such as atherosclerosis? How do the alterations in smooth muscle cell phenotype contribute to development/progression of vascular disease? As should be evident in reading this book, the answers to most, if not all, of these questions have not been adequately addressed.

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Architecture of the Smooth Muscle Cell

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I. Introduction

The mechanical properties of smooth muscle cells derive from two mutually interactive systems of filaments: the cytoskeleton and the contractile apparatus. By definition, smooth muscle cells are "smooth" or unstructured in the normal light microscope, so that efforts to elucidate their structural organization first utilized electron microscopy (reviewed in Bagby, 1983). Using this technique, actin filaments, myosin filaments, and intermediate filaments could be identified in ultrathin cross sections of smooth muscle tissue. However, the lack of any obvious filament order in two-dimensional ultrastructural images, such as groups of actin and myosin filaments, called for other approaches to resolve the architecture of the smooth muscle cell. Along with improvements in immunocytochemical techniques, high resolution fluorescence microscopy has been making important contributions in this area. By focusing on the localization of specific contractile and cytoskeletal proteins, new information has come to light about the organization of the contractile machinery and its mode of interaction with the cytoskeleton. This information now allows us to present a more complete structural model of the smooth muscle cell. In this new model, the cytoskeleton is attributed a central role in maintaining the organization of the contractile apparatus.

II. The Cytoskeleton

Our working definition of the smooth muscle cytoskeleton will be that part of the structural lattice which is not directly involved in the production of contractile force. The definition is, by necessity, a rather loose one because the cytoskeleton is itself coupled to the contractile machinery so that a number of structural components will be common to both systems.

When the actin filaments and myosin filaments are extracted from isolated smooth muscle cells using concentrated salt solutions, a framework of intermediate filaments is left (as well as some residual actin) that maintains the original, spindle-like form of the cells (Small and Sobieszek, 1977). It has thus been concluded that the intermediate filament network forms the backbone of the smooth muscle cytoskeleton (Fig. 1a). The major constituent of smooth muscle intermediate filaments is desmin (Lazarides, 1982), but depending on tissue or species, smooth muscle cells may also express variable combinations of desmin, vimentin, and cytokeratins (Jahn *et al.,* 1987; Osborn *et al.,* 1981). The intermediate filaments are not homogeneously distributed in the cell but are predominantly organized in loosely packed bundles that are mainly oriented in parallel to the cell axis but which also show branched interconnections; these bundles are most readily seen in confocal immunofluorescence microscope images of whole cells (Draeger *et al.,* 1990; Fig. 1a) or in ultrathin sections (Small *et al.,* 1986; Fig. 1c) labeled with desmin antibodies. In general, the bundles are heterogeneous in diameter and in some cases a large coaxial bundle of intermediate filaments may also be observed (Stromer and Bendayan, 1988). Two proteins, synemin (230 kDa; see reviews by Lazarides, 1982; Traub, 1985) and epinemin (44 kDa; Lawson, 1983), have been isolated from smooth muscle and colocalize with intermediate filaments of vimentin and desmin in skeletal muscle and nonmuscle cells, but no clues as to the function of these proteins have yet emerged.

The noted bundling of intermediate filaments correlates with their

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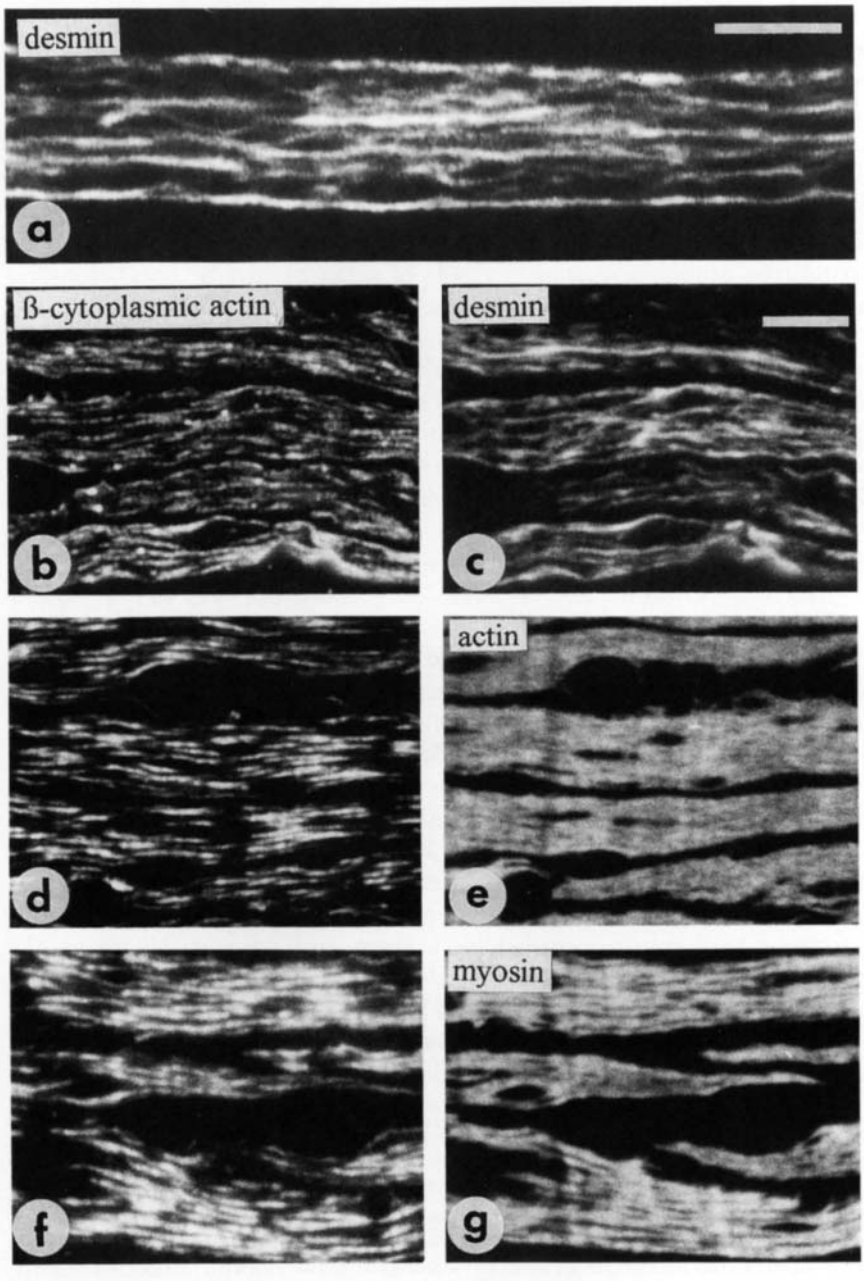
FIG. 1. The cytoskeleton of smooth muscle cells, as visualized by immunofluorescence microscopy. (a) The intermediate filaments. Confocal microscope image of an optical section of part of a chicken gizzard smooth muscle cell labeled with antibodies to desmin. Micrograph courtesy of Dr. A. Draeger. Bar, 5 μm. (b–g) Ultrathin, longitudinal cryosections of chicken gizzard smooth muscle, double labeled with antibodies against β-cytoplasmic actin (b, d, f) and desmin (c), muscle actin (e), and myosin (g). Note codistribution of β-actin with the desmin-containing cytoskeleton. The β-actin label in f lies in the dark channels in g that are unlabeled with myosin antibodies. Bar, 5 μm.


desmin
a
ß-cytoplasmic actin
b
desmin
c
d
actin
e
f
myosin
g

common grouping around the cytoplasmic dense bodies, the ovoid, and electron-dense structures (Figs. 2 and 3a) that are uniformly distributed throughout the cytoplasm (see, e.g., Draeger *et al.,* 1989; Kargacin *et al.,* 1989). Like the Z-discs of striated muscle, the dense bodies harbor the actin cross-linking protein α-actinin (Schollmeyer *et al.,* 1976; Geiger *et al.,* 1981; Fig. 2) and have therefore been generally taken to be anchorage sites for the actin filaments of the contractile machinery. It turns out (see later and Section IV) that these structures probably anchor two sets of actin filaments, one belonging to the contractile apparatus and the other to the cytoskeleton.

In addition to desmin and its associated proteins, filamin is also resident in the cytoskeletal network of smooth muscle cells. Filamin is an actin cross-linking protein (Hartwig and Kwiatkowski, 1991) that occurs in amounts in smooth muscle approaching 40% of the myosin content (Wang, 1977). Using immunocytochemical methods (Small *et al.,* 1986), filamin has been localized to the regions occupied by the intermediate filaments, as well as to the adhesion plaques on the sarcolemma that will be scrutinized in more detail in the following section. Filamin is, however, excluded from the dense bodies (Small *et al.,* 1986).

The colocalization of filamin and desmin was originally taken to suggest the existence of a "cytoskeletal domain" in the smooth muscle cell (see Fig. 5), composed of intermediate filaments together with a filamin–actin filament complex (Small *et al.,* 1986). At that time, however, a distinct actin component in this domain that was separate from the contractile apparatus could not be demonstrated. Studies on gizzard have now revealed that a specific, cytoskeletal actin component does in fact exist; thus, antibodies against a nonmuscle actin isoform, β-cytoplasmic actin, have been shown to specifically label the cytoskeleton domain occupied by filamin and desmin (North *et al.,* 1994b; Fig. 1). The same study also shows that the β-cytoplasmic actin enters the dense bodies (Fig. 2b).

To a first approximation then, the cytoskeleton is made up of filaments of cytoplasmic actin, of unknown length, that are tethered into

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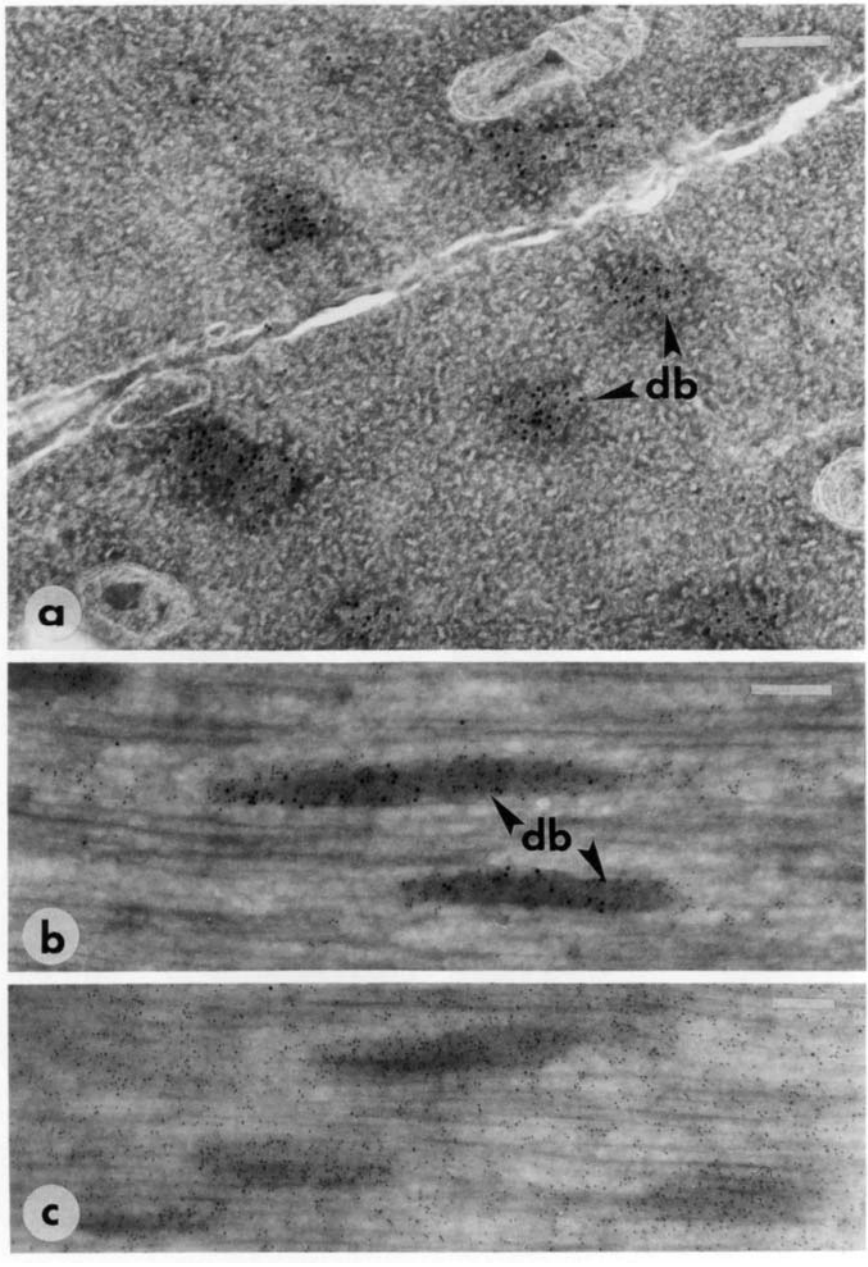
FIG. 2. Components of the dense bodies, as demonstrated by cryosection immunoelectronmicroscopy. (a and b) The specific localization of α-actinin (10-nm gold particles) in the dense bodies (db), in a transverse section (a; polyvinylalcohol embedding), and a longitudinal section (b; sucrose embedding). (b) Labeled with antibodies against β-cytoplasmic actin (5-nm gold particles), which colocalizes with α-actinin in the dense bodies and is also present in the cytoskeletal channels that link them. (c) Longitudinal section labeled with antibodies to calponin; this protein is localized in the dense bodies as well as in the cytoskeleton and contractile apparatus. Bars, 0.2 mm.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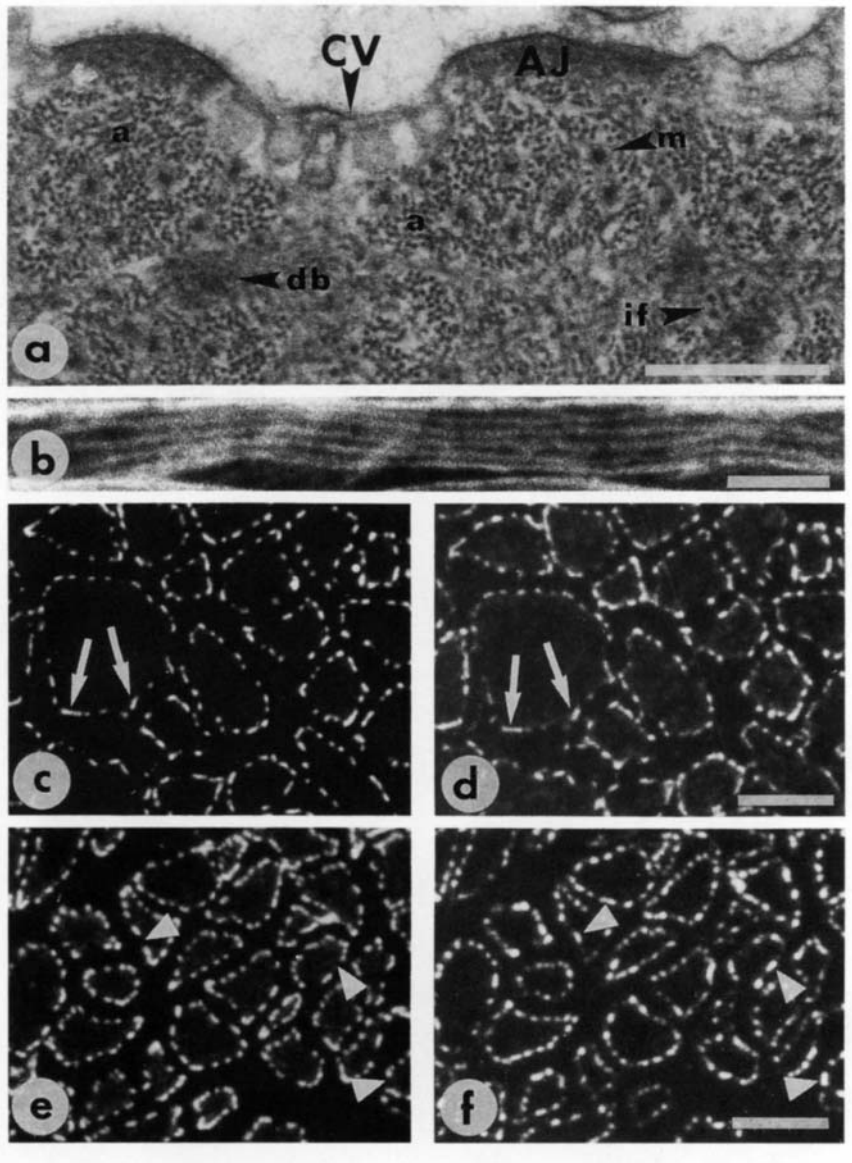

longitudinal fibrils by the dense bodies. These fibrils contain filamin and are accompanied by colinear arrays of intermediate filaments. Branching of some intermediate filaments between fibrils and attachment to the membrane skeleton serves to maintain the three-dimensional integrity of the cytoskeleton.

As well as providing coupling sites for components of the contractile apparatus, the cytoskeleton may be attributed a role in preventing overextension or undue shortening of the contractile machinery. This is suggested by the proportional increase in the number of intermediate filaments as well as of filamin content in smooth muscles subjected to increased mechanical stress *in vivo* (Berner *et al.,* 1981; Malmquist and Arner, 1990; Malmquist *et al.,* 1991). A constraining function of the cytoskeleton during contraction is indicated by the contractile properties of cells in which the cytoskeletal components are partially degraded by proteolysis; unrestrained shortening, or supercontraction, of the contractile apparatus then occurs that results in an artifactual penetration of actin filaments through the cell surface (Small *et al.,* 1990 and later). This supercontraction is only possible through an uncoupling of the contractile apparatus from its normal anchorage sites in the cytoskeleton. In addition to these structural roles, the cytoskeleton may serve more significant functions in the organization of the contractile machinery and in tension maintenance. Speculations about these roles will be reserved for a later section.

III. The Membrane Skeleton and Filament Anchorage

When viewed in cross section in the electron microscope, smooth muscle cells exhibit submembranous dense patches or plaques around 0.2μm thick and 0.5μm wide that are more or less regularly distributed around the cell periphery. These prominent plaque structures are separated by approximately equi-sized, uncoated membrane regions that bear many vesicular invaginations or caveolae (Gabella and Blundell, 1978; Bagby, 1983; Fig. 3a). In mammals, the plaques and intervening vesicle-rich regions are organized in continuous and parallel alternating channels that are oriented along the cell axis (Gabella and Blundell, 1978; Small, 1985; Draeger *et al.,* 1989, Fig. 5).

FIG. 3. The membrane skeleton. (a) Electron micrograph of a cross section of a smooth muscle cell from the guinea pig taenia coli showing dense plaques (or adherens junctions; AJ) at the cell surface and the intervening caveolae-rich domains (CV). m, myosin filament; a, actin filaments; if, intermediate filaments; db, dense body. Bar. 0.2 mm. (b) Fluorescence micrograph of a smooth muscle cell in a whole mount preparation of guinea pig taenia coli labeled with an antibody to vinculin. The adherens junctions, containing vinculin, are arranged in rib-like arrays. Bar, 5 μm. (c–f) Fluorescence microscope images of ultrathin cross sections of guinea pig taenia coli smooth muscle double labeled with antibodies against vinculin and β-integrin (c, d) and vinculin and dystrophin (e, f). Integrin is colocalized with vinculin in the adherens junctions (arrows in c, d) whereas dystrophin is localized in the vinculin-negative (caveolae-rich) regions between the adherens junctions (arrowheads in e, f). Bars, 5 μm.

Structural and immunocytochemical evidence places the dense plaques in the family of junctions of the adherens type (Geiger and Ginsborg, 1991; Small *et al.,* 1992) that are involved in anchoring actin filaments to the cell membrane. Proteins known to reside in the adherens junctions of smooth muscle and that are typical of actin-membrane anchorage sites in other cells include vinculin, talin, paxillin, filamin, and tensin (for a more complete list see Geiger and Ginsborg, 1991). Antibodies against these proteins clearly mark the adherens junctions and demonstrate their geometrical organization in longitudinal bands at the surface of the smooth muscle cell (Small, 1985; Draeger *et al.,* 1989; Fig. 3b). The adherens junctions of smooth muscle are of the cell matrix type and are characterized by the presence of transmembrane matrix receptors or integrins (Hynes, 1992; see chapter by Glukhova and Koteliansky in this volume, Figs. 3c and 3d) that couple the cytoskeleton components linking actin on the inside of the cell to the matrix on the outside. It has also been shown that matrix fibronectin is specifically concentrated on the extracellular side of the adherens junctions in smooth muscle; this distribution contrasts with that of laminin, which is more uniformly distributed around the cell perimeter (North *et al.,* 1993), consistent with the presence of a continuous basal lamina. The restricted localization of fibronectin in the matrix would seem to be determined directly by the integrins that are confined to the adherens junctions (North *et al.,* 1993; Fig. 3d).

Do the adherens junctions bind the actin filaments of the contractile apparatus, the cytoskeleton, or both? It had been assumed in the past that the filaments of the contractile apparatus are directly anchored in these plaque-like structures (see reviews by Bagby, 1983, 1986; Small *et al.,* 1992). However, new results from immunoelectron microscopy have shown the presence of β-cytoplasmic actin in the adherens junction domains (A. J. North *et al.,* unpublished results). This finding underlines the striking homology between the adherens junctions of smooth muscle and the focal adhesions of fibroblasts (Burridge *et al.,* 1988). The latter are sites of anchorage of cytoplasmic actin to the cell membrane and contain almost the same complement of proteins as found in smooth muscle adherens junctions (see, e.g., Small *et al.,* 1992), including filamin (Pavalko *et al.,* 1989).

It has not yet been demonstrated whether actin filaments of the contractile apparatus are also anchored within the adherens junctions. The possibility may be entertained, however, that "contractile" actin filaments are bound to the periphery of the dense plaques and only the cytoplasmic actin is linked to transmembrane integrins. The intermediate filaments of the cytoskeleton also appear to bind to the

adherens junctions (see, e.g., Bagby, 1983; Tsukita *et al.,* 1983), a supposition that is supported by the additional presence of the intermediate filament-associated protein plectin at these sites (Wiche *et al.,* 1983). Thus, the adherens plaques of smooth muscle may be viewed as bifunctional structures involved in the anchorage of both actin and intermediate filaments. Whether or not integrin-type molecules specific for intermediate filament linkers (see Magee and Buxton, 1991) exist in smooth muscle plaques is currently unknown. As with actin filaments of the contractile apparatus, the intermediate filaments may also bind to the periphery of the adherens junctions and therefore remain remote from the membrane.

The caveolae-rich sarcolemma regions between the adherens junctions have been described in some detail by Gabella (1984). Filament anchorage does not occur in these domains, as is most clearly illustrated by the intracellular face views of the caveolae-rich channels obtained by high resolution scanning electron microscopy (Sawada, 1981). It has been shown that these membrane regions are the sites of localization of dystrophin (Byers *et al.,* 1991; North *et al.,* 1993; Figs. 3e and 3f), the gene product of the Duchenne muscular dystrophy locus that is expressed in smooth as well as in striated muscle (Hoffmann *et al.,* 1988). From the predicted presence of an actin-binding domain at the N terminus of dystrophin and the demonstrated association of the C-terminal part with membrane glycoproteins (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya *et al.,* 1992), it has been suggested that a dystrophin–actin complex may add stability to the sarcolemma of striated muscle; the same proposal may also be forwarded for smooth muscle. The membrane-skeleton protein spectrin has been identified in skeletal muscle (e.g., Coleman *et al.,* 1989; Vybiral *et al.,* 1992) and could collaborate there with dystrophin in conferring membrane stability. However, attempts to identify spectrin in smooth muscle, using a wide spectrum of antibodies, have so far failed (North *et al.,* 1993). Caveolin, a protein shown to be specifically localized in fibroblast caveolae (Rothberg *et al.,* 1992), is also found in the caveolae-rich domains of smooth muscle (North *et al.,* 1993). However, the function of caveolin as well as of caveolae in smooth muscle is at present unclear.

In summary, the sarcolemma of the smooth muscle cell expresses two distinct structural domains, each containing its own complement of cytoskeletal proteins. The adherens junctions are involved in filament anchorage and form part of the smooth muscle cytoskeleton. The caveolae-rich domains appear to provide the cell with an extra membrane pool that allows changes in surface area, or conformation, on shortening (e.g, Gabella, 1984).

IV. The Contractile Apparatus: Nailing Down the Contractile Unit

Models of muscle structure are generally based on the idea that the thick and thin filaments are fixed both in length and in relative position. We cannot be certain that this is true for smooth muscle, although, for the sake of simplicity, most models have been based on this assumption. Smooth muscle cells express significant amounts of proteins known to influence the polymerization state of actin, namely gelsolin (Hinssen *et al.,* 1984) and profilin (Buss and Jockusch, 1989), and share with nonmuscle cells a type of myosin that readily dissociates from filaments to monomers at physiological ionic strength (see, e.g., Trybus, 1991). Although electron microscope studies have shown that myosin filaments are present in smooth muscle rapidly frozen in states of both relaxation and contraction (Somlyo *et al.,* 1981; Tsukita *et al.,* 1982), the existence of a dynamic equilibrium between the monomer and polymer states of myosin, which may play a role in transiently remodeling the contractile apparatus, cannot be excluded. With these reservations in mind, we shall discuss data that have provided information about the three-dimensional arrangement of the contractile elements.

One primary problem has been to define the length of the thick myosin and thin actin filaments in smooth muscle in the absence of an obvious sarcomere organization. High voltage electron microscopy of serial semithick sections of rabbit portal vein have yielded an average thick filament length in this tissue of around 2.2 μm. Experiments with cells isolated from chicken gizzard have provided independent values for the lengths of the thick filaments as well as for the first direct measurements of thin filament length (Small *et al.,* 1990). In the latter studies, it was shown that cell fragments in which the cytoskeleton is partially degraded undergo a "supercontraction" on ATP addition (Fig. 4a) that yields uniformly long actomyosin assemblies as end products (Fig. 4b). These assemblies are composed of

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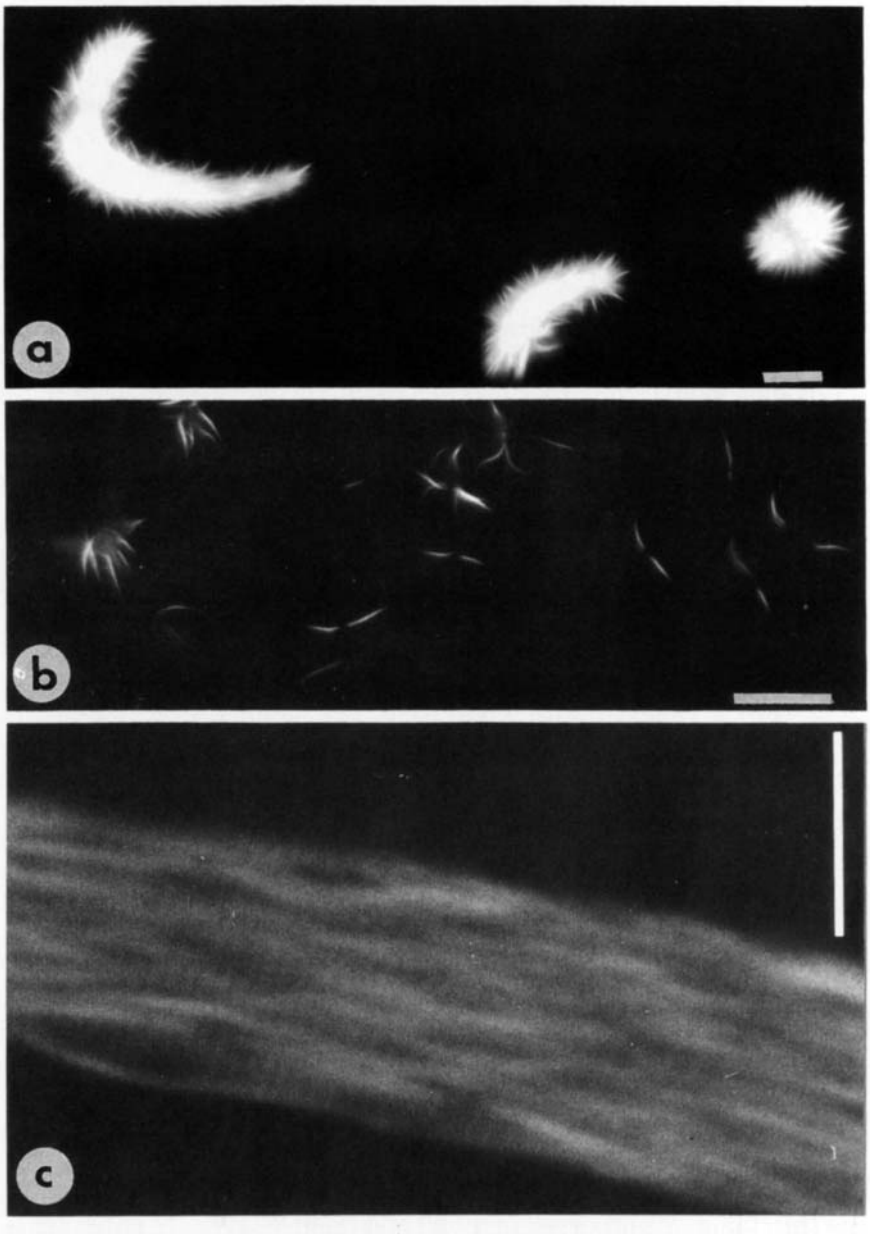
FIG. 4. The contractile apparatus. (a) Fluorescence microscope image of isolated gizzard smooth muscle cells that were prelabeled with rhodamine-conjugated phalloidin to mark the actin filaments and then induced to supercontract by the addition of ATP. The artifactual penetration of actin filaments throughout the whole cell surface is consistent with an oblique orientation of the contractile elements. (b) Isolated end products of supercontraction, obtained by fragmentation of cells such as shown in a. Bundles of actin filaments around 5 mm long are commonly seen as colinear dimers flanking an actin-free region; this central region is occupied by myosin filaments (see text). (c) Confocal microscope image of an optical section of part of a chicken gizzard smooth muscle cell labeled with antibodies to myosin, showing oblique arrangement of fibrils. Micrograph courtesy of Dr. A. Draeger. All bars, 10 mm.


a
b
c

bundles of myosin filaments around 1.5 μm long that are flanked by actin filaments of 3 to 6 μm or more in length (Small *et al.,* 1990). These findings support earlier suggestions (reviewed by Bagby, 1983) that the thin filaments may be much longer than the thick filaments, which explains the high actin-to-myosin filament number ratio in cross sections and the slower rate of shortening of smooth muscle (e.g., Murphy, 1979).

Further results, obtained mainly from studies of isolated cells, indicate that the contractile elements are organized obliquely with respect to the cell axis. This was first suggested by the observation of obliquely arranged birefringent fibrils in isolated and demembranated cells (Small, 1974) that were later shown to be labeled with antibodies to myosin (Draeger *et al.,* 1989; Fig. 4c). The angle of these fibrils, relative to the cell axis, increases at progressively shorter cell lengths (Small, 1974) and causes a corresponding change in the orientation of birefringence (Fisher and Bagby, 1977). The appearance of membrane blebs over most of the surface of smooth muscle cells that were induced to contract *in vivo* (Fay and Delise, 1973) was also taken as evidence for an oblique arrangement of contractile elements. In supercontracted cell fragments, this reorientation of contractile filaments on shortening is particularly pronounced (Small *et al.,* 1990). Figure 4a shows examples of supercontracted cells and cell fragments that were prelabeled with fluorescent phalloidin (to label F-actin). Actin filament bundles penetrate through the cell surface in all directions, consistent with a gross reorientation of the contractile elements. Electron micrographs of longitudinal sections of smooth muscle also indicate an oblique orientation of the myofibrils relative to the intermediate filament bundles (Bond and Somlyo, 1982), which are themselves mainly oriented parallel to the long axis of the cell (see Fig. 1a and Draeger *et al.,* 1990).

What is the nature of the contractile unit? Bond and Somlyo (1982) and Tsukita *et al.* (1983) have provided the clearest ultrastructural evidence for the insertion of actin filaments into the cytoplasmic dense bodies; they also showed that the polarity of the actin filaments is opposite on either side of the dense body as it is on either side of the Z-disc in skeletal muscle. Since muscle actin-containing filaments are more stable to fixation and embedding procedures than nonmuscle-type actin filaments (like those found in the cytoskeleton domain), the thin filaments observed by Bond and Somlyo (1982) to enter dense bodies were probably active filaments of the contractile apparatus and not of the cytoskeleton. Current evidence therefore supports the existence of I band-like structures as components of the contractile apparatus. However, a segregation of the myosin filaments into dis-

crete A band-like groups or arrays has not yet been demonstrated convincingly. In confocal microscope optical sections or ultrathin cryosections of cells labeled with myosin antibodies, a periodic arrangement of myosin is not evident (Draeger *et al.,* 1990); A. J. North *et al.,* unpublished data; Figs. 1i and 4c). In addition, it has not been possible to follow myosin-positive fibrils from one side of a cell to the other (see Draeger *et al.,* 1990), suggesting that individual contractile fibrils do not span the cell.

We should recall that the myosin filaments of smooth muscle are assembled in a unique fashion that has been referred to, descriptively, as "face polar," whereby molecules on opposite edges of a myosin filament are oriented in opposite directions (reviewed by Trybus, 1991; see also Fig. 5). This assembly mode would allow optimal interaction of one actin filament along the whole length of a myosin filament and would be consistent with a telescopic-like shearing between the thin and the thick filaments during shortening (see, e.g., Small and Squire, 1972; Small, 1977). The myosin filaments would then not be constrained in a position (such as in striated muscle) so that actin filaments may slide only to the center of each thick filament. In fact, they could be distributed relatively homogenously, as long as they in-

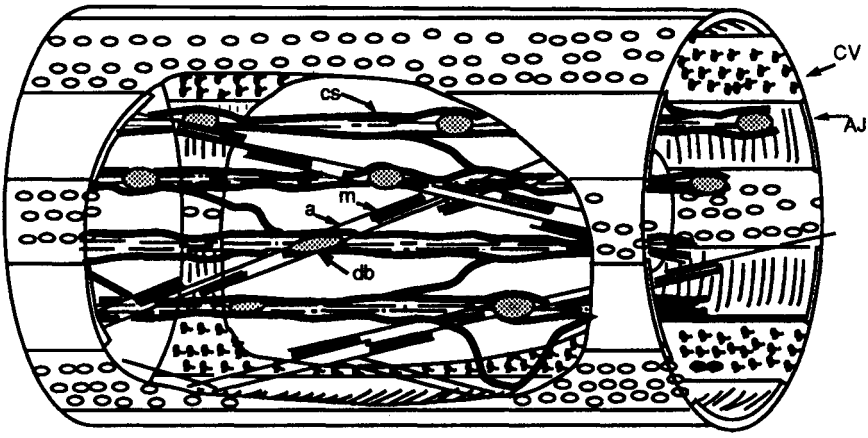


FIG. 5. Organization of the contractile and cytoskeletal apparatus of the smooth muscle cell. The cytoskeleton (cs) is composed of mainly longitudinal fibrils, composed of intermediate filaments (thick, solid lines), cytoplasmic actin (dashed and dotted lines), and dense bodies (db). Branching of intermediate filaments between adjacent fibrils also occurs. The contractile apparatus is composed of obliquely arranged arrays of face-polar myosin filaments (m) and muscle actin filaments (a) that are anchored at the cytoplasmic dense bodies and at the adhesion plaques or adherens junctions (AJ) of the membrane skeleton. CV, caveolae domains.

teract with oppositely polarized actin filaments from different dense bodies. They could even change actin filament partners during shortening, as has been proposed for helically striated muscle, when undergoing large changes in length (De Eguileor *et al.*, 1988). More results are needed, possibly from overextended smooth muscle, to shed more light on the detailed spatial distribution of the myosin filaments.

An intriguing feature of smooth muscle cells, worthy of final mention, is their ability to form contraction bands at regular intervals along their length. These were first observed by Kölliker (1849) and have been shown to be focal concentrations of myosin filaments (Draeger *et al.*, 1990; Bennett *et al.*, 1988). The contraction bands themselves are clearly artifacts but appear to reflect a hierarchical organization of the contractile elements (see also Kargacin *et al.*, 1989).

V. Organization of the Smooth Muscle Cell

Table I lists the various major components of the smooth muscle cell according to their location in the different subcellular compartments that have been discussed. This information, as well as other

TABLE I

Localization of Cytoskeletal and Contractile Components in Smooth Muscle Cells

Protein	Dense body	Dense plaque	Cytoskeletal channel	Contractile domain
βNM-actin	+	+	+	−
Desmin	−	−	+	−
Filamin	−	+	+	−
α-Actinin	+	+/−	−	−
Vinculin	−	+	−	−
γSM-actin	−	−	?/−	+
Myosin	−	−	−	+
Caldesmon	−	−	−	+
Tropomyosin	−	−	?/+	+
Calponin	+	+	+	+

Note: Proteins colocalized in the plasma membrane-associated dense plaques with vinculin (e.g., talin, tensin, metavinculin, integrin, etc.) are not listed. Smooth muscle γ-actin (γSM-actin) is present in the contractile apparatus but its presence in the cytoskeletal channels, due to overlap with the contractile filaments, cannot be excluded. Likewise, tropomyosin has not been shown to be definitely associated with βNM-actin in the cytoskeletal channels. α-Actinin appears to be associated with the periphery of the dense plaques but occurs here in much lower amounts compared to the dense bodies. Calponin occurs in all of the regions indicated, but is most concentrated in the cytoskeleton. "Cytoskeletal channel" corresponds to that part of the cytoskeletal domain occupied by desmin. Reproduced from North *et al.* (1994b) with permission of Company of Biologists Ltd.

data considered in the foregoing sections, forms the basis of the model of the smooth muscle cell presented in Fig. 5. The finding of the nonmuscle β-actin in the cytoskeleton consolidates earlier evidence (Small *et al.,* 1986) indicating the presence of two spatially and functionally distinct actin components, one belonging to the contractile apparatus and the other to the cytoskeleton. The cytoskeleton has been shown to contain at least three major components: desmin (or vimentin), β-actin, and filamin. Both of the latter components have also been localized to the adherens junction, suggesting that the structural framework of the cytoskeleton pervades not only the cell interior but also coats the sarcolemma, where it serves to anchor the cell surface, via transmembrane molecules, to the surrounding matrix.

Coupling between the contractile apparatus and the cytoskeleton is envisaged to occur primarily at the cytoplasmic-dense bodies, which are presumed to anchor both species of actin, contractile and cytoskeletal. In our studies (North et al., 1994b), we have localized β-actin to the dense bodies and have shown that calponin, a component of the contractile filaments, also resides in these structures (North *et al.,* 1994a; Fig. 2c). The linking of contractile units between dense bodies in different cytoskeletal channels (Fig. 5) is consistent with the observed, oblique, and sometimes zig-zag organization of the contractile fibrils in shortened cells. Details about the precise organization of the contractile fibrils remain to be established.

VI. Filament Cross-Linking and Tone

What new information on smooth muscle function can we derive from these structural observations? In this last section we will take the liberty of speculating about the possible role of filament cross-linking in the modulation of the contractile activity of smooth muscle.

The demonstrated colocalization of filamin and actin in the cytoskeleton raises the possibility, suggested earlier (Small *et al.,* 1986), that the cross-linking of actin by filamin in the cytoskeleton domain could contribute to the maintenance of tone. By analogy with nonmuscle cells, such as fibroblasts, we may expect that the cytoskeletal actin domains in smooth muscle contain, in addition to filamin, a number of other nonmuscle-type actin-associated proteins. In particular, the nonmuscle myosin isoform identified in smooth muscle (Larson *et al.,* 1984; Gaylinn *et al.,* 1989) is very likely resident in the cytoskeleton (we intend to test this idea using discriminating antibodies). The smooth muscle cytoskeleton would then possess the potential to produce and maintain tension, independent of the contractile apparatus. More work needs to be done to test this idea. Nevertheless, the possibility exists

that signaling processes leading to sustained tension maintenance and involving, for example, protein kinase C or a small G-protein (Rasmussen *et al.,* 1987; Kitazawa *et al.,* 1991; Hirata *et al.,* 1992) act mainly on the cytoskeleton rather than on the contractile machinery. Modulation of the mechanical properties of the cytoskeleton could be effected via the reversible cross-linking of filaments by filamin and through the dynamic turnover of actin filaments mediated by gelsolin (Hinssen *et al.,* 1984) and profilin (Buss and Jockusch, 1989). Nonmuscle myosin in the cytoskeleton could serve to maintain the longitudinal integrity of the cytoskeleton by mediating the sliding of cytoskeletal actin filaments in concert with the contractile apparatus, as the cell shortens.

In other studies (reviewed by Sobue and Sellers, 1991), the actin- and calmodulin-binding protein caldesmon has been attributed a role in maintaining tone in smooth muscle, either by directly cross-linking the thick and thin filaments (Ikebe and Reardon, 1988; Sutherland and Walsh, 1989; Marston, 1989; Chalovich *et al.,* 1990) or by modulating the myosin cross bridge cycle to produce a "latch" state (Hai and Murphy, 1989). Caldesmon is specifically localized in the contractile apparatus and is excluded from the cytoskeleton (Fürst *et al.,* 1986). Filament cross-linking, whether in the contractile apparatus, in the cytoskeleton, or both, could thus explain tension maintenance in the smooth muscle cell.

VII. Concluding Remarks

As discussed at length by Bagby (1986), conventional electron microscopy of smooth muscle thin sections has not proved adequate, as it has for striated muscle, to define the arrangements of the contractile and cytoskeletal elements. In smooth muscle, a three-dimensional structural organization exists that is not easily deciphered from two-dimensional images. We have thus had to resort to the use of other approaches, involving the manipulation of isolated cells (reviewed in Small and Sobieszek, 1980; Bagby, 1983, 1986; Fay *et al.*, 1989) and the use of high resolution immunocytochemistry to map the distribution of contractile and cytoskeletal proteins.

These approaches have been rewarding, for they have yielded enough new information to allow us to piece together a realistic model of the smooth muscle cell. Inevitably, these new insights have raised many new questions, not least about the relative contributions of the contractile apparatus and the cytoskeleton to the mechanical properties of smooth muscle tissue. We await new developments in this area with interest.

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Molecular Basis of Smooth Muscle Contractility Myosin Heavy Chains: Gene Structure and Expression

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I. Introduction

Myosin is a major component of the contractile apparatus in both sarcomeric and nonsarcomeric muscle tissues. A myosin molecule is composed of a pair of heavy chains (approximately 200 kDa) and two pairs of light chains (15–27 kDa). Myosin heavy chain (MHC) is an asymmetric protein with a globular head and an α-helical coiled-coil rod that is responsible for the assembly of myosin into filaments. The myosin head has an ATPase activity, whereby myosin transforms the

chemical energy of ATP to the mechanical function of muscle contraction. In vertebrates there are more than 10 different MHC isoforms encoded by a large multigene family (Nguyen *et al.*, 1982; Robbins *et al.*, 1986). MHC isoforms differ in their ATPase activities and seem to correlate with physiological functions, such as the velocity of fiber shortening and with the economy of muscle contraction (Pagani and Julian, 1984; Alpert *et al.*, 1983). In mammalian skeletal and cardiac muscle, the expression of MHC isoforms is regulated developmentally, in a tissue-specific manner, and by a variety of pathophysiological conditions including hormonal and mechanical stress (Huszar, 1972; Lompre *et al.*, 1982, 1984; Periasamy *et al.*, 1984, 1985; Izumo *et al.*, 1986; Nagai *et al.*, 1987). MHC isoforms are also molecular markers of muscle development, differentiation, and diseases.

Although vertebrate smooth muscle and nonmuscle myosins resemble sarcomeric myosins in many respects, nonsarcomeric myosins have several distinct features; for example, the myosin-linked regulation of actin–myosin interactions is accompanied by phosphorylation of the regulatory light chain, and the dynamic assembly and disassembly of the thick filament *in vitro*. In smooth muscle the tension produced during contraction can be equal to or greater than that produced in striated muscle despite the presence of a lower myosin/actin ratio. Although vertebrate smooth muscles display a functional diversity which far surpasses that of striated muscles, there is no comparable evidence for multiplicity in the myosin molecule. The diversity of smooth muscle myosin isoforms has been extensively investigated using molecular biology and protein mapping techniques (Rovner *et al.*, 1986a,b; Sartore *et al.*, 1994; Yanagisawa *et al.*, 1987; Nagai *et al.*, 1988, 1989; Kuro-o *et al.*, 1989, 1991; Babij and Periasamy, 1989; Babij *et al.*, 1991, 1992; Katsuragawa *et al.*, 1989; Saez *et al.*, 1990; Kawamoto and Adelstein, 1991; Simons *et al.*, 1991). These studies revealed that smooth muscle cells express at least four types of MHC, two of which are smooth muscle specific and two that are nonmuscle type MHCs. This chapter describes the molecular basis of MHC diversity in smooth muscles, their expression in development and disease, and MHC gene structure in both animals and humans.

II. Myosin Heavy Chain Isoforms in Smooth Muscle

A. cDNA Cloning Analysis

cDNA clones encoding smooth muscle MHC were first isolated from an embryonal chicken gizzard cDNA library (Yanagisawa *et al.*, 1987). Subsequently, cDNA clones corresponding to smooth muscle MHC

were isolated from rabbit, rat, and human smooth muscle tissues (Nagai *et al.*, 1989, 1991; White *et al.*, 1993; Babij and Periasamy, 1989; Aikawa *et al.*, 1993) in our laboratory. cDNA cloning analysis revealed that there are two types of cDNA encoding two distinct but very closely related MHC molecules, SM1 and SM2 MHC, respectively. As described later, SM1 and SM2 are products of the same MHC gene generated by alternative RNA splicing (Babij and Periasamy, 1989).

The complete nucleotide and amino acid sequences have been obtained for chicken gizzard (1979aa) and rabbit (1972aa) smooth MHC (Yanagisawa *et al.*, 1987; Babij *et al.*, 1991). The primary structure of the smooth muscle MHC is very similar to that of other sarcomeric MHCs of skeletal and cardiac muscles. Smooth muscle MHC has a globular head and an α-helical rod. Comparing the S1 head region of the rabbit uterus MHC, chicken gizzard, and chicken nonmuscle, MHCs indicate that the overall amino acid sequences are highly conserved, showing 90% sequence identity (Fig. 1). The putative Mg^{2+}-ATPase and actin-binding domains are virtually identical for all three MHCs. In contrast, a homology of only 48 to 49% exists in the head region between smooth muscle MHC and the sarcomeric MHC (Yanagisawa *et al.*, 1987).

The characteristics of the myosin rod domain are also maintained in smooth muscle MHC; that is, an α-helical coiled-coil structure and a periodic seven-residue repeat, in which hydrophobic amino acids appear at alternate intervals of three and four residues (Yanagisawa *et al.*, 1987; Nagai *et al.*, 1988). This characteristic amino acid distribution facilitates the intertwining of two α-helical molecules through hydrophobic interactions. The light meromyosin (LMM) region of the rod also has a 28 residue repeat pattern in which clusters of positively and negatively charged residues alternate every 14 residues, which favors the adherence of adjacent myosin LMMs through electrostatic interactions.

A prominent characteristic of the smooth muscle or nonmuscle myosin is the presence of a nonhelical tailpiece at the carboxyl-terminal end (Yanagisawa *et al.*, 1987; Nagai *et al.*, 1988). In skeletal and cardiac MHC, the α-helix-breaking amino acid proline does not appear in the rod region, whereas proline exists in the tailpiece of the rod in smooth muscle and nonmuscle MHC. Removal of the 4-kDa nonhelical tailpiece of the SM1 MHC by chymotryptic cleavage has been shown to activate the Mg^{2+}-ATPase activity of phosphorylated gizzard actomyosin (Horowitz and Trybus, 1992). This suggests that the C-terminal tailpiece in smooth muscle myosin may play an important role in modulating ATPase activity similar to that seen in nonmuscle myosin. Moreover, removal of the C-terminal tail of the SM1

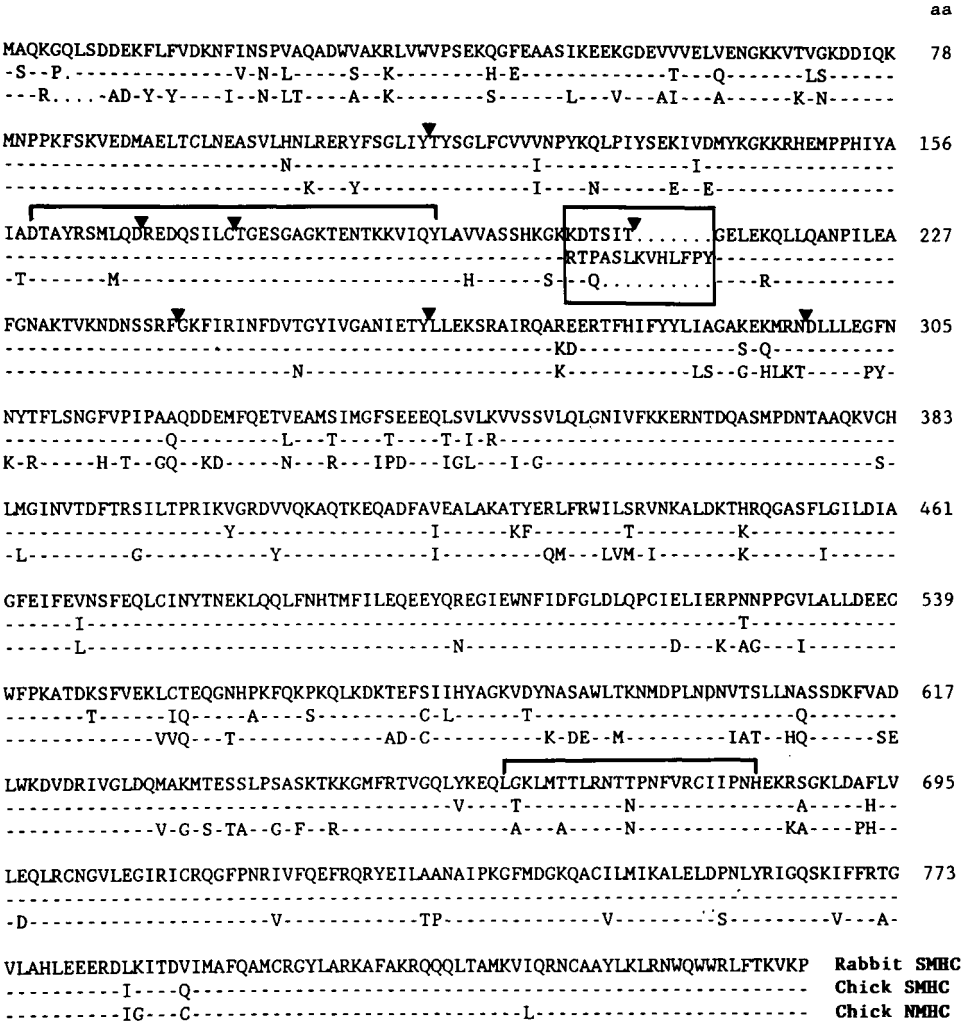


Fig. 1. Comparison of deduced amino acid sequences of S1 globular head region of rabbit uterus smooth muscle MHC (SMHC), chicken gizzard SMHC, and nonmuscle MHC (NMHC). Boxed area identifies unique region in the MHC molecule where each sequence diverges. Bracket shows putative Mg^{2+}-ATPase domain (residues 159–194) with respect to the rabbit uterus sequence.

MHC also promotes the 10S to 6S transition and filament formation (Horowitz and Trybus, 1992). It has also been reported that binding of a monoclonal antibody near the carboxyl terminus inhibited filament assembly and actin-activated ATPase activity (Ikebe *et al.*, 1991). A functional role for the differential expression of smooth muscle MHC

isoforms is also suggested by a threefold increase in unloaded shortening velocity associated with an increase in the SM1 MHC isoform content of permeabilized fibers from rat myometrium (Hewett *et al.*, 1993).

B. Molecular Basis of Smooth Muscle MHC Diversity

The presence of at least two smooth muscle MHC isoforms was first suggested from sodium dodecyl sulfate (SDS)–polyacrylamide gel analysis. Rovner *et al* (1986a) found that MHC protein from various smooth muscle tissues can be separated into two bands of SM1 (approximately 204 kDa) and SM2 (approximately 200 kDa) on a porous SDS–polyacrylamide (3.5%) gel electrophoresis, both of which cross-reacted with a polyclonal smooth muscle myosin antibody. The two MHC bands were thought to represent two different MHC isoforms because limited proteolysis and peptide mapping of purified myosin showed a difference in the LMM rod portion of the myosin molecule (Eddinger and Murphy, 1988). Direct evidence for a difference in the primary structure of SM1 and SM2 was provided by cDNA cloning analyses (Nagai *et al.*, 1988, 1989; Babij and Periasamy, 1989). We demonstrated, by cDNA cloning, that there are two types of SMHC cDNA clones (SMHC40 and SMHC29 in rabbit) corresponding to SM1 and SM2, respectively (Nagai *et al.*, 1989). These two MHC cDNA clones are identical in nucleotide and amino acid sequences in the LMM region, except for the carboxyl terminus and a portion of the 3′-untranslated region. SM1 mRNA encodes a unique stretch of 43 amino acids at the carboxyl terminus, whereas SM2 mRNA contains a shorter carboxyl terminus of 9 unique amino acids which is the result of a 39 nucleotide insertion (Fig. 2). Further, we generated antibodies against the unique carboxyl-terminal amino acids of SM1 and SM2 (deduced from cDNA sequences) and established, by immunoblotting, that the two types of cDNA encode the SM1 and SM2 MHC molecules (Fig. 3).

It has been suggested that additional smooth muscle MHC isoforms may exist. Using a nuclease protection assay, Hamada *et al.* (1990) demonstrated that aortic smooth muscle MHC mRNA could be distinguished from the gizzard MHC mRNA in the 5′-coding region. Similarly, Babij *et al.* (1991) reported a difference in the primary structure of SM1 between rabbit uterus and chicken gizzard at the junction of the 25- and 50-dKa proteolytic fragments of the myosin head. Both uterus and gizzard SM1 are highly homologous up to amino acid 205, but then diverge completely at amino acid 206. From this point of divergence, the rabbit uterus MHC encodes 6 unique amino acids, whereas chicken gizzard MHC encodes a stretch of 13 unique amino

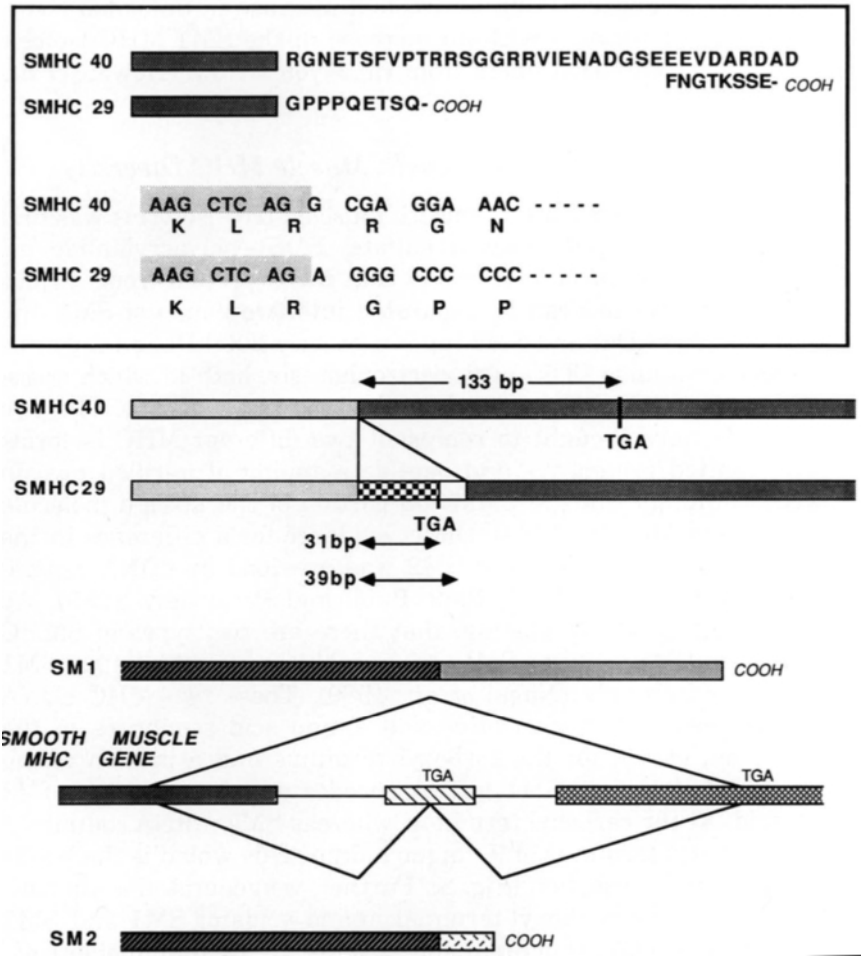


Fig. 2. Two types of smooth muscle MHC mRNAs. The organization of rabbit SM1 (SMHC 40) and SM2 (SMHC 29) cDNA clones and an alternate RNA splicing mechanism are shown. SM2 mRNA includes a 39 nucleotide insertion not found in SM1 mRNA, and is the result of alternate splicing of a unique exon containing a stop codon. Note that there are two stop codons in the SMHC gene.

acids before the high degree of homology is restored (Fig. 1). These results suggested that the smooth muscle MHC 25/50-kDa junction differs among smooth muscle tissues and may represent a functionally important domain in the MHC molecule. Further cloning analyses led to the identification of two rat SMHC cDNA clones (SMHC-11 and SMHC-5) that differ precisely at the 25/50-kDa junction of the myosin

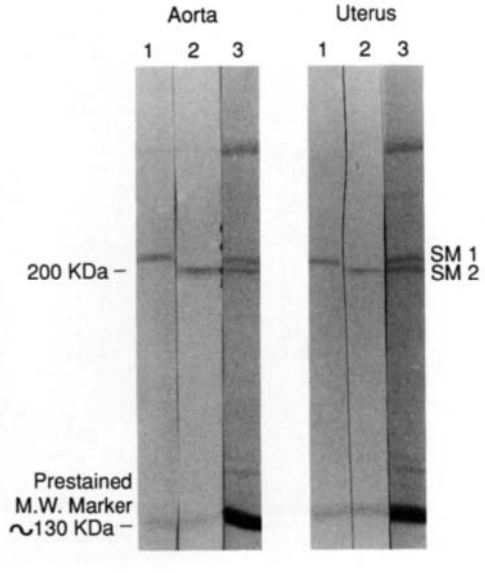
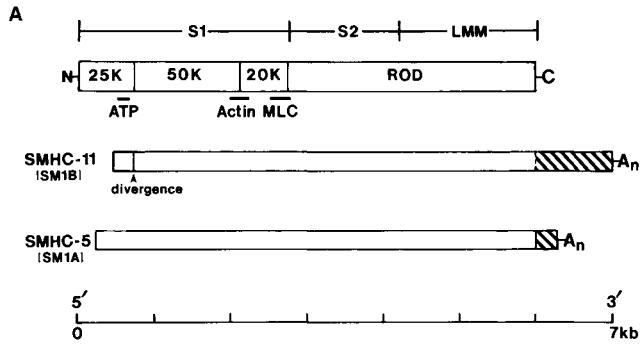


Fig. 3. Immunoblot analysis of purified smooth muscle myosin. The purified myosin from aorta and uterus was separated on a 3.5% polyacrylamide gel. Specific antibodies raised against the carboxyl-terminal peptides of SM1 and SM2 distinguish each isoform. Lane 1, anti-SM1; lane 2, anti-SM2; and lane 3, amido black staining.

head (Fig. 4A). Sequence comparison of the two clones revealed that they are identical in their LMM regions of the molecule and encode the SM-1 type myosin at the carboxyl end (White *et al.*, 1993). However, a divergence occurs in the region encoding the 25/50-kDa junction of the myosin head, where the SM1B isoform (SMHC-11) contains an additional seven amino acids (Fig. 4B). It has been shown that this sequence heterogeneity results from alternate RNA splicing of a unique exon containing seven amino acids in rabbits (Babij, 1993). This divergent region is located adjacent to the Mg^{2+}-ATPase site, and differences in this region may be of functional importance. Ribonuclease protection analysis demonstrates that the corresponding SM1B and SM1A mRNA are coexpressed in most smooth muscle tissues; however, the proportion of the two mRNAs present differs significantly between tissues (White *et al.*, 1993; Babij, 1993). The SM1A-type mRNA predominates in most smooth muscle tissues, with the exception of intestine and urinary bladder, which contain greater proportions of the SM1B message. The differential distribution of these two isoforms may provide important clues toward understanding differences in smooth muscle contractile properties.

C. Nonmuscle Myosin Heavy Chain Isoforms

In addition to smooth muscle MHC isoforms, cultured smooth muscle cells and embryonic smooth muscle tissues express nonmuscle-type MHCs. Kawamoto and Adelstein (1987) first reported that SM1



B

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Rat    SM 1B  DTAYRSMLQDREDQSILCTGESGAGKTENTKKVIQYLAVVASSHKGKKDSSIT QGPSFAY GELEKQLLQANPILEAFGNA
Rat    SM 1A  ---------------------------------------------------- ....... -------•-------------
Rabbit SMHC   ----------------------------------------------------T--- ....... --------------------
Chick  SMHC   ----------------------------------------------RTPASL KVHLFPY --------------------
Chick  NMHC   -------M----------------------------H------S---Q... ....... ----R---------------
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Fig. 4. (A) Rat smooth muscle MHC cDNA clones SMHC-11 (SM1B) and SMHC-5 (SM1A). The location of a 21 nucleotide divergence in SMHC-11 is indicated by a solid line. (B) Comparison of an amino acid sequence in the area of the 25/50-kDa junction between smooth and nonmuscle MHCs.

and SM2 disappeared in cultured rat aortic smooth muscle cells and were replaced by a 196-kDa nonmuscle MHC (NM-MHC) which was recognized by an antiplatelet myosin antibody. The expression of nonmuscle MHC isoforms both in developing and in atherosclerotic rabbit aortas was elegantly demonstrated by immunoblotting and indirect immunofluorescence techniques (Kuro-o *et al.*, 1989; Zanellato *et al.*, 1990). cDNA cloning analyses of these MHCs in smooth muscle confirmed that they are identical to NM-MHC-A and NM-MHC-B characterized by Katsuragawa *et al.*, (1989) and Kawamoto and Adelstein (1991). The nonmuscle MHC isoforms A and B are encoded by two separate genes distinct from the SMHC gene and have been localized to two different chromosomes (Simons *et al.*, 1991). Using an antibody against the carboxyl terminus of NM-MHCB, Kuro-o *et al.* (1991) described that developing and proliferating arterial smooth muscle cells express high levels of NM-MHC-B.

Similarly, the NM-MHC isoforms were found to be expressed in developing human aortic smooth muscle (Frid *et al.*, 1993; Aikawa *et al.*,

1993). MHC heterogeneity in the S1 head region was also reported for nonmuscle myosin by alternate RNA splicing events. Takahashi *et al.* (1992) demonstrated that NM-MHC-B has a 63 nucleotide insertion (encoding 21 amino acids) located in the head region near the actin-binding site and a 30 nucleotide insertion encoding 10 amino acids near the ATP-binding site (both of which are expressed in cells of the nervous system but are absent in other nonmuscle cells).

D. SMHC Gene Structure and Organization

Compared to the vertebrate sarcomeric MHC isoforms, the smooth muscle-specific MHC isoforms are encoded by a single gene. The structure of the smooth muscle MHC gene has been investigated in rabbits and rats. To date, only portions of the gene have been isolated and characterized as the gene could be as large as 200 kb (Babij and Periasamy, 1989; Babij *et al.*, 1991). The direct evidence showing that SM1 and SM2 myosin isoforms are generated from a single gene was provided by characterizing the 3′ end of the rat SMHC gene (Babij and Periasamy, 1989). A short exon specifying the 39 nucleotides unique to SM2 mRNA was identified in this gene. The 39 nucleotide exon is flanked on either side by two relatively large introns of approximately 2.6 and 2.7 kb. Inclusion of this short exon generates a shorter carboxyl terminus of SM2 because of the presence of a stop codon in it, whereas exclusion results in the longer carboxyl terminus of 43 amino acids of SM1.

Characterization of the 5′ end of the SMHC gene is very important because it may contain *cis*-acting DNA regulatory elements controlling smooth muscle-specific expression of this gene. Babij *et al.* (1991) described the isolation of the rabbit SMHC gene. The rabbit smooth muscle MHC gene is large (estimated to be ~200 kb) and has an unusual exon/intron organization at the 5′ end. The first eight contiguous exons are located within a region of genomic DNA covering at least 70 kb (Fig. 5). Some introns were found to span several kilobases of DNA, and others at the 5′ end show a high degree of positional conservation in the Mg^{2+}-ATPase domain when compared with sarcomeric MHC genes. The first noncoding exon is 78 bp long, and the remaining 17 bp of the 5′-untranslated sequence is contained in the first coding exon (exon 2; 366 bp) separated by >20 kb of intervening sequence. A unique exon encoding 7 aa, responsible for MHC heterogeneity at the 25/50-kDa junction, has been mapped between exon, 5 and 6 in the rabbit gene (Babij, 1993). The rabbit SMHC gene has a single transcription initiation site and a canonical TATAAA sequence 26 nucleotides upstream of the putative start site. Interestingly, this same initiation site is used to transcribe MHC mRNA in

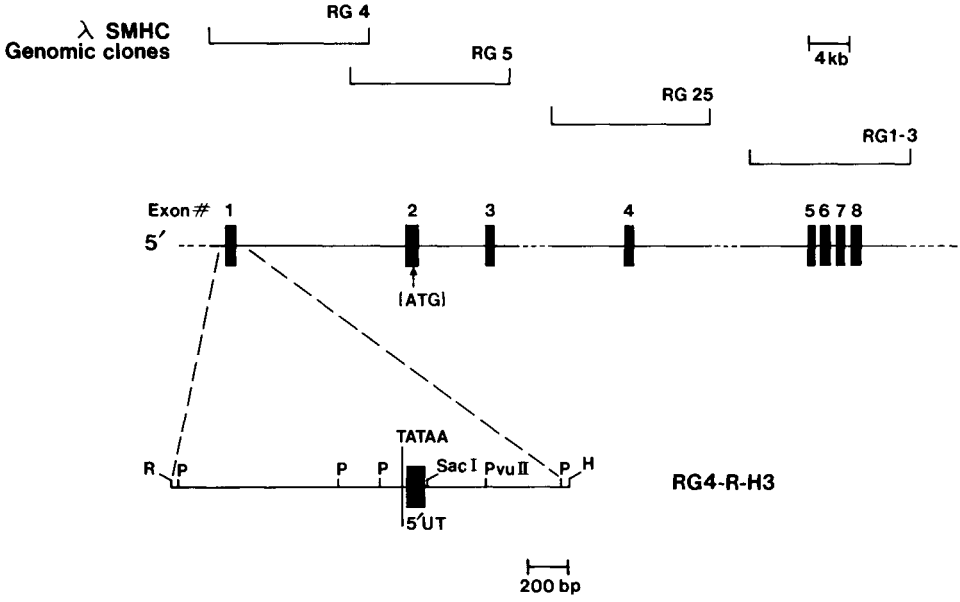


Fig. 5. Exon/intron organization at the 5′ end of the rabbit SMHC gene. Black boxes represent exons and horizontal line represents intervening sequence. The putative promoter site is identified as TATAA.

different smooth muscle cell types (Babij *et al.*, 1991), strongly suggesting a single promoter for this gene.

III. Myosin Heavy Chain Gene Expression

Other chapters in this book provide a more general overview of patterns of contractile and cytoskeletal protein expression during smooth muscle differentiation (chapters by Glukhova and Koteliansky and Gittenberger-de Groot *et al.*). The chapter by Glukova and Kofeliansky describes in detail that several smooth muscle proteins, including contractile and extracellular matrix proteins, show developmental and pathological regulation, some of which are controlled by alternate mRNA splicing. Therefore, this section focuses on the myosin heavy chain.

A. Smooth Muscle Myosin (SM1 and SM2) Expression Is Highly Restricted to Smooth Muscle Cells

A number of studies using antibodies have examined the expression of smooth muscle myosins in a number of species during development

(reviewed in Sartore *et al.*, 1994). Although these studies have provided important information, they do not thoroughly examine the question of whether SM myosin is expressed only in smooth muscle cells. It was reported that SM α-actin mRNA is expressed in developing cardiac and skeletal muscle (Ruzika and Schwartz, 1988; Sawtell and Lessard, 1989). Miano *et al.* (1994) carried out *in situ* hybridization analyses to determine whether SMHC transcripts are expressed in other tissues, including developing cardiac and skeletal muscle. *In situ* hybridization and RNase mapping analysis (Miano *et al.*, 1994; White *et al.*, 1993) showed that SM-MHC transcripts are not present in cardiac and skeletal muscle. The expression of SM-MHC transcripts was also not detectable in cell types, including endothelial cells, fibroblasts, skeletal muscle cell lines, and several rhabdomyosarcoma cell lines.

B. Smooth Muscle Development

We earlier reported that most adult smooth muscle tissues express both SM1 and SM2 myosins, although their ratio might differ between tissues (Nagai *et al.*, 1989). The developmental transition of MHC isoforms has been extensively investigated in rabbit vascular smooth muscle tissues (reviewed in Sartore *et al.*, 1994). Using RNase mapping and immunoblotting analyses, Kuro-o *et al.* (1991) have demonstrated that SM1 is expressed exclusively in developing fetal rabbit aorta. SM2 myosin appears only after birth and increases with development (Fig. 6). This differential expression of SM1 and SM2 has also been demonstrated by immunofluorescence histology using specific antibodies against the carboxyl termini (Fig. 7).

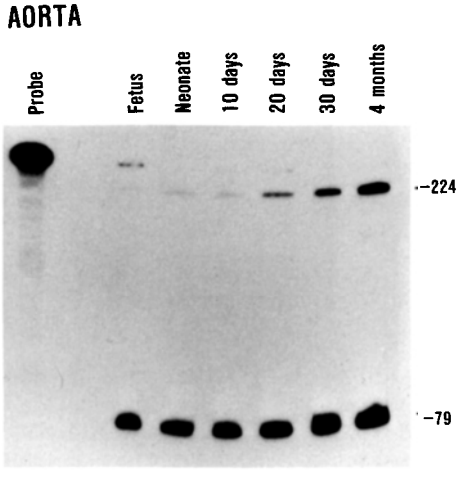


Fig. 6. Developmental expression o SM1 and SM2 mRNA in rabbit aort as determined by S1 nuclease mappin with a cDNA probe discriminatin these transcripts. SM1 mRNA is pos tive (represented as a band of 79 nt but SM2 mRNA appears after birt (bands at 224 nt).

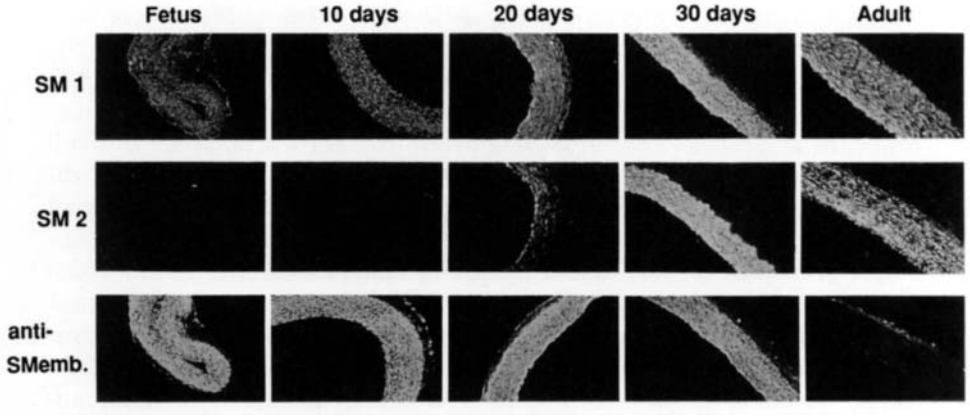


Fig. 7. Expression of three MHC isoforms in developing rabbit aorta. Antibodies specific for SM1, SM2, and NM-MHC-B/SMemb were utilized.

In addition, the nonmuscle MHC isoform (NM-MHC-B/SMemb) is predominantly expressed in embryonic and perinatal aortas (Kuro-o *et al.*, 1991; Zanellato *et al.*, 1990). In rabbits, a decrease in NM-MHC-A expression occurs after birth concomitant with an increase in SM2 expression (Fig. 7). Polyacrylamide gel analysis of myosin is not a useful technique to show the developmental changes in the smooth muscle MHC isoforms because NM-MHC isoforms comigrate with SM2 myosin on the gel around 200 kDa (Fig. 8). Immunoblot analysis using specific antibodies is necessary to distinguish between them. It is also clear from these studies in rabbit fetal aortas that NM-MHC-B is more abundant than SM1 and that the nonmuscle MHC isoform NM-MHC-A (196-kDa NMHC) is not present in significant amounts.

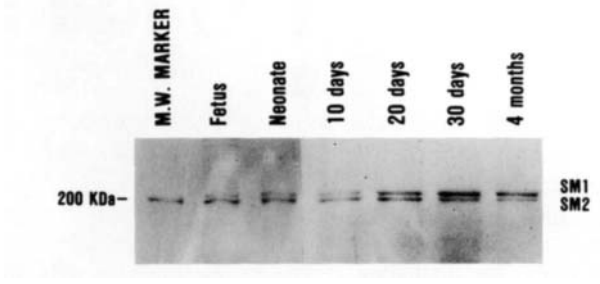


Fig. 8. Analysis of MHC isoforms in developing rabbit aorta on a 4% polyacrylamide gel. Although SM2 appears predominant in fetal aorta, the lower band is actually NM-MHC-B/SMemb which comigrates with SM2 in adult. NM-MHC-A (196 dKa) is not expressed at high levels in the fetus.

The physiological significance of MHC isoform transition in smooth muscle development is not understood. However, ultrastructural examination has revealed that embryonic vascular smooth muscles are rich in subcellular organelles, such as free ribosomes, and contain much less myofilaments than in adults. Therefore it is likely that switching from nonmuscle-type MHC to SM1 and SM2 reflects increased contractility in SM1- and SM2-positive smooth muscles.

As discussed in the chapter by Gittenberger de-Groot and colleagues, the ductus arteriosus shows rapid changes in the smooth muscle phenotype in the perinatal period. This may represent a molecular basis for the proposal relating the ultrastructure of smooth muscle cells to the cells ability to contract versus synthesis of matrix proteins (Campbell and Campbell, 1990). During vascular development, the ductus arteriosus SM2 myosin is expressed as early as the late fetal stage, when SM2 is negative in other arteries (Kim *et al.*, 1993). These data indicate that the differentiation of ductus smooth muscle cells is much earlier compared to other arteries, which may be part of the mechanism causing early closure of the ductus at birth.

The developmental regulation of smooth muscle MHC isoforms is not necessarily unique to the vascular system. The differential expression of SM1 and SM2 isoforms also occurs in developing intestinal smooth muscles (M. Periasamy and S. White, unpublished observations) (Fig. 9). In the intestinal smooth muscles, SM2 myosin appears earlier than in aorta.

C. Myosin Expression in Smooth Muscle Cultures

The expression of MHC isoforms in cultured smooth muscle cells differs according to the method of cell preparation and culture conditions. When smooth muscle cells were cultured by the explant method, they expressed exclusively nonmuscle MHC, and SMHC gene transcripts are undetectable (Kuro-o *et al.*, 1991). On the other hand, when primary cultures of smooth muscle cells were established by an enzymatic digestion method, SM1- and SM2-specific mRNA were detectable (Babij *et al.*, 1992). This study reported, however, that in primary cultures of smooth muscle cells the SM1 mRNA level decreased by 30% and that SM2 mRNA decreased by 80% when cells were grown in 10% fetal calf serum for 3–5 days (Fig. 10). SM1 and SM2 mRNA expression in these cells was not influenced by confluency or serum withdrawal. In contrast, nonmuscle type MHC (NM-MHC-A) was increased 10-fold in subconfluent cultures but increased only 3-fold higher than controls in quiescent cells. Effects of cell culture on smooth muscle phenotype are discussed at more length in the chapter by Gabbiani and colleagues. Of particular importance, this group has

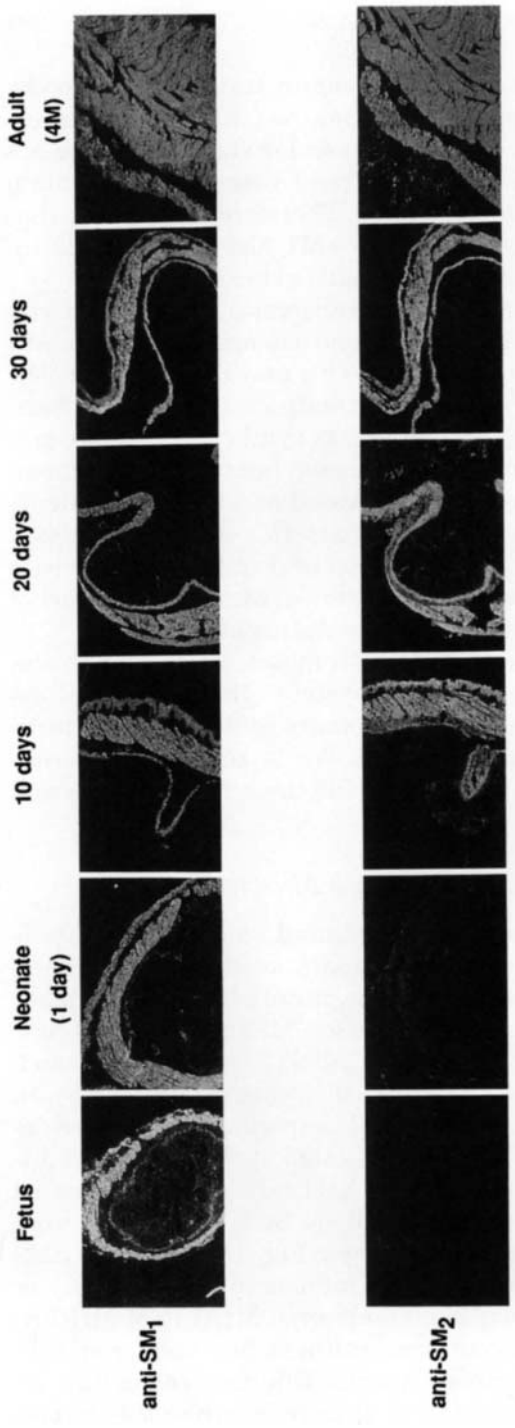


Fig. 9. Immunofluorescent histology for SM1 and SM2 in developing large intestines. As demonstrated in aorta, intestinal smooth muscles show differential expression of SM1 and SM2 during development.

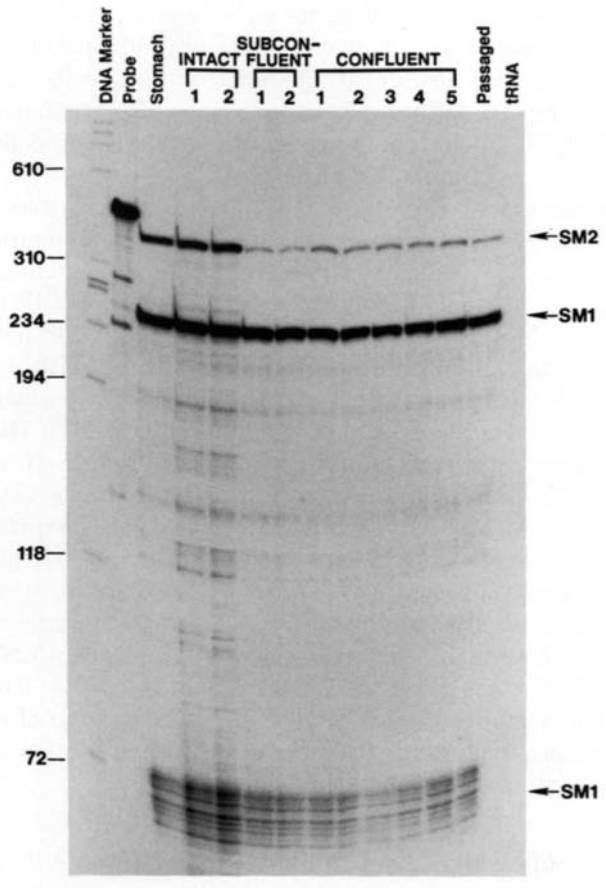


Fig. 10. RNase mapping analysis of SM1 and SM2 mRNA expression in rat aortic smooth muscle cells. SM2 mRNA expression is markedly decreased in culture.

isolated and cloned a smooth muscle cell line from newborn aortas. These clones maintain myosin expression even when passaged.

D. Myosin Expression in Vascular Disease

In skeletal and cardiac muscles, the expression of fetal-specific MHC genes is reinduced during certain pathophysiological conditions, and are considered as pathological markers for diseased muscles (Bandman, 1985). A similar reversal to fetal phenotype is also observed when smooth muscle cells proliferate in response to vascular injury. Smooth muscle cell proliferation is one of the most characteris-

tic features of arterio- and atherosclerosis, as well as in vascular restenosis following percutaneous transluminal coronary angioplasty (Austin *et al.*, 1985). Proliferating smooth muscle cells show a distinct phenotype from those in quiescence *in vivo*. The former is referred to as the synthetic phenotype whereas the latter is the contractile phenotype (Chamley-Campbell *et al.*, 1979).

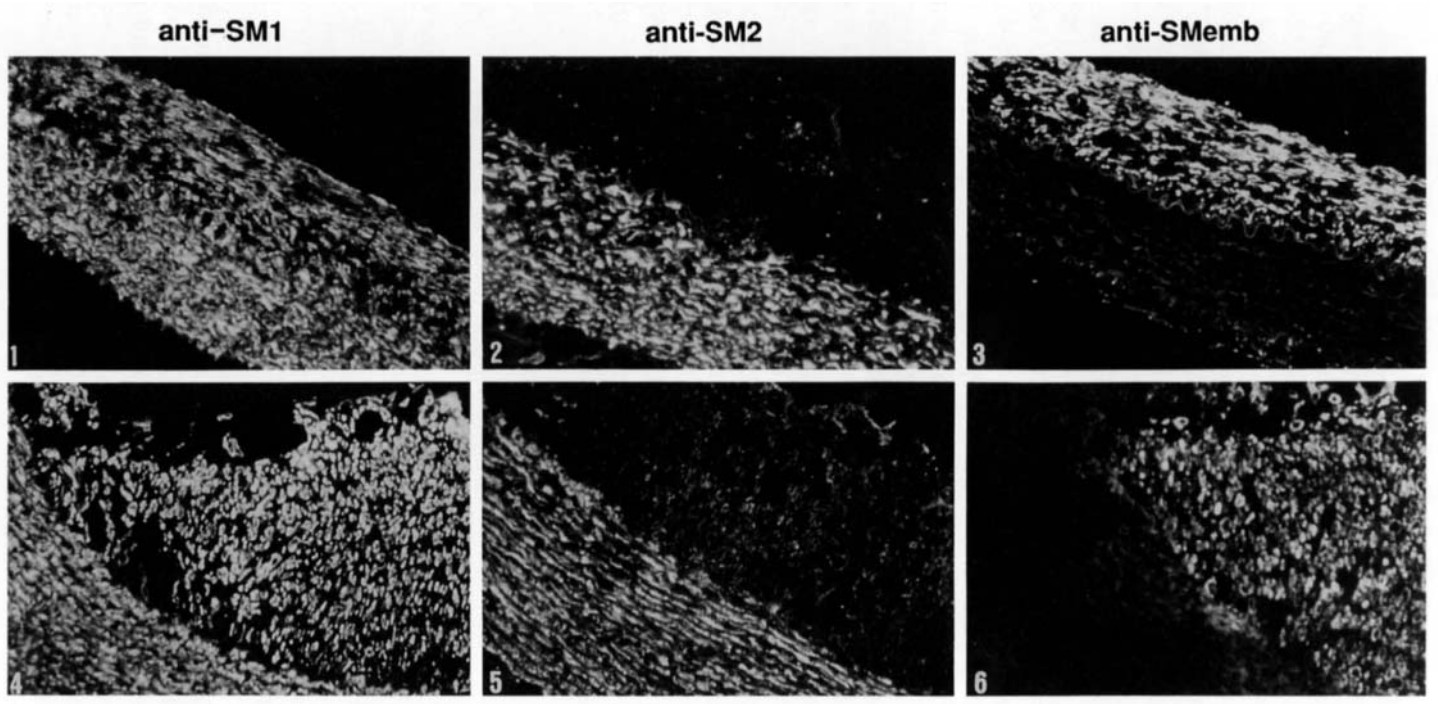
In a rabbit model, Zanellato *et al.* (1990) and Kuro-o *et al.* (1991) examined the MHC isoform expression in aortas following either balloon injury or 1% cholesterol feeding. Figure 11 demonstrates immunohistological analysis of three MHC isoforms (SM1, SM2, and NM-MHC-B/SMemb) in injured rabbit aortas. After balloon injury, smooth muscle cells proliferate in the neointima within a week and the neointimal thickness reaches maximum at 2–3 weeks. Interestingly, neointimal cells express only SM1 and NM-MHC-B/SMemb but not SM2, showing a marked similarity to embryonic smooth muscles. In contrast, rabbit medial smooth muscles express both SM1 and SM2 but not NM-MHC-B/SMemb. Therefore, MHC expression in rabbit vascular smooth muscle is a useful marker in distinguishing the contractile from the synthetic smooth muscle cells. In high cholesterol-fed rabbits, the proliferation of smooth muscle cells is also prominent in the neointima, and they also express both SM1 and NM-MHC-B/SMemb but not SM2. These studies on MHC isoforms in injured rabbit aortas suggest that the dedifferentiation of smooth muscles toward the embryonic phenotype is involved in the mechanisms underlying arterio- or atherosclerosis.

IV. Myosin Heavy Chains in the Human Vascular Smooth Muscle System

A. *Identification of Myosin Heavy Chains*

The studies described in this chapter on MHC expression in rats and rabbits clearly demonstrate that MHC isoforms might play important roles in vascular development and pathophysiology. However, these studies were carried out on animal models and may not be entirely applicable to humans. It is well known that the expression pat-

Fig. 11. Immunohistological expression of SM1, SM2, and NM-MHC-B/SMemb in injured rabbit aortas. Vascular injury was produced by endothelial denudation using a balloon catheter (top) or 1% cholesterol feeding (bottom). In both injury models, medial smooth muscles are positive for anti-SM1 and anti-SM2 antibodies but are negative against the anti-SMemb antibody. In contrast, intimal smooth muscles are positive for anti-SM1 and anti-SMemb antibodies, but negative against anti-SM2 antibodies.


anti-SM1
anti-SM2
anti-SMemb
1
2
3
4
5
6

tern of sarcomeric MHC isoforms often shows species difference between animals and humans as seen with cardiac ventricular MHC isoforms. In the rat, β-MHC is predominantly expressed in the fetal ventricle but is replaced by α-MHC in adult heart. On the other hand, in the human ventricle, β-MHC is constitutively expressed from the fetal stage to adult and α-MHC expression is not detectable. Therefore, in order to understand the pattern of MHC expression during normal human vascular development, characterization of human smooth muscle and nonmuscle MHC isoforms is essential. We have isolated and characterized three cDNA clones from human smooth muscle: SMHC94 (SM1), SMHC93 (SM2), and HSME6 (SMemb/NM-MHC-B) (Aikawa *et al.*, 1993). The cDNA clones SMHC94 (SM1) and SMHC93 (SM2) encode part of the light meromyosin region of the myosin molecule and have identical nucleotide and amino acid sequences except at the very carboxyl terminus where SMHC93 has a stretch of 39 extra nucleotides (encoding 9 amino acids). SMHC94 and SMHC93 are highly homologous to rabbit SM1 and SM2, showing 92 and 93% identity at the nucleotide level in the coding regions and 96 and 97% identity at the amino acid sequence level, respectively. As shown for rabbit smooth muscle MHC, the SMHC93 clone encodes a shorter carboxyl terminus with 9 unique amino acids specific for SM2 myosin and the SMHC94 cDNA encodes the SM1 myosin with 43 unique amino acids at the carboxyl end. The cDNA clone HSME6 (NM-MHC-B) has 92% nucleotide and 98% amino acid sequence identity with rabbit NM-MHC-B.

B. Myosin Expression in Normal and Diseased Human Blood Vessels

SMHC expression in developing vascular smooth muscle was examined by the S1 nuclease mapping analysis. This analysis showed that SM1 mRNA is constitutively expressed from the fetal to adult stage, but that the SM2 mRNA level is significantly low in the fetal aorta but increases after the perinatal stage (Aikawa *et al.*, 1993). On the other hand, the expression level of nonmuscle/SMemb mRNA in aortic smooth muscle was found to decrease after birth but seemed to increase again in the elderly. Using indirect enzymatic immunohistochemistry and antibodies, both SM1 and NM-MHC were detectable from the fetal stage to adult life in the media of the aorta and extramural coronary arteries, whereas a positive signal for SM2 was obtained only after birth. NM-MHC was not detected in the medial smooth muscles of smaller arteries, such as the vasa vasorum or intramural coronary arteries.

The intimal smooth muscles in coronary arteries reacted positively with SM1, SM2, and SMemb antibodies. However, the expression of SM2 was reduced in smooth muscles of nonatheromatous as well as atheromatous intima. These observations suggest that (1) human smooth muscle MHC isoforms are differentially expressed during vascular development, (2) the phenotype of medial smooth muscle cells may differ depending on the size and the type of arteries, and (3) phenotypically modulated intimal smooth muscle cells can be identified by altered MHC isoform expression, although it remains to be investigated whether the altered MHC expression correlates with the activation of cytokine or growth factor production. Simons *et al.* (1993) reported that abundant NM-MHC-B mRNA was expressed in human coronary atherosclerosis or restenotic lesions after PTCA treatment. Furthermore, using cultured human smooth muscle cells *in vitro*, they demonstrated that antisense to NM-MHC-B mRNA dramatically inhibited the cell growth (Simons and Rosenberg, 1992). These results suggest that nonmuscle myosin plays a pivotal role in smooth muscle cell proliferation.

V. Conclusion and Perspectives

Identification of smooth muscle-specific MHC isoforms and analysis of their expression have made significant contributions to our understanding of smooth muscle phenotypes in development and disease. Although the physiological significance of each of the myosin isoforms is not completely elucidated, their differential expression enables us to suggest that they may have distinct roles in smooth muscle development and physiology. The mechanisms regulating smooth muscle development and differentiation are poorly understood. We expect that the cloning and identification of MHC genes in smooth muscle will contribute to the dissection of the molecular mechanisms regulating smooth muscle myogenesis and gene expression. Molecular mechanisms regulating skeletal muscle determination and differentiation have been better understood with the discovery of a set of master regulatory genes, namely the MyoD family, which includes MyoD, myogenin, myf-5, and MRF-4, which are capable of inducing myogenesis when expressed in embryonic fibroblasts (Olson, 1990). However, the MyoD gene family or related helix-loop-helix transcription factors have not yet been identified in smooth muscles. This raises the possibility that there are other types of transcription factors involved in smooth muscle myogenesis and possibly even angiogenesis. Chandrashekhar *et al.* (1992) identified a homeobox transcription factor expressed in blood vessels at all stages of development. The precise role

of this homeobox factor on smooth muscle development and gene expression needs to be studied.

It is also important to investigate the mechanisms responsible for transcriptional activation of nonmuscle MHC genes NM-MHC-B and -A since their expression is tightly associated with smooth muscle cell proliferation and differentiation. It is anticipated that future research on the regulatory mechanisms of these MHC genes will contribute toward understanding the fundamental mechanisms regulating smooth muscle differentiation and the vascular disease process.

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Vascular Smooth Muscle-Specific Gene Expression

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I. Introduction

Among the important issues awaiting exploration in vascular smooth muscle are the identification of vascular smooth muscle-specific gene products and analysis of their tissue-specific regulation. Efforts to identify proteins unique to vascular smooth muscle have brought limited rewards to date, and studies addressing the fundamental nature of vascular smooth muscle tissue at the level of gene regulation remain preliminary. Nonetheless, there are several prominent protein products of vascular smooth muscle characteristically understood to mark this tissue type. The first portion of this chapter presents proteins associated with vascular smooth muscle as phenotypic markers; the second portion discusses several transcription factor families of possible importance in vascular smooth muscle cell gene regulation.

II. Smooth Muscle-Specific Genes

A. α-Smooth Muscle Actin

The α-smooth muscle actin is a characteristic and highly expressed protein in all smooth muscle tissues, including vascular smooth muscle tissues. The α-smooth muscle actin gene provides an appealing model for the study of vascular smooth muscle regulation of gene expression. The gene has been cloned in several vertebrate species, and several features are well conserved between species. There is no alternative splicing to increase the complexity of message generation. The mRNA is present in abundance in vascular smooth muscle tissues, and vascular smooth muscle cells in culture and changes in steady-state levels are therefore easily seen. Finally, this mRNA and protein has often been used as a marker of the vascular smooth muscle phenotype, and there is an abundance of data regarding agents which will alter α-smooth muscle actin steady-state mRNA levels and whose actions at the transcriptional and/or post-transcriptional levels may therefore be examined.

1. α-Smooth Muscle Actin and the Actin Gene Family

The actin family of proteins is highly conserved in amino acid sequence in all eukaryotes (Vandekerckhove and Weber, 1981, 1984). Despite the structural and functional similarities of these proteins, most organisms possess multiple actin isoforms with a unique cell type, an intracellular compartment, and developmental stage-specific distributions. At least seven actin isoforms are present in the chicken (Chang *et al.*, 1984; Bergsma *et al.*, 1985) and six in higher mammals (Weber, 1978). The three α-actins are the major isoforms found in sarcomeres of skeletal, cardiac, and vascular smooth muscle tissues. They are named after the tissues in which they were originally associated in the greatest abundance: α-cardiac, α-skeletal, and α-(vascular) smooth muscle actin. Each of the actin isoforms is encoded by a separate gene, and each gene is controlled by its own DNA regulatory sequences. These genes probably evolved by a series of gene duplication events of a primitive α-cardiac gene during the appearance of amphibia and early reptilia. The α-actin genes represent dispersed actin genes found on separate chromosomes: in the human, α-smooth muscle actin is on chromosome 10 (Ueyama *et al.*, 1990), α-skeletal actin is on chromosome 1, and α-cardiac actin is on chromosome 15 (Gunning *et al.*, 1984).

Carroll *et al.* (1986) published the sequence of the chicken α-smooth muscle actin gene and it remains the only published fully sequenced α-smooth muscle actin gene. Genomic clones of α-smooth muscle actin

have been reported from mouse (Min *et al.*, 1990), rat (Blank *et al.*, 1992), and human (Nakano *et al.*, 1991; Reddy *et al.*, 1990). These have been characterized sufficiently to show that the overall genomic organization is well conserved. The principal features include a small, untranslated first exon with a well-conserved primary sequence, a >-kb first intron, and a small second exon combining a further untranslated sequence and the initial coding sequences. There are nine exons overall, and the common origin of this gene with the other α-actin genes is demonstrated by several shared intron–exon borders. In the chicken the mRNA 3′ UTR is known to have multiple polyadenylation signals of no known regulatory significance, a feature not shared by the other α-smooth muscle actin genes examined to date. The primary sequence of the 3′ UTR is not well conserved between chicken α-smooth muscle actin and the mammalian α-smooth muscle actins.

Several authors have reported cell culture data showing a variation in α-smooth muscle actin mRNA and/or protein content in response to manipulations of vascular smooth muscle cells in culture (Table I). As of this time there are no studies localizing these effects to specific genomic regulatory elements. Some of the observed effects are likely to be post-transcriptional in nature (Corjay *et al.*, 1992).

TABLE I

KNOWN MODULATORS OF α-SMOOTH MUSCLE ACTIN PROTEIN OR MRNA LEVELS IN VASCULAR SMOOTH MUSCLE CELL CULTURE

Agent	Observed effect	Ref.
Angiotensin II	Increased protein and mRNA levels	Turla *et al.* (1991)
Arginine vasopressin	Increased protein and mRNA levels	Turla *et al.* (1991)
Epidermal growth factor	Decreased protein levels	Björkerud (1991)
Fetal bovine serum	Decreased protein levels	Owens *et al.* (1986); Blank *et al.* (1988)
Fibronectin substrate	Decreased protein and mRNA levels	Hedin *et al.* (1990)
Growth arrest	Increased protein levels	Owens *et al.* (1986)
Heparin	Increased protein and mRNA levels	Desmouliere *et al.* (1991); Clowes *et al.* (1988)
Heparinase	Decreased mRNA and protein levels	Campbell *et al.* (1992)
Platelet-derived growth factor (AB and BB)	Decreased protein and mRNA levels	Blank *et al.* (1988); Corjay *et al.* (1992)
Transforming growth factor β	Increased protein levels; substrate-dependent effects on mRNA levels	Blank *et al.* (1988); Kocher and Madri (1989); Björkerud (1991)

2. α-Smooth Muscle Actin Promoter Mapping

The proximal promoter region of α-smooth muscle actin genes shows 70 to 90% homology between species with 100% homology of some readily defined *cis* elements. These include two serum response elements (SREs) and the TATA box, all three of which are found in the first 120 bp. There is complete core and immediate flanking sequence conservation of the SRE elements and the interelement spacing is within 1 bp of identical in all four species. This striking degree of conservation suggests that the spatial relationships of the two SREs with each other and with the TATA box may be of great importance. Transcriptional dependence on multiple SRE elements appears to be a common feature of α-actin promoters (Chow and Schwartz, 1990; Lee *et al.*, 1991; Miwa and Kedes, 1987).

Initial studies of chicken α-smooth muscle actin promoter function were performed in chick embryo fibroblasts and skeletal myoblasts (Carroll *et al.*, 1988). The presence of an important negative regulatory element (NRE1) as well as tissue specificity of regulation of sequences upstream of NRE1 has been demonstrated. Blank *et al.* (1992) subsequently studied the chicken α-smooth muscle actin promoter in rat aortic vascular smooth muscle cells (VSMCs), bovine endothelial cells, and chicken aortic VSMCs derived from adult and day 19–20 embryonic chickens. Their findings indicated that the chicken α-smooth muscle actin promoter is tissue restricted in its expression (no expression in bovine aortic endothelial cells) and that upstream elements in the promoter are differentially utilized between cell types. In particular, they showed that NRE1 appeared to have no regulatory effect in chicken VSMCs and that rat VSMCs appeared to be especially sensitive to a positive regulatory element between -209 and -257.

Promoter mapping of the mouse α-smooth muscle actin gene has also been reported (Foster *et al.*, 1992; Min *et al.*, 1990). These studies indicated the presence of upstream sequences between -374 and -1074 that mediated upregulation of the promoter in response to cell density increases in BC3H1 cells. This region of the mouse promoter is in large part dissimilar in sequence to the chicken promoter. However, they also pointed to a multipartite *cis* element between -191 and -224 which possessed separable elements, one of which restricted expression in AKR-2B fibroblasts and the other of which promoted expression in BC3H1 cells. This region of the mouse promoter is well within the highly conserved region noted in all species and may indicate a region important for vascular smooth muscle-specific regulation of this gene.

In related analysis of the mouse α-smooth muscle actin promoter in BC3H1 cells, Foster *et al.* (1992) showed that the ~400-bp span be-

tween -330 and -724 appears to confer upregulation at growth arrest, which mimics the endogenous pattern of a α-smooth muscle actin gene regulation in these cells. In a previous publication from the same laboratory, Min *et al.* (1990) showed that the first intron also contained regulatory sequences capable of increasing expression after transient transfection into BC3H1 cells.

A possibly important insight into the regulation of the α-smooth muscle actin gene was contributed by the observation that *ras*-transformed fibroblast cells repress α-smooth muscle actin expression and lose repression upon reversion (Kim *et al.*, 1994; Kumar *et al.*, 1992). This effect could be overcome by forced expression of serum response factor (SRF, see later), the prototype cognate-binding factor for the serum response element. These findings seem to confirm the importance of SRF for α-smooth muscle actin expression and may also implicate a specific intracellular intermediate, Ras, for signals mediating downregulation of the gene.

3. Distribution of α-Smooth Muscle Actin Expression

One of the difficulties associated with α-smooth muscle actin as a model gene for analysis of vascular-smooth muscle specific gene expression is that it is not a tightly restricted gene in its pattern of expression (Table II). Inspection of Table II leads to the conclusion that this gene is capable of being induced in at least a large subset of mesenchymal tissues, including myofibroblasts, under the appropriate conditions. The conditions most highly associated with expression outside of smooth muscle tissues are transitional embryological states (especially of differentiating striated muscle tissues), injury responses of differentiated tissues, and growth of cells in culture. The propensity of multiple mesenchymal cells in culture to express this gene makes the interpretation of transcriptional studies of α-smooth muscle actin in vascular smooth muscle cells difficult to assess; is the expression of α-smooth muscle actin in smooth muscle cells *in vitro* a reflection of a differentiated smooth muscle cell phenotype or is it reflective of a more primitive "stem cell" phenotype? Will the transcriptional pathways to α-smooth muscle actin gene expression prove to be different between differentiated vascular smooth muscle cells and other α-smooth muscle actin-expressing cells? Other α-actin genes expressed in multiple tissue types do indeed have separate transcriptional pathways in separate tissues that only become apparent with very close *cis* element analysis (Moss *et al.*, 1994). Data showing distinctive pathways of promoter utilization between AKR-2B fibroblasts and BC3H1 cells are suggestive that similar mechanisms are present in the α-smooth muscle actin promoter as well.

TABLE II

SITES OF α-SMOOTH MUSCLE ACTIN GENE EXPRESSION

Location	Ref.
Smooth muscle tissues	Vandekerckhove and Weber (1981)
Embryonic tissues	
Heart	
Myocardium	Ruzicka and Schwartz (1988); Sawtell and Lessard (1989); Sugi and Lough (1992)
Somites	Sawtell and Lessard (1989)
Lung	Leslie *et al.* (1990); Roman and McDonald (1992)
Ventrolateral mesoderm (Xenopus)	Saint-Jeannet *et al.* (1992)
Soft tissue tumors	Nanni *et al.* (1991); Jones *et al.* (1990)
Myofibroblasts	Sappino *et al.* (1990)
Cells in culture	
Smooth muscle cells	Vandekerckhove and Weber (1981)
Embryonic fibroblasts	
Chick embryo fibroblasts	Vandekerckhove and Weber (1981)
NIH-3T3	Sharp *et al.* (1992)
10T1/2	Sharp *et al.* (1992)
Cardiac myocytes	Eppenberger-Eberhardt *et al.* (1990)
Skeletal myoblasts	
BC3H1	Strauch and Rubenstein (1984)
C2C12	Sharp *et al.* (1992)
L6	Pinset and Whalen (1984)
Miscellaneous	
Bone marrow stroma	Peled *et al.* (1991); Galmiche *et al.* (1993)
Astrocytes	Lecain *et al.* (1991)
Lens forming cells	Schmitt-Gräff *et al.* (1990)
Hepatic Ito cells	Ramadori *et al.* (1990)
Cardiac fibroblasts	Brouty-Boye *et al.* (1992)
P 19 embryonal carcinoma cells	Rudnicki *et al.* (1990)
Mesangial cells	Elger *et al.* (1993)
Transformed fibroblasts	Segura and Saborio (1982); Nanni *et al.* (1991); Sappino *et al.* (1990)

B. Elastin

The expression of elastin in the great vessels is interesting for several reasons. The onset of elastin expression in the great vessels of chick embryos has been proposed to derive from an interaction between neural crest-derived mesenchymal cells and the "myocardial cuff" of the truncus arteriosus (Rosenquist *et al.*, 1990a,b). Elastin mRNA production is initiated at the level of the truncus arteriosus and then proceeds distally through the great vessels in the chick embryo (Selmin *et al.*, 1991). Elastin protein content in the mammalian

(sheep) aorta increases dramatically in the immediate perinatal period, prior to the actual increase in blood pressure associated with adaptation to the postnatal circulation (Bendeck and Langille, 1991). Elastin gene expression is known to decline with aging in the aorta and a possible relationship between declining elastin levels with declining levels of IGF-1 has been proposed, as IGF-1 upregulates elastin gene expression *in vitro* (Rich *et al.*, 1992) and *in vivo* (Foster *et al.*, 1990); circulating IGF-1 levels are known to vary in parallel with elastin gene production in rats (Foster *et al.*, 1990) and chick embryos (Foster *et al.*, 1989). The upregulation induced by IGF-1 is at least partially transcriptional in origin as determined by both transient transfection assays (Rich *et al.*, 1992) and nuclear run-on assays (Badesch *et al.*, 1992). Interestingly, the influence of IGF-1 on elastin gene expression may be restricted to vascular tissue during development (Foster *et al.*, 1989).

1. Elastin Gene Structure

Elastin is encoded by a single gene in all animals analyzed to date (cow, chicken, human, rat) (Yeh *et al.*, 1989; Pollock *et al.*, 1990; Bashir *et al.*, 1989; Pierce *et al.*, 1992). Several structural features of elastin genes are shared between species and deserve comment. Elastin genes are unusual in that they have a large number of individually small exons interspersed among large introns; consequently, there is a much higher ratio of intron DNA:exon DNA (roughly 20:1) over the 40–45 kb of an elastin gene than is normally seen. Although elastin exons are not uniform in size, all elastin exons contain multiples of three nucleotides and all end on the second nucleotide of a codon and begin with the third nucleotide of a codon. This permits the exons to undergo multiple splicing in a cassette-type fashion without fear of incompatibility in splice sites. Finally, the intronic sequences are approximately 60% G+C rich, an unusually high amount. The elastin protein contains multiple hydrophobic domains and cross-linking domains, and exons encoding for hydrophobic protein domains generally alternate with exons encoding cross-linking domains.

There is significant conservation of sequence in elastin promoter regions (Manohar *et al.*, 1991). The elastin promoters are G+C rich and demonstrate multiple transcriptional start sites. Controversy exists whether the ATAA sequence 30 bp upstream of the principal transcriptional start site is a functional TATA box. If not, then the elastin gene would be an unusual example of a TATA-less tissue-restricted promoter.

2. Elastin Gene Expression and Regulation

The principal sites of elastin gene expression are the smooth muscle cells of large blood vessels and the fibroblasts of skin, lung, and ligament. Multiple isoforms of elastin mRNA are demonstrable in these tissues, but none are restricted to a specific tissue. Elastin mRNA isoform distribution in specific tissues also varies according to the developmental stage of the organism, but once again there are no transcripts unique to a specific stage of development (Heim *et al.*, 1991). Elastin isoforms are generated by alternative splicing (Pierce *et al.*, 1992; Heim *et al.*, 1991). Elastin mRNAs are well conserved in primary sequence between mammalian species, including the 3′ UTR sequences (Rosenbloom *et al.*, 1991). Sequence conservation in noncoding sequences suggests the presence of a regulatory role, and 3′ UTR sequences are most likely to be involved in mRNA stabilization. In this light it is interesting to note that phorbol ester downregulates elastin mRNA levels by shortening the mRNA half-life in chondrocytes from 20 to 2.2 hr (Parks *et al.*, 1992) whereas transforming growth factor B (TGF-β) increases elastin mRNA half-life in human skin fibroblasts (Kähäri *et al.*, 1992a).

A number of transcription regulatory motifs have been identified in the sequence of the human elastin promoter, including multiple putative Spl sites, cyclic AMP response element (CRE) sites, glucocorticoid-responsive elements, and TPA (phorbol ester)-responsive elements. Deletion mutations in this promoter have been assayed in rat aortic smooth muscle cells. An alternating pattern of negative and positive regulatory sequences was found, with the highest level of promoter activity found in a fragment encompassing bp -380 to -1. An internal deletion of a 47-bp span containing three presumed Spl sites (from -87 to -134) resulted in a 80 to 90% downregulation of promoter function when compared to a promoter without the deletion. The largest segment of 5′ flanking DNA examined for regulatory function was 2.2 kb in length; as appreciable expression of this construct was seen in two cell lines, NIH-3T3 and HeLa, which do not express the endogenous elastin gene, it was inferred that tissue specificity was a function determined by as yet unidentified *cis* elements (Novy *et al.*, 1990).

IGF-I has been associated with increased elastin protein synthesis and steady-state mRNA levels in embryonic rat aorta tissue culture, rat aorta smooth muscle cells, and bovine pulmonary artery smooth muscle cells in culture (Foster *et al.*, 1987; Rich *et al.*, 1992; Badesch *et al.*, 1992). *In vivo* steady-state mRNA levels of elastin have been shown to vary in parallel with the normal raising and lowering of serum IGF-I levels during embryogenesis and aging (Foster *et al.*,

1989). IGF-I added exogenously *in vivo* increases aortic elastin mRNA content and protein content (Foster *et al.*, 1990). The upregulation of elastin mRNA levels *in vitro* is accomplished by physiologic levels of IGF-I (from 0.2 to 3×10^{-9} *M*) in vascular smooth muscle cells only; pulmonary fibroblasts also respond to IGF-I with increased elastogenesis but require exaggerated doses ($0.5-1 \times 10^{-7}$ *M*) (Rich *et al.*, 1992; Noguchi and Nelson, 1991).

The effect of IGF-I on elastin steady-state mRNA levels is exerted at the transcriptional level (Wolve *et al.*, 1993). Two negative *cis* elements located between -165 and -137 of the human elastin promoter were shown to be the presumptive sites through which regulation was achieved. A deletion construct lacking these sites was constitutively active. In the absence of IGF-I, constructs containing these sites were expressed at low levels. The addition of IGF-I caused the same promoters to be activated to levels seen when the sites were deleted. Footprint analysis showed that two sites that were protected in the promoter in the absence of IGF-I administration were no longer protected in the presence of IGF-I, suggesting that one or both of the proteins responsible for the footprints were negative regulators of elastin gene expression.

Several other agents have been shown to alter elastin steady-state mRNA levels in aortic smooth muscle cells. TGF-β in particular has been shown to upregulate elastin mRNA levels by transcriptional and post-transcriptional mechanisms (Kähäri *et al.*, 1992a,b; Katchman *et al.*, 1994; Marigo *et al.*, 1993). Glucocorticoids reportedly upregulated elastin synthesis in aortic tissue culture (Eichner and Rosenbloom, 1979) whereas 1,25- dihydroxyvitamin D_3 and EGF have both been shown to downregulate elastin mRNA levels (Hinek *et al.*, 1991; Ichiro *et al.*, 1990). Interleukin-1β and interleukin-10 have been reported to upregulate the elastin promoter in dermal fibroblasts *in vitro* and *in vivo* (Mauviel *et al.*, 1993; Reitamo *et al.*, 1994)

3. Elastin, Williams Sydrome, and Supravalvar Aortic Stenosis

Abnormalities of the elastin locus on the long arm of human chromosome 7 (Fazio *et al.*, 1991) have been linked to two human diseases, supravalvar aortic stenosis and Williams syndrome. SVAS is characterized by abnormal narrowing of the sinotubular junction of the aorta; other vessels, especially the pulmonary arteries, may also be affected. The histologic findings have been shown to include disorganized smooth muscle hyperplasia and an abnormal connective and elastic tissue matrix with loss of the normal highly organized and parallel nature of elastic fiber and smooth muscle layers. The vascula-

ture of individuals with Williams syndrome is similarly affected, but they also suffer additional features which include infantile hypercalcemia, a characteristic facial appearance, and mental retardation (Jones, 1990).

Supravalvar aortic stenosis is in most cases a hereditary vascular disorder with an autosomal dominant mode of inheritance. Ewart *et al.* (1993b) demonstrated linkage of SVAS to the ELN (elastin) locus in two unrelated families, making elastin a candidate gene for the etiology of this condition. Curran *et al.* (1993) subsequently demonstrated a translocation breakpoint disrupting the elastin gene at exon 28 in an affected family with SVAS and, thereby, directly linked an elastin gene abnormality to the SVAS phenotype.

Because of the similarities in cardiovascular pathology found in Williams syndrome and SVAS, it has often been proposed that these conditions were genotypically similar. Individuals meeting the minimal diagnostic criteria for Williams syndrome were present in two of the three kindreds described by Ewart *et al.* (1993b) and Curran *et al.* (1993) in their work linking the ELN locus to the SVAS phenotype, leading to the conjecture that the two conditions were both specifically related to elastin gene defects. This conjecture was strengthened by the demonstration that both sporadic and familial Williams syndrome patients are hemizygous for abnormal elastin alleles (Ewart *et al.*, 1993a). The elastin locus was hemizygous in nine out of nine William syndrome patients reported, and the evidence suggested that the abnormal allele was a microdeletion of the elastin gene and flanking regions, at least 140 kb in size, creating a contiguous gene syndrome.

The implications of these two genotypes resulting in similar phenotypes are challenging to explain. In SVAS there are two alleles, one of which is abnormal. The mutated elastin locus of SVAS presumably leads to either abnormal or insufficient mRNA production. The result is therefore either an insufficient quantity and/or qualitatively abnormal elastin protein production with subsequent abnormalities in elastin fiber formation. This model has been well characterized in molecular analysis of the phenotypic abnormalities of Marfan's syndrome, another autosomal dominant disease with a prominent vascular phenotype due to an abnormal connective tissue protein (fibrillin) allele (Dietz *et al.*, 1991).

In Williams syndrome, one allele is presumably normal and the second allele is deleted in its entirety, along with contiguous DNA with an unknown number of additional genes. This implies that a single functional elastin gene is unable to produce enough elastin mRNA for normal elastin fiber production in the aorta and that the relative deficiency of elastin fibers is the initiating factor for the phenotype.

These two diseases, therefore, have in common pathologic abnormalities of arterial vasculature and demonstrated anomalies of elastin gene loci. The pathologic abnormalities are seen in complete deletion as well as subtler abnormalities of elastin loci indicates that either qualitative or quantitative defects in elastin gene expression may be mechanisms through which elastin gene abnormalities may create the phenotype of SVAS. Left unanswered, however, is the question of why the phenotype is limited to the ascending aorta and other large arterial structures when the expression of the elastin gene is present at appreciable levels in other tissues that are phenotypically indistinguishable from normal in these individuals. A partial answer may be suggested from the apparent function of the elastin network in the great vessels. These vascular tissues are subjected to high levels of pulsatile wall stress and the elastin network, in combination with other extracellular matrix elements, is an adaption to maintain structural integrity in this mechanically stressed environment. A suggested hypothesis is that the lack of a normal elastin network may result in the transmission of abnormal mechanical stresses and/or abnormal extracellular matrix signaling to the smooth muscle cells of the large vessels, leading to abnormal growth characteristics. In support of this conjecture, abnormal extracellular matrix molecule production has been linked *in vivo* to an abnormal vascular smooth muscle proliferative state by the association of deletions of the α5 (IV) and α6 (IV) collagen genes with diffuse leiomyomatosis in Alport's syndrome (Zhou, J., *et al.*, 1993a). Specific evidence of an abnormal trophic environment for the affected tissues in supravalvar aortic stenosis is provided by the observation of increased quantities of PDGF in these tissues (Ewart *et al.*, 1993b).

C. *Smooth Muscle Myosin Heavy Chain*

Myosin heavy chains are encoded by a large multigene family [for a review of myosin diversity, see Titus (1993)]. Most of the isoforms display tissue and developmental stage specificity of expression. Two isoforms appear to be limited in their *in vivo* expression to smooth muscle tissues (Babij and Periasamy, 1989). These two isoforms, SM1 and SM2, are encoded by the same gene and thus represent products of alternative splicing (Babij and Periasamy, 1989). SM1 has also been shown to exist in two alternatively spliced isoforms (SM1B and SM1A) differing by the presence of an additional seven amino acids at the 25/50-kDa junction region in the SM1B isoform (White *et al.*, 1993). SM1 and SM2 encode proteins of 204 and 200 kDa respectively. The two isoforms are developmentally regulated, with the SM1 iso-

form predominating in early development and the SM2 isoform becoming predominant subsequently (Kuro-o *et al.*, 1989). The isoforms switch in relative abundance in atherosclerotic intimal proliferation, in which the SM1 isoform again predominates (Kuro-o *et al.*, 1991). The difference in function between the two isoforms is unknown.

The smooth muscle myosin heavy chain promoter region has been cloned. It is a GC-rich promoter with a classic TATA sequence. The initial analysis suggests the presence of a smooth muscle-specific enhancer region between -1223 and -1548 bp from the transcription initiation site (Katoh *et al.*, 1994). A protein–DNA interaction was identified within this region over a site with similarities to a MEF2 binding site (see later). However, data suggest that this protein was not MEF2 in that a typical MEF2-binding site from the MCK (muscle creatine kinase) enhancer was unable to compete for binding and a MEF2 antibody did not interact with the protein in gel shift analysis.

D. Miscellaneous Gene Products Associated with Vascular Smooth Muscle

Several muscle-associated proteins produce vascular smooth muscle-specific isoforms through alternative splicing, including vinculin/*meta*-vinculin (Koteliansky *et al.*, 1992). Na^+,K^+ ATPase α-1 (Medford *et al.*, 1991), α-tropomyosin (Wieczorek *et al.*, 1988), β-tropomyosin (Helfman *et al.*, 1986), and L-type dihydropyridine-sensitive voltage-dependent calcium channel (Koch *et al.*, 1990), to name but a few. Alternative splicing as a mechanism of generating vascular smooth muscle-specific mRNA isoforms has been examined using the α-tropomyosin gene as a model (Goodling *et al.*, 1994). In the α-tropomyosin gene the presence of exon 2 and the absence of exon 3 is specific for vascular smooth muscle, whereas the presence of exon 3 and the absence of exon 2 is the pattern in all other tissues. In their analysis of this pattern of expression, Goodling *et al.* (1994) showed that vascular smooth muscle cells exclude exon 3 even in the absence of exon 2 and further defined two intronic regions flanking exon 3 which were both required for directing smooth muscle-specific splicing.

The number of cloned gene products from vascular smooth muscle is increasing steadily and each of them is a possible source of insight into vascular smooth muscle-specific transcription. However, none of these gene products have been definitively demonstrated to be absent from myofibroblasts [reviewed in Sappino *et al.* (1990) and this volume] or other nonmuscle cells; some of them have alternatively spliced products that in part distinguish between smooth muscle-spe-

cific expression and nonmuscle expression (particularly caldesmon). Nonetheless, the following cloned products at the present time seem promising candidates for analysis: calponin (Takahashi and Nadal-Ginard, 1991; Gimona *et al.*, 1990, 1992), caldesmon (Wang and Rubenstein, 1992; Bryan *et al.*, 1989; Haruna *et al.*, 1993), calvasculin (Watanabe *et al.*, 1992), myosin regulatory light chain 2 (Grant *et al.*, 1990; Kumar *et al.*, 1989), SM22 (Gimona *et al.*, 1992), and CHIP28 (Shanahan *et al.*, 1993).

III. Transcription Factors of Potential Importance in Regulation of the Smooth Muscle Phenotype

No transcription factors at this time are demonstrated to be specifically limited to or fundamentally important in the regulation of the vascular smooth muscle phenotype. However, considerable progress has been made in the understanding of the transcriptional regulation of the skeletal muscle phenotype, and some important insights have been gained in understanding the transcriptional regulation of the cardiac muscle phenotype. Some of the concepts and specific transcription factors that have been demonstrated in these tissues are expected to be important in the regulation of vascular smooth muscle phenotype. This section discusses the transcription factors and conceptual models found to be important in understanding striated muscle differentiation, which might be expected to be important in vascular smooth muscle cell differentiation as well.

A. Transcriptional Models of Striated Muscle Differentiation

The cell type-specific transcription of muscle-specific promoters has been shown to rely on a complex interaction between tissue-specific transcription factors and ubiquitous transcription factors. A limited number of families of transcription factors have been identified which contribute to muscle specificity. Members of the MADS box transcription factor family are dependent on A/T-rich *cis* elements for creating muscle-specific transcription. In skeletal muscle-specific transcription, the MADS box proteins appear to be of secondary importance to the family of basic helix-loop-helix (hBLH) transcription factors. Data show that homeodomain proteins are likely to play a fundamental role in differentiation in cardiac muscle and that MADS box transcription factors may also be of some importance. bHLH proteins are present in the early heart but their importance is unclear (Srivastava *et al.*, 1994).

B. MADS Box Transcription Factors

1. SRF AND THE SRE

Serum response factor (SRF) is a widely occurring 67- kDa phosphoprotein which homodimerizes prior to binding to DNA (Norman *et al.*, 1988). Phosphorylation occurs at multiple sites, and phosphorylation by casein kinase II is specifically important for permitting DNA binding. SRF is capable of activating transcription *in vivo* and *in vitro* through amino acid sequences in its C terminus (Lee *et al.*, 1992; Prywes and Zhu, 1992).

SRF was the first vertebrate member of the MADS box transcription factor family to be characterized. The MADS box is a protein motif found in all three kingdoms of eukaryotic life. The MADS acronym refers to the transcriptional regulating proteins MCM1, ARG80/AG, DEFA, SRF; these proteins were described in yeast, yeast/snapdragon, Arabidopsis, and human, respectively (Jarvis *et al.*, 1989; Ma *et al.*, 1991; Dubois *et al.*, 1987; Sommer *et al.*, 1990; Norman *et al.*, 1988). All share a high degree of homology across a 56 amino acid domain which provides both DNA-binding capacity and dimerization capacity to these proteins.

The serum response element (SRE) is the target binding site of SRF. It was originally recognized as the essential core of that portion of the c-*fos* promoter associated with serum responsive transcription. Continued analysis of DNA–protein interactions of this sequence resulted in the identification and cloning of SRF (Norman *et al.*, 1988). The sequence motif CC $(A/T)_6GG$ defines the core of the SRE, and SREs have subsequently been identified in a large number of promoters of two general categories of genes. As might be anticipated, SREs are important in immediate early response promoters such as the c-*fos* promoter. They are also essential elements of the α-actin striated muscle-specific genes (Miwa and Kedes, 1987; Moss *et al.*, 1994; Lee *et al.*, 1991). In both settings, SREs have been associated with a high degree of complexity of transcription factor association.

The complexity of the c-*fos* SRE has been reviewed by Treisman (1992). This element consists of overlapping binding sites for no less than six independent proteins. In addition to SRF there are binding sites for the transcription factors YY1, Phox1, NF-IL6, SRE-ZBP, and E12. With the exception of [Phox1 + SRF], the binding of these proteins is presumably mutually exclusive to the binding of SRF and possibly with each other. *In vivo*, two additional Ets transcription factor family proteins, Elk-1 and SAP-1, bind to the SRE in SRF-dependent fashion; the association of Phox1 with SREs *in vivo* may also be SRF dependent (Grueneberg *et al.*, 1992). Although discrete roles for this

panoply of transcription factors are not in all cases known, it is clear that the c-*fos* SRE has promoter regulatory features more complex than simply providing "the place where SRF sits."

The α-actin SREs similarly demonstrate significant complexity in transcription factor association. Both the α-skeletal muscle actin and the α-cardiac muscle actin promoters possess multiple SREs (Lee *et al.*, 1991; Moss *et al.*, 1994). The SREs are nonequivalent in structure and function. In the α-skeletal actin SRE1, a binding site competition exists between YY1 and SRF, and YY1 functions as a repressor of gene activation which is overcome when SRF occupies the SRE (Lee *et al.*, 1992). This mechanism appears to be an essential switch for upregulation of the α-skeletal actin gene during myogenesis. In the chicken α-cardiac actin promoter, two of the SRE elements are overlapped with binding sites (E-boxes) for bHLH factors. The resulting complex elements are recognized by different factors between skeletal muscle and cardiac muscle cells and appear to provide different pathways for promoter activation between the two lineages (Moss *et al.*, 1994).

SRF is present in vascular smooth muscle tissues from early in development (Schwartz and Croissant, unpublished data), and the SRE motif appears to be an indispensable *cis* element for α-smooth muscle actin promoter activation in embryonic vascular smooth muscle cells (McQuinn and Majesky, unpublished observations). SRF is associated with the muscle-specific transcription of at least two other promoters. SRF is highly expressed in precardiac mesoderm and the developing heart (Croissant and Schwartz, unpublished observations) and appears to be an essential transcription factor for cardiac expression of the α-cardiac muscle actin promoter. SRF is required for the skeletal muscle-specific regulation of the α-skeletal actin promoter as well. At the present time it seems reasonable to speculate that SRF is required for the basal expression of α-smooth muscle actin in vascular smooth muscle as well as a more general role as a mediator of the immediate early response in vascular smooth muscle tissues.

2. MEF2

MEF2 transcription factors are a subset of the MADS box family of DNA-binding proteins. They are defined by the presence of a 29 amino acid domain continuous with the carboxy-terminal end of the MADS box domain. The discrete function of the MEF2 domain is unknown. The consensus sequence recognized by all MEF2 proteins to date conforms to the consensus sequence $CTA(A/T)_4TAG$ (Pollock and Treisman, 1991).

MEF2 was originally described as a DNA-binding activity present in differentiating myotubes. The specific recognition sequence used to identify this activity was an A/T-rich sequence from the MCK enhancer. The presence of this DNA-binding protein was induced at myotube fusion and by serum withdrawal, and could be blocked by cycloheximide and growth factor treatment by TGF-β or bFGF. When monomers of the binding site were combined with a minimal promoter element, they did not enhance muscle-specific transcription. However, multimers of this sequence were successful in dramatically upregulating muscle-specific transcription in both myoblasts and myotubes (Gossett *et al.*, 1989). Subsequent studies of the MEF2-binding activity showed that MEF2 could be induced by myogenin in cells that could be transformed into a myogenic phenotype as well as in cells that could not be transformed into a myogenic phenotype (Cserjesi and Olson, 1991). The roles of MEF2 proteins in cardiac and smooth muscle-specific transcription are less well established. MEF2 mRNA is present in smooth muscle tissue, and MEF2-like sites have been implicated in the regulation of smooth muscle-specific transcription of the smooth muscle myosin heavy chain promoter (Katoh *et al.*, 1994). In transgenic mice, mutagenesis of MEF2 site in the α-cardiac myosin heavy chain promoter has been associated with an increased expression of the reporter gene in the heart and ectopic expression of the promoter in the aorta (Adolph *et al.*, 1993).

Just as the SRF:SRE association is not exclusive, it has been shown that MEF2 sites are capable of being occupied by several other transcription factors. Examples identified to date include TARP and BBF-1, neither characterized at the sequence level as yet (Horlick *et al.*, 1990; Zhou *et al.*, 1993); HF-1b (Zhu *et al.*, 1993); and several homeodomain transcription factors: MHox, Oct-1, Gtx, and Clox (Cserjesi *et al.*, 1994; Komuro *et al.*, 1993; Andres *et al.*, 1992). Of these proteins, Oct-1, Clox, Gtx, and TARP have distributions that are not muscle specific, and MEF2-like binding sites are conversely present in several nonmuscle promoters (Pollock and Treisman, 1991). In the case of HF-1b, analysis of the rat cardiac MLC-2 gene demonstrated that transcription of the promoter in cardiac myocytes was dependent on a multipartite *cis* element containing an A/T-rich sequence that was initially identified as a probable MEF2 binding site by several criteria (Navankasattusas *et al.*, 1992); Yu *et al*, (1992) subsequently showed that MEF2 indeed binds to this sequence. However, a protein species that bound this site was identified in heart and skeletal muscle nuclear extracts and was named HF-1b. HF-1b was cloned and shown not to be a MADS box protein, but rather a zinc finger protein of the C_2H_2 class, restricted in its expression to heart, skeletal, mus-

cle, and brain (Zhu *et al.*, 1993). This finding underscores the complexity of transcription factor/*cis* element interactions in that two positive regulatory proteins of entirely different structures both specifically recognize the same *cis* element and may in fact be present in the same cells.

A particularly challenging report of MEF2-binding site interactions demonstrated overlapping binding sites between MEF2 proteins and the TATA box-binding protein TFIID in the promoter of one of the two Xenopus MyoD genes (*XMyoDa*) (Leibham *et al.*, 1994). In this study a TATA box mutation which abolished TFIID binding but not MEF2 binding was equally as capable of directing transcription in skeletal muscle as was the original sequence, but was significantly less efficient than the wild-type sequence injected into Xenopus oocytes. Cotransfection of a frog MEF2 cDNA (XMEF2) into nonmuscle cells failed to transactivate those promoters unable to bind TFIID, suggesting that XMEF2 was an incorrect isoform of MEF2 or that additional unrecognized muscle-specific transcription-associated factors were required for MEF2 proteins to somehow substitute for TFIID.

Four MEF2 genes so far have been recognized in higher eukaryotes, although there appears to be only one in Drosophila (Lilly *et al.*, 1994). This fits well with the original estimate of four or five possible human MEF2 loci detected by Yu *et al.* (1992). The terminology for the MEF2 isoforms used in this chapter reflects the effort to standardize the nomenclature of these genes as *MEF2A*, *MEF2B*, *MEF2C*, and *MEF2D* (Breitbart *et al.*, 1993). Table III shows the relationships between current terminology and the original descriptions. *MEF2A*, *MEF2C*, and *MEF2D* have been mapped in the mouse genome to cen-

TABLE III
CURRENT TERMINOLOGY FOR MEF2 FAMILY PROTEINS

Current name	Original name	Source	Ref.
MEF2A	RSRFC4, RSRFC9	Human	Pollock and Treisman (1991)
	MEF2	Human	Yu *et al.* (1992)
	SL-2	Xenopus	Chambers *et al.* (1992)
	aMEF2, a*MEF2	Human	Yu *et al.* (1992)
MEF2B	RSRF2	Human	Pollock and Treisman (1991)
	xMEF2	Human	Yu *et al.* (1992)
MEF2C	MEF2C	Human	Leifer *et al.* (1993)
	MEF2C	Mouse	Martin *et al.* (1993)
MEF2D	SL-1	Xenopus	Chambers *et al.* (1992)
	MEF2D	Human	Breitbart *et al.* (1993)
	MEF2D	Mouse	Martin *et al.* (1994)

tral chromosome 7, distal chromosome 13, and central chromosome 3, respectively (Martin *et al.*, 1994). *MEF2A*, *MEF2C*, and *MEF2D* gene products are capable of undergoing alternative splicing to provide multiple protein isoforms; all isoforms identified to date have proven capable of homodimerization and heterodimerization with other MEF2 proteins and so the cumulative complexity of potential MEF2 protein products brought to bear on a MEF2-binding site is considerable (Fig. 1). All of the mammalian MEF2 isoforms display some mRNA species, which are several kilobases larger than the cloned cDNAs, and it is believed that the discrepancy reflects large unidentified segments of 5′ UTR. No data have been published describing the genomic organization of any of the MEF2 genes.

a. MEF2A. The first MEF2 transcription factor to be cloned was identified by Pollock and Triesman (1991) using a MADS box DNA sequence to probe a library at reduced stringency. Since the MADS box domain was derived from the protein SRF, the clones that were recovered were labeled related to SRF or RSRF proteins. Three cDNAs were recovered. Two of them, RSRFC4 and RSRFC9, were alterna-

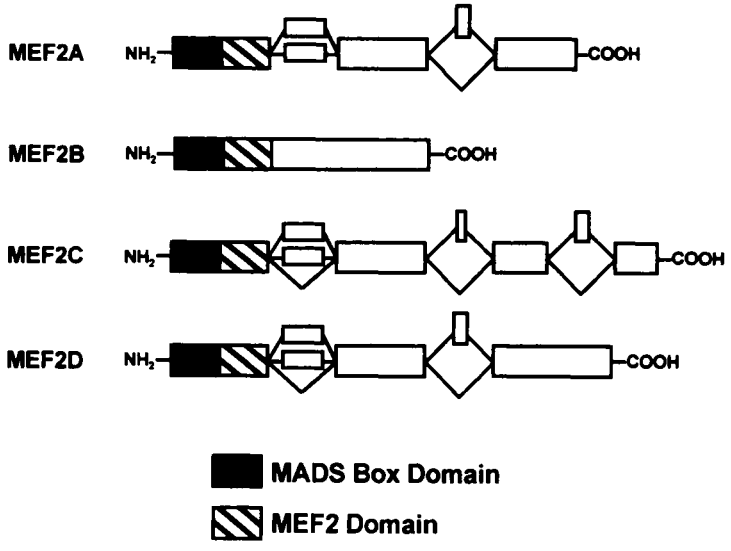


FIG. 1. MEF2 protein isoforms identified to date (after Martin et al. 1994). The diversity of isoforms for each individual MEF2 gene is amplified by the ability of all tested MEF2 proteins to freely heterodimerize with each other; if the various isoforms and heterodimers have differing functional consequences, a MEF2-based transcriptional regulatory system with great variety and subtlety of function may result.

tively spliced products of a single gene. The mRNA for these factors was found to be ubiquitously expressed and, on the basis of band shift data, the DNA-binding activity of these proteins was felt to be ubiquitous as well. The correspondence of RSRFC4 with MEF2 was determined on the basis of a binding-site analysis which revealed binding-site preferences and diethylpyrocarbonate interference data identical to that reported by Gossett *et al.* (1989) for MEF2. However, Pollock and Triesman (1991) found that reporter plasmids with RSRF-binding sites could not be stimulated by cotransfection with RSRF expression plasmids.

Subsequently, the same gene products were cloned and characterized with the names MEF2, aMEF2, and a *MEF2 (Yu *et al.*, 1992). MEF2 and a *MEF2 corresponded to RSRFC4 and RSRFC9, respectively. All three MEF2A isoforms were found to have ubiquitously expressed mRNA species, but were more abundant in brain, heart, and skeletal muscle. However, the protein products were only identifiable in skeletal muscle, cardiac muscle, and smooth muscle tissues, suggesting an important role for post-transcriptional mechanisms in controlling expression. In gel mobility shift assays, only complexes containing the aMEF2 protein isoform were identified. An additional alternatively spliced exon was found to be specific to skeletal muscle, heart, and brain tissue. Forced expression of MEF2 was not capable of converting 10T1/2 fibroblasts into a myogenic phenotype, indicating that it was a transcription factor downstream of transcriptional commitment to muscle cell differentiation. However, forced expression of any of the MEF2A isoforms did result in the sequence-specific upregulation of plasmids containing multimers of the MEF2 recognition sequence.

Cotransfection of MEF2 and aMEF2 into pulmonary artery vascular smooth muscle cells, cardiac myocytes, and skeletal muscle myotubes was unable to augment expression in the reporter plasmids used. However, baseline expression of these reporter plasmids in these cell types was already extremely high, and the authors comment that these findings are probably the result of saturating levels of endogenous MEF2.

SL-2 is a MEF2A homolog from Xenopus (Chambers *et al.*, 1992). SL-2 expression begins in the early neurula, after SL-1 (MEF2D). SL-2 mRNA expression was restricted to the somitic mesoderm and myotomes in the embryo, but was much less restricted in the adult. The expression of SL-2 in the adult was seen at high levels in the heart and muscle, with low levels in the brain and spleen and less easily detected levels in other tissues examined.

b. MEF2B. RSRFR2 is a MADS box protein also identified by Pol-

lock and Triesman (1991). Unlike RSRFC4, RSRFR2 was found to have a tissue-specific expression largely limited to B cells (Pollock and Treisman, 1991). RSRF2 was also cloned by Yu *et al.* (1992) as xMEF2. The mRNA for this transcription factor was found in and restricted to brain, heart, skeletal muscle, and myotubes. Neither Pollock and Triesman (1991) nor Yu *et al.* (1992) were able to demonstrate any DNA-binding capacity for the xMEF2 protein. Nonetheless, forced expression of xMEF2 resulted in the sequence-specific upregulation of plasmids containing multimers of the MEF2 recognition sequence. The authors proposed that xMEF2 may be heterodimerizing with other MEF2 proteins in order to become a transactivator, as Pollock and Triesman (1991) had shown previously that RSRFR2 and RSRFC4 were capable of forming stable heterodimers in solution.

c. MEF2C. Two groups reported the cloning of a cDNA corresponding to the third MEF2 gene, MEF2C (Martin *et al.*, 1993; McDermott *et al.*, 1993). Both groups reported a high level of mRNA to be present in skeletal muscle and brain, with a low level of mRNA detected by reverse transcriptase polymerase chain reaction in the heart. The appearance of this transcription factor, mRNA and protein, was a late event in myotube formation in C2C12 cells and did not correspond to the early appearance of the MEF2-binding activity originally described by Gossett *et al.* (1989). Immunologic detection of hMEF2C showed it to be present in only a specific subset of cortical neurons in the brain, and in cell culture it was expressed in only a subset of differentiated myotube nuclei (McDermott *et al.*, 1993). MEF2C could be upregulated by the forced expression of myogenin (Martin *et al.*, 1993).

In situ hybridization studies in developing mouse embryos showed an entirely different pattern of temporal expression than that in C2C12 cells (Edmondson *et al.*, 1994). MEF2C mRNA *in vivo* is expressed earlier than MEF2A and MEF2D in both myocardial and skeletal muscle lineages.

d. MEF2D. In Xenopus the search for SRF-related genes resulted in the cloning of two MEF2 domain proteins, SL-1 and SL-2. SL-2 was a MEF2A homolog and has been described earlier. SL-1 was a unique MEF2 protein. Both of these transcription factors were expressed as maternal RNAs in the oocyte. SL-1 expression in the developing embryo began in the late gastrula stage, before SL-2. Expression of SL-1 was restricted to the somitic mesoderm and myotomes in the embryo, but was much less restricted in the adult. The expression of SL-1 in the adult was present in all tissues examined and enriched in the heart, muscle, brain, and spleen, with corresponding DNA-binding activities also ubiquitously seen (Chambers *et al.*, 1992). Ectopic expres-

sion of SL-1 in Xenopus animal pole explants results in ectopic expression of the cardiac-specific *XMLC2* (a myosin light chain) gene mRNA, suggesting a possible role in cardiac muscle specification (Chambers *et al.*, 1994). Despite a 90% sequence similarity in the DNA-binding domain, injections of SL-2 mRNA neither transactivated *XMLC2* gene expression nor competed with the transactivation ability of SL-1.

The subsequent cloning of human and murine MEF-2D cDNAs (Breitbart *et al.*, 1993; Martin *et al.*, 1994) showed that the mammalian homologs were also widely expressed with tissue-specific differences in mRNA sizes and abundance. The protein products, however, were restricted to tissue previously associated with the presence of MEF2 DNA-binding activity and MEF2-sensitive promoter activity, suggesting an important role for post-transcriptional mechanisms in the regulation of MEF2D function. Alternatively spliced exons in the mRNA were shown to demonstrate tissue specificity and perhaps to predict the presence of the translated protein product.

Breitbart *et al.* (1993) demonstrated the existence of MEF2D protein before expression of myogenin in C2C12 myoblasts and prior to the expression of other MEF2 proteins. *In situ* analysis of mouse embryo MEF2 mRNAs failed to show such a relationship (Edmondson *et al.*, 1994), with MEF2D mRNA expression trailing both MEF2C and myogenin in the somites. It is unclear whether the discrepancy reflects a peculiarity of cell culture or whether the *in situ* detection of mRNA is an unreliable indication of MEF2 protein expression.

e. MEF2 Transcription Factors in Vascular Smooth Muscle. MEF2 was originally described as a DNA-binding activity present in differentiating myotubes. The presence of this DNA-binding protein was induced at myotube fusion and by serum withdrawal, and could be blocked by cycloheximide and growth factor treatment by TGF-β or bFGF. When monomers of the binding site were combined with a minimal promoter element, they did not enhance muscle-specific transcription. However, multimers of this sequence were successful in dramatically upregulating muscle-specific transcription in both myoblasts and myotubes (Gossett *et al.*, 1989). Additional studies of the MEF2-binding activity showed that MEF2 could be induced by myogenin in cells that could be transformed into a myogenic phenotype as well as in cells that could not be transformed into a myogenic phenotype (Cserjesi and Olson, 1991). Subsequent studies showed that MyoD could also upregulate MEF2A in cultured cells (Yu *et al.*, 1992).

A dimer of MEF2-binding sites confers a six- to eightfold increase to a heterologous promoter in pulmonary smooth muscle cells, and MEF2 sites have been implicated in the regulation of smooth muscle-

specific transcription of the smooth muscle myosin heavy chain promoter (Yu *et al.*, 1992; Katoh *et al.*, 1994). In transgenic mice, mutagenesis of a MEF2 site in the α-cardiac myosin heavy chain promoter has been associated with an increased expression of the reporter gene in the heart and ectopic expression of the promoter in the aorta (Adolph *et al.*, 1993). The only MEF2 isoform demonstrated to date to be expressed as a protein in vascular smooth muscle tissues is MEF2A (Yu *et al.*, 1992). However, Edmondson *et al.* (1994) demonstrated the presence of MEF2A, MEF2C, and MEF2D mRNA species in vascular smooth muscle tissues during the course of their developmental study of MEF2 gene expression. MEF2 isoforms were reported to be present in vascular smooth muscle at all stages of development, with MEF2C showing particularly high levels of expression in the aorta of late gestation embryos and in the coronary arteries of newborn animals. All three species were also present in some migratory neural crest cells; as other neural crest-derived neural structures did not express MEF2 transcripts, it was suggested that the expression of MEF2 may mark the neural crest population destined to become vascular smooth muscle cells. In some instances of early blood vessel formation, it appeared that MEF2A transcripts predominated in abundance. Interestingly, it was noted that MEF2D was the most abundant isoform detected in gastrointestinal smooth muscle tissues in early development.

Since at least the MEF2A protein is present in vascular smooth muscle, what might the role of MEF2 transcription factors be for the determination of the vascular smooth muscle phenotype? Comparisons between cardiac muscle and skeletal muscle expression of MEF2 isoforms lead in different directions. In skeletal muscle tissues the timing of MEF2 mRNA expression places all known isoforms of MEF2 downstream of the earliest expression of the bHLH proteins myf-5 and myogenin (Edmondson *et al.*, 1994), notwithstanding the presence of an essential MEF2 site in the myogenin promoter (Cheng *et al.*, 1993). This suggests a subsidiary role for MEF2 proteins in muscle-specific transcription as "amplifiers" and/or "stabilizers" of the myogenic differentiation program, a conclusion that also results from the inability of forced MEF2 factor expression to convert pluripotential cells such as 10T1/2 cells into a myogenic phenotype (Yu *et al.*, 1992).

In cardiac cells, however, MEF2C mRNA is expressed at day 7.5 (Edmondson *et al.*, 1994) and it thus appears as early as Nkx 2.5 does in the precardiac mesoderm (see later). This suggests the potential for MEF2 proteins to be more fundamental in establishing the cardiac muscle phenotype than their role in establishing the skeletal muscle phenotype.

The hierarchical position of MEF2 in invertebrate striated somatic muscle development seems to provide yet another picture. In Drosophila the single MEF2 homolog D-MEF2 is expressed downstream of *twist* and *snail*, two transcription factors required for mesoderm formation, and upstream of the single Drosophila myogenic bHLH protein nautilus in the development of somatic muscle. In Drosophila cardiac development the expression pattern is more congruent with that described in vertebrates. D-MEF2 expression in the Drosophila precardiac lineage is coincident with and independent of the cardiac-specific homeobox gene *tinman* (Lilly *et al.*, 1994).

C. *Basic Helix-Loop-Helix Proteins*

1. bHLH Transcription Factors

The myogenic bHLH protein transcription factor family has been reviewed (Weintraub *et al.*, 1991; Weintraub, 1993; Sassoon, 1993). As a group they are essential regulatory genes of the skeletal muscle cell phenotype, and ectopic expression of these genes is capable of inducing the skeletal muscle phenotype in some, but not all, nonmuscle cells in culture. These proteins were initially suspected and later proven to be transcription factors with downstream targets known to include their own promoters (Thayer *et al.*, 1989; Braun *et al.*, 1989a; Edmondson and Olson, 1989) and regulatory elements of multiple muscle-specific genes including creatine kinase (Lassar *et al.*, 1989), cardiac α-actin (Sartorelli *et al.*, 1990; Moss *et al.*, 1994), desmin (Li and Capetanaki, 1994), and the α-subunit of the acetylcholine receptor (Piette *et al.*, 1990).

MyoD (myoblast determinant gene) is the prototype member of the bHLH transcription factor group (Davis *et al.*, 1987). All are expressed exclusively in skeletal muscle. Myogenin, the second member of the family to be cloned, was discovered while attempting to define factors involved in the second stage of myogenesis, the decision to terminally differentiate (Write *et al.*, 1989; Edmondson and Olson, 1989). The general conservation of the basic helix domain of these muscle-specific transcription factors has been used to isolate the additional regulatory factors MRF4/herculin/myf6 (Rhodes and Konieczny, 1989; Miner and Wold, 1990; Braun *et al.*, 1990) and myf5 (Braun *et al.*, 1989b).

The basic HLH protein motif is not limited to muscle-specific transcription factors but has also been identified in a growing number of proteins implicated in transcriptional regulation. These include several genes regulating cell fate in Drosophila, such as *achaete-scute*

(Villares and Cabrera, 1987), *daughterless* (Caudy *et al.*, 1988), and *twist* (Thisse *et al.*, 1988), the E2A gene products E12 and E47 (Mellentin *et al.* 1989), and members of the myc family of oncogenes (Blackwood and Eisenman, 1991) (also referred to as bHLH-Zip proteins because of their additional leucine zipper domain). The bHLH proteins have been divided into three classes, A, B, and C, according to their anatomic distributions and their heterodimerization abilities (Murre *et al.*, 1989). Class A proteins are exemplified by the E2A gene products in vertebrates or *daughterless* in Drosophila; they are ubiquitous in distribution and are able to heterodimerize with each other and Class B proteins. Class B proteins include, among others, MyoD, DRF4, myf5, and myogenin in vertebrates, *achaete-scute* in Drosophila, and the mammalian *achaete-scute* homolog hASH1 (Ball *et al.*, 1993). Unlike the Class A proteins, the Class B proteins have tissue-restricted distributions. Class C includes the oncogenes myc, max, and mad; proteins in this group contain an additional leucine zipper domain and will heterodimerize with each other but not with Class A or Class B proteins. Transcriptional activation of the muscle-specific phenotype requires the heterodimerization of one of the E2A gene products E12/E47 with one of the muscle-specific bHLH proteins, as homodimers of MyoD fail to transactivate (Lassar *et al.*, 1991); in the case of E47, an additional cellular factor may be necessary to enable DNA binding (Thayer and Weintraub, 1993).

Class A and Class C bHLH proteins are present in vascular smooth muscle tissues but Class B bHLH proteins are not. The ectopic expression of MyoD in rat aorta vascular smooth muscle cells in culture results in the expression of a variety of skeletal muscle genes, including skeletal α-actin and myosin isoforms, accompanied by morphological evidence of myoblast and myotube formation (roughly 2% of the cell population) (van Neck *et al.*, 1993). Interestingly, when transfected into cells derived from adult rat aorta, basic fibroblast growth factor stimulated conversion, but when newborn rat aorta vascular smooth muscle cells were exposed to bFGF, myogenic conversion was extinguished.

In the bHLH motif the helix-loop-helix domain is associated with the ability to form dimers (Murre *et al.*, 1989). The basic region is responsible for sequence-specific DNA binding of the consensus sequence known as an E box (CANNTG) in all family members (Murre *et al.*, 1989). The basic region is also associated with transactivation, but the abilities to bind DNA specifically and to transactivate are separable functions; E12 dimers cannot transactivate a muscle-specific promoter (Lassar *et al.*, 1991). It has been shown in a series of experiments with MyoD/E12 fusion proteins and E12 mutation experiments

(summarized in Davis and Weintraub, 1992) that myogenic transactivation ability can be transferred to E12 by the mutation of only three amino acid residues in the E12 bHLH domain to their MyoD counterparts. Two of the critical amino acid residues are present in the basic region and one is in the junctional region between the basic region and the HLH domain. In MyoD to bHLH region is necessary and sufficient for transcriptional activation (Tapscott *et al.*, 1988). In the case of myogenin, however, the bHLH region is necessary but insufficient. Additional activation domains were identified at both the N and C termini which required interaction with a specific amino acid sequence in the basic region in order to demonstrate their function (Schwarz *et al.*, 1992).

Multiple developmental studies and transgenic animal experiments involving bHLH genes and their products provide evidence that an intricate regulatory hierarchy with significant functional redundancy is present in normal development. During skeletal muscle development, myf5 and myogenin mRNAs are both expressed in the somites at day 8.5 (although mf5 may be sclerotomal in its distribution at this stage), MRF4 mRNA is additionally present at day 9.5, and MyoD mRNA is expressed last at day 10.5 (reviewed in Sassoon, 1993). The expression of myf5 mRNA is lost in the somitic musculature by day 17.5, but expression of the other myogenic bHLH mRNAs continues to be detectable through adult life. The expression in developing forelimb muscle is slightly different; myf5 mRNA is present at day 10.5, myogenin and MyoD mRNAs are additionally present by day 11.5, and MRF4 mRNA is only first noted on day 13.5, by which time myf5 expression can no longer be detected. Myogenin, MyoD, and MRF4 mRNAs continue to be expressed at low levels in limb muscle through adult life.

Considering the early expression of myf5 mRNA in the development of both somitic and forelimb musculature, it was with some surprise that it was shown that loss of myf5 through targeted disruption of the myf5 gene (Braun *et al.*, 1992) was associated with apparently normal muscle formation. Similarly, targeted disruption of the MyoD gene resulted in histologically normal muscle development (Rudnicki *et al.*, 1992); in the MyoD knockout animals, the myf5 gene function was upregulated in possible compensation for the loss of MyoD function. Evidence has also been presented for a compensatory role for MyoD in myf5 -deficient myogenesis (Braun and Arnold, 1994). In embryos homozygous for targeted disruption of the myogenin gene, however, there was a severe reduction of skeletal muscle formation and some indication of an inability to undergo conversion from MyoD-containing myoblasts to multinucleated myotube (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Breeding for double homozygotes of the myf5

and MyoD gene disruptions resulted in embryos with a total absence of skeletal muscle and no detectable myoblasts (Rudnicki *et al.*, 1993). Taken together, these data have been used to suggest that MyoD and myf5 occupy basically redundant roles in mammalian skeletal myogenesis and that one of the essential steps which they mediate is the upregulation of myogenin expression (Weintraub, 1993).

MyoD has also been introduced into transgenic mice in gain of function experiments (Faerman *et al.*, 1993). When directed in its expression by the β-actin promoter, MyoD expression results in embryonic lethality, although the great majority of embryos examined demonstrated normal germ layer differentiation. For unknown reasons, MyoD transcripts could not be identified in the endoderm. Transgenic embryos demonstrated ectopic expression of myogenin and myosin light chain 2 mRNAs without demonstrating ectopic formation of skeletal muscle morphologically. Most of the transgenic embryos displayed evidence of retarded growth, suggesting a link between MyoD expression and cell proliferation (see later). In transgenic animals where ectopic MyoD expression was directed exclusively to the heart, a similar local phenotype was observed: myogenin and skeletal muscle-specific mRNAs (and some proteins) were induced, no histologic skeletal muscle transformation was observed, and the growth characteristics of the tissue were abnormal (Miner *et al.*, 1992).

2. Id Proteins, bHLH Proteins, and Cell Cycle Regulation

There is an additional ubiquitous group of bHLH-related proteins which lacks the basic region associated with DNA binding and transactivation functions but nonetheless contains HLH domains. Because of the presence of the HLH domain they remain capable of heterodimerizing with Class A or Class B bHLH proteins. The prototype protein of this group is Id (Inhibitor of differentiation) (Benezra *et al.*, 1990). By heterodimerizing with Class A bHLH proteins, Id prevents the Class A:Class B heterodimerization required for transcriptional activation, and the Id proteins have therefore been considered as transcriptional repressors. In addition, expression of Id is associated with proliferating, undifferentiated cells, and there is evidence of an association between Id-2 protein expression and the maintenance of the proliferative state through interaction with the hypophosphorylated form of the Rb gene product (pRb) (Iavarone *et al.*, 1994). pRb contributes to growth arrest in part by the ability to bind and sequester cell cycle stimulatory proteins such as E2F and D-type cyclins. These interactions are mediated via the so-called "pocket" do-

main, which is the domain utilized in the pRb:Id interaction. Therefore, the pRb:Id association may effectively prevent the sequestration of cell cycle stimulatory proteins and thereby prevent growth arrest. The portion of the Id molecule responsible for this association is the HLH domain. Similar findings for Class A (E2) and Class B (MyoD) proteins had been previously shown by Gu *et al.* (1993) in demonstrating bHLH–pRb complex formation; in addition, they were able to show evidence that pRb or the structurally and functionally similar protein p107 may be interacting with E-box related transcription factors in muscle-specific DNA–transcription factor complexes. As in the case of Id-2, MyoD interacts with the pocket region of the pRb protein.

Some evidence of the functional consequences of these interactions for cell cycle regulation has been presented. Overexpression of the Id protein stimulates cellular proliferation (Iavarone *et al.*, 1994). Conversely, microinjection of MyoD into fibroblasts results in growth arrest temporally consistent with a block at the G1 to S transition and prevents the increase in hyperphosphorylated pRb that is associated with transition into the S phase (Thorburn *et al.*, 1993). [Interestingly, the closely related pocket protein p 107 is capable of interacting with MyoD but is incapable of associating with Id or E2 (Schneider *et al.* 1994)]. Taken together, the evidence is strong that Id and the myogenic bHLH proteins are directly involved with cell cycle regulation through their interactions with pRb and/or p 107. If so, these transcription factors provide an attractive link between the arrest of the cell cycle and the expression of tissue-specific gene products, two of the most typical features of terminally differentiated cells. However, Kemp *et al.* (1991) were able to document the loss of smooth muscle myosin heavy chain mRNA expression in aortic smooth muscle cell cultures in the absence of detectable Id mRNA expression; in their hands, Id mRNA expression correlated very well with the serum induction of cell cycle progression and cellular proliferation. This suggests that in vascular smooth muscle cells the coordination of Id expression with the regulation of the cell cycle may be tighter than its association with regulation of differentiation.

While it is clear that none of the skeletal muscle-specific bHLH proteins are present in smooth muscle tissue, there is reason to believe that proteins with similar dimerization properties may play a role in vascular smooth muscle cell transcriptional regulation. The evidence for this suspicion is the expression of Id (Kemp *et al.*, 1991; Evans and O'Brien, 1993) and E2 gene products in vascular smooth muscle cells. These proteins are presumed to have functions in vascular smooth muscle cells such as regulation of tissue-specific gene expres-

sion and regulation of the cell cycle similar to the roles described for them in other differentiated tissue types.

D. The Homeodomain and Homeobox Genes

The "homeodomain" is a 61 amino acid DNA-binding domain encoded by a "homeobox" gene sequence. This motif is present in a continually expanding superfamily of DNA-binding proteins, some of which are present in vascular smooth muscle cells. The homeodomain is highly conserved in evolution from yeast to plant to man. This motif was originally noted in Drosophila transcriptional regulatory proteins encoded by loci associated with "homeotic" mutations; a homeotic mutation is one in which "one part of the body develops in the likeness of another" (Scott *et al.*, 1989). A striking example of this type of mutation is the Antennapedia mutation of Drosophila in which the antenna are transformed into an extra set of legs. Homeobox genes were first identified through investigation of the body plan organization in Drosophila, but it has since become apparent that homeodomains are also present in transcriptional regulators associated with pattern formation, cell-type specification, signal transduction, and possibly cell cycle regulation.

The structure of the homeodomain has been reviewed by Gehring *et al.* (1994) and Scott *et al.* (1989). The homeodomain tends to be located near the C termini of proteins. Homeodomain-containing proteins can bind DNA as monomers (Gehring, 1992), but some require that they be in complex with nonhomeodomain DNA-binding proteins to demonstrate high degrees of sequence specificity (Smith and Johnson, 1992). Others have been shown to occupy paired binding sites cooperatively (Galang and Hauser, 1992). The homeodomain consists of an N-terminal arm of flexible structure followed by well-defined α-helices I, II, and III; helix III is extended by a fourth more flexible short segment of α-helix (helix IV). A short "turn" of three amino acids is present between helix 2 and helix 3, and this portion of the homeodomain creates a "helix-turn-helix" structure. The third helix is the recognition helix and it fits into the major groove of DNA as it associates with its specific binding site. An important feature of the binding-site DNA sequence in common with most homeodomain major groove recognition sites is 5′-ATTA-3′/5′-TAAT-3′. Additional minor groove contracts are made by the N-terminal arm of the homeodomain.

Multiple classes of homeodomain proteins have been identified on the basis of similarities in the sequence of the homeodomain, especially helix 3, and the presence or absence of additional protein motifs in combination with the homeodomain [reviewed in Scott *et al.* (1989)].

1. *Hox* Genes and Vascular Smooth Muscle

The first widely recognized role for homeodomain proteins was in Drosophila segmental development. Analysis of the genetic basis of homeotic mutations led to the description of *Antennapedia*-class homeobox (*Hox*) genes. In Drosophila the *Hox* genes are arranged in two sets of clusters, ANT-C and BX-C, which can be conceptualized as one continuous string of homeobox genes. The position of somatic expression from anterior to posterior in the fly embryo corresponds to the position the gene occupies in the cluster from 5′ to 3′, this feature has been termed spatial colinearity. The presence of spatially colinear Hox genes is highly conserved in evolution, having been identified to date in nematodes, arthropods, and vertebrates. In the evolution of vertebrates, extensive duplication of the "ancestral" *Hox* cluster has occurred and the vertebrate genome consequently contains four functional and distinct *Hox* clusters. The position of a vertebrate Hox gene in a cluster predicts not only its spatial expression (spatial colinearity) but also its temporal expression (temporal colinearity) and its degree of transcriptional responsiveness to retinoic acid stimulation (Fig. 2). *Hox* genes occupying similar positions in their respective

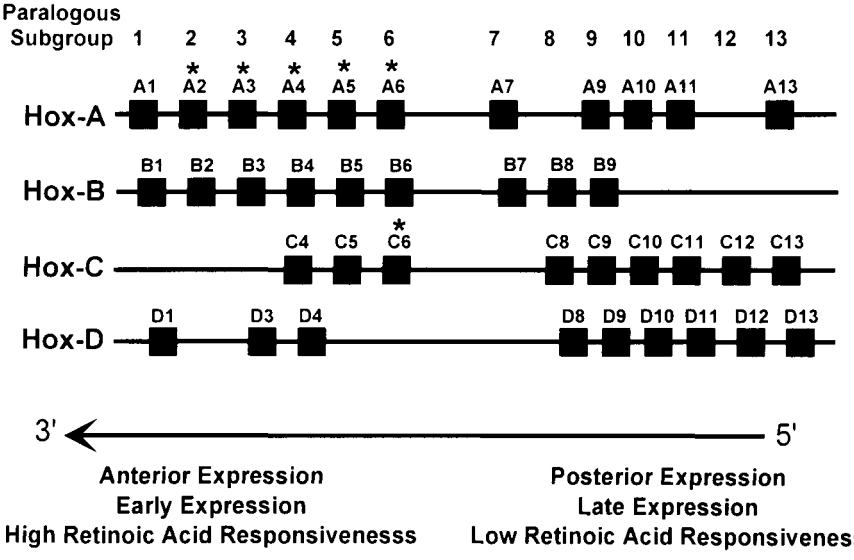


FIG. 2. The vertebrate (murine) *Hox* gene clusters are diagrammed (after Krumlauf, 1993). The asterisks indicate *Hox* genes known to be expressed in vascular smooth muscle tissues (see also Table IV), specifically in the neural crest-derived ectomesenchymal vascular smooth muscle. At least two members of paralogous subgroup 6 are expressed in vascular smooth muscle, although in the Hox-A group the highest level of expression seems to occur in the most 3′ members of the cluster.

clusters are termed paralogous, and sequence analysis reveals that the paralogous groups in the mammalian embryos are structurally related to the homeobox gene in the same relative position in the Drosophila ANT-C and BX-C clusters.

Individual *Hox* genes in vertebrates are typically limited in their expression to discrete subsets of tissues. Of relevance to the topic of this chapter is the expression of several members of the *Hox-A* cluster in embryonic and/or mature smooth muscle tissues (Table IV). *Hox-A2* has been described as limited to smooth muscle cells in the mature animal (Patel *et al.*, 1992). A targeted knockout of the *Hox-A3* gene in mice was reported to develop anomalies of the great vessels (Chisaka and Capecchi, 1991) with an overall pattern of features in the mutant mice indicating abnormal neural crest-derived structures. In general, there is little information regarding the specific target genes for *Hox* transcription factors and this is also true for the subset expressed in smooth muscle.

TABLE IV

HOMEODOMAIN PROTEINS KNOWN TO BE EXPRESSED IN VASCULAR SMOOTH MUSCLE TISSUES

Homeodomain protein	Notes	Ref.
Hox-A6 (Hox-1.2)	Scattered expression	Gaunt *et al.* (1988)
Hox-A5 (Hox-1.3)	Scattered expression	Gorski *et al.* (1993); Gaunt *et al.* (1988)
Hox-A4 (Hox-1.4)	Higher level expression than *Hox-A6, Hox-A5,* and *Hox-C6*	Gorski *et al.* (1993); Gaunt *et al.* (1988)
Hox-A3 (Hox-1.5)	Knockout leads to cardiovascular defects	Chisaka and Capecchi (1991)
Hox-A2 (Hox-1.11)	Expression in adult limited to aorta and lung	Patel *et al.* (1992)
Hox-C6 (Hox-3.3)	Scattered expression	Gaunt *et al.* (1988)
R1b		Gorski *et al.* (1993); Falzon and Chung (1988)
Mox-1	Neural crest mesenchyme, cardiac outflow tract	Candia *et al.* (1992)
Msx-1 (Hox-7)	Scattered expression in aortic arch mesenchyme up to HH stage 37	Chan-Thomas *et al.* (1993); Suzuki *et al.* (1991)
MHox	See text	Cserjesi *et al.* (1992) Kuratani *et al.* (1994)
Gax	Putative cell cycle regulator cloned from smooth muscle cells	Gorski *et al.* (1993)

2. Homeodomain–MADS Box Interactions

Homeodomain proteins also display roles in cell-type determination in animals as primitive as yeast. Yeast do not display sequential morphologic differentiation but they do differ one from another according to their mating type. There are three potential yeast cell types, **a**, α, and **a**/α, and they are determined at the level of transcription in response to a pheromonal signal encountered at the cell surface (reviewed in Kurjan (1993). In **a** cells, the **a** set of genes is turned on and creates the **a** phenotype; in α or **a**/α cells the **a**-specific genes are turned off. This relatively simple system is established transcriptionally by the highly cooperative interactions of the MADS box yeast protein MCM1 with three other transcription factors: two homeodomain proteins, MATα1 and MATα2, and the STE12 transcription factor (Primig *et al.*, 1991). Expression of MCM1 occurs in all three cell types. Expression of MATα2, present only in cells with an α allele, results in MCM1/MATα-2 complex binding to **a**-specific promoters and repression of **a**-specific genes. Expression of MATα1 results in MATα1/MCM1 complex formation on α-specific gene promoters with activation of α-specific genes. STE12 activates **a**-specific promoters by binding cooperatively with MCM1 in the absence of MATα-2. The presence of MCM1 in the MATα2/MCM1 complex has been shown to be necessary for the accurate identification of the DNA target sequence by the MATα2 protein (Smith and Johnson, 1992).

The association between homeodomain proteins and MADS box proteins has been conserved in higher organisms as well. The human homeodomain protein phox1 interacts with the MADS box protein SRF and potentiates DNA binding by SRF through a mechanism requiring phox1/SRF association, but not phox1 binding to DNA (Grueneberg *et al.*, 1992). phox1 was capable of activating c-*fos* promoter transcription by itself and also potentiated the transcriptional response of that promoter to SRF. The interactions between SRF and phox1 could be duplicated by two fusion proteins, one containing only the evolutionarily conserved MADS box and the second a homeodomain, further highlighting the analogy with the MCM1/homeodomain protein system in yeast cell-type specification.

phoxl was independently cloned from mouse and described by Cserjesi *et al.* (1992) as MHox (For Muscle Homeobox protein). MHox/phoxl is a member of the "paired-like" homeodomain group in common with the protein S8 (DeJong and Meijlink, 1993; Opstelten *et al.*, 1991; De Jong *et al.*, 1993), and the consensus binding site for the *paired*-like homeodomain has been shown to be ANC/TC/TAATTAA/GC (note the typical ATTA core). There is no evidence of clustering for the *paired*-like class of homeobox genes in the

murine genome. MHox was shown to be restricted to mesoderm in the adult mouse, with the mRNA most highly expressed in heart, skeletal muscle, and uterus. *In situ* hybridization studies in mouse embryos showed labeling in a variety of mesenchymally derived tissues, including low levels in the dorsal aorta and other ectomesenchymal neural crest-related structures (Kuratani *et al.*, 1994). A clear role for MHox in muscle-specific transcription is as yet undetermined. The MHox protein binds in a sequence-specific manner to a subset of A/T-rich sequences capable of being bound by MEF-2 and weakly upregulates transcription mediated through MEF-2 sites *in vitro* (Cserjesi *et al.*, 1994). A novel mouse homeodomain protein, Gtx (found in brain and germ cells of testis), has also been shown to bind to MEF-2 sites *in vitro*, to compete with MEF-2 for DNA binding *in vitro*, and to repress a serum-induced increase in transcription of a MEF-2 site reporter construct (Komuro *et al.*, 1993); interference with MEF-2-mediated upregulation of an enhancer from the β-myosin heavy chain gene has also been shown for the *cut* homeodomain protein Clox (Andres *et al.*, 1992).

3. NK Class Homeodomain Proteins

Striated muscle determination also appears to be a role played by homeodomain proteins in both flies and vertebrates. The Drosophila NK-4 gene product [*msh-2* (Bodmer *et al.*, 1990) or *tinman* (Azpiazu and Frasch, 1993)] is critical for dorsal mesoderm formation; in the absence of *tinman* the fly has no heart, the visceral musculature is nearly absent, and skeletal muscle formation is abnormal. A second NK homeobox gene, *bagpipe* (Azpiazu and Frasch, 1993) or NK-3 (Kim and Nirenberg, 1989), is closely linked with *tinman* in the Drosophila genome and is expressed in segmental clusters of dorsal mesodermal cells. *bagpipe* seems to be downstream of tinman in a cascade of mesodermal determination factors. An absence of *bagpipe* expression leads to deficiencies in the midgut musculature of fly larvae. The NK family of homeobox genes is partially clustered in that NK-1, NK-3, and NI-4 are closely associated whereas NK-2 occupies a separate chromosomal location.

Vertebrates also possess NK class homeodomain proteins (Fig. 3). At least six *tinman* Nkx genes have been identified to have significant homology to NK-2 (Price *et al.*, 1992; Lints *et al.*, 1993), some of which have similarly been implicated in lineage determination. *Nkx-2.1* has been previously identified as TTF-1 and is suggested to be an important regulator of thyroid, pulmonary, and possibly brain-specific gene expression during embryogenesis (Edmondson *et al.*, 1992). *Nkx-2.2* is restricted in its expression to brain (Price *et al.*, 1992). *Nkx-2.5* (Lints

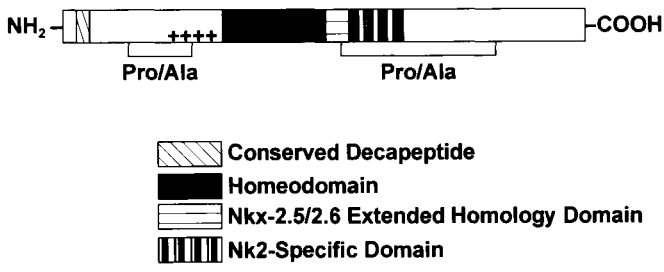
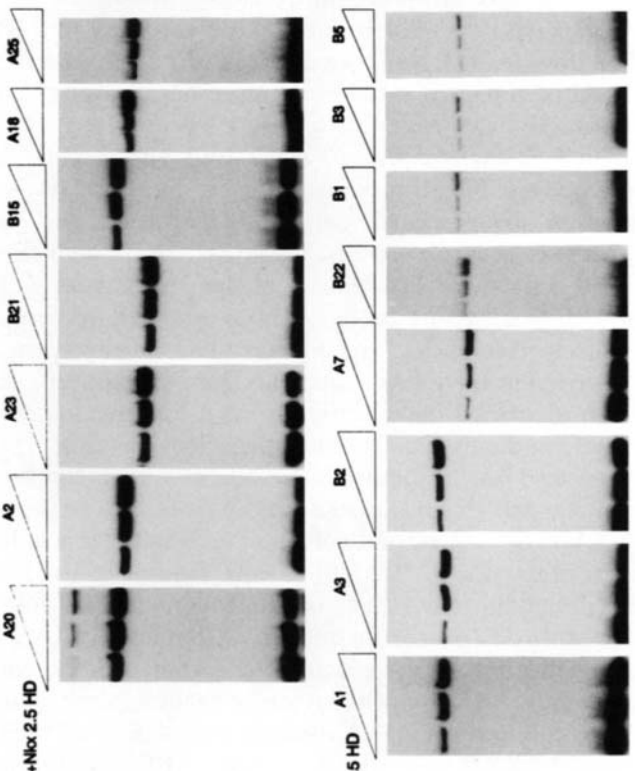


FIG. 3. This schematic representation of the Nkx-2.5 protein illustrates the position of the important domains. These domains include proline/alanine-rich regions (Pro/Ala), charged regions (++++), the homeodomain region, a region of high homology between Nkx-2.5 and Nkx-2.6, and the Nkx-2-specific domain. Also shown near the amino terminus is a decapeptide motif conserved between at least two murine Nkx proteins (Nkx-2.1 and Nkx-2.5) and the Drosophila protein NK4/*msh-2*/*tinman*.

et al., 1993) or *Csx* (Komuro and Izumo, 1993) encodes a homeodomain transcription factor in large part restricted to the precardiac and cardiac myocyte cell lineages. *Nkx-2.1*, *Nkx-2.2*, and *Nkx-2.3* have been mapped to separate mouse chromosomes, making colinear clustering an unlikely feature of this homeobox group.

1. NK Class Homeodomain Proteins and Target gene Expression. In mouse embryos, Nkx-2.5 transcripts are localized to early cardiac cell progenitors, prior to cardiogenic differentiation, and Nkx-2.5 continues to be expressed in cardiac myocytes throughout development. Nkx-2.5 is also detected in a subset of the pharyngeal endoderm tissue immediately adjacent to the cardiac mesoderm, tongue muscle, visceral muscle (stomach), and spleen. The downstream targets of Nkx-2.5 expression have been unclear. The DNA-binding site of Nkx-2.5 has been identified by selection of DNA-binding sequences from a population of randomly generated oligonucleotides (Fig. 4). The bacterially expressed Nkx-2.5 homeodomain bound with the highest avidity to sequences which contained a binding site also preferred by Nkx-2.1: 5′-TNAAGTG-3′. A second subset of selected, but weaker, binding sequences contained a 5′-TTAATT-3′ core similar to the binding sites of the Antennapedia (*Hox*) class of homeodomains as well as the AT-rich central core of the serum response element (CC[A/T]$_6$GG). Following these observations, we determined that Nkx-2.5 could bind to each of the four SREs on the avian α-cardiac actin promoter and could form combinatorial DNA-binding complexes with SRF. Forced expression of Nkx-2.5 in combination with SRF resulted in the transcriptional activation of the α-cardiac actin promoter in 10T1/2 fibroblasts. Transactivation of the α-cardiac actin promoter with Nkx-2.5



Group A: Nkx 2-like binding sequences

Clone	Sequence		Activity
Nkx 2.1	CACTGCCCAG	**TCAAGTG** TTC	++++
A20	**CAATTAAC**	**TCAAGTG**	++++
A23	CGGTTTGG	**TTAAGTG**	+++
B21	TTT	**AAAAGTG** CTTGG	+++
B15	GAC	**TCAAGTG** TGCATTAACA	+++
A18	TGTCGGAG	**GTAAGTG** GTCAG	++
A25	GGATAG	**TCAAGTA** TG	++
Consensus		**TAAGTG**	

Group B: TAAT-like sequences

A2	GAGTCCA	**CATAATTA**		+++
A1		**CTTAATTG**	CTTAGGT	++
A3	ATGGG	**CATAATTG**	AA	++
B2	ACTAGTA	**CTTAATAC**		++
Consensus		**C(A/T)TAATTN**		
A7	T	**CATAATCT**	TGCTCG	+
B22	T	**ATTAATAG**	TTTAAT	+
B1		**CATAATAT**	TGAACCT	+
B3	TATAAC	**TATAATAT**	A	+
B5	TACGCTCAT	**CATAATGA**		+

and SRF in fibroblasts was dependent on having intact multiple SREs in the promoter region. Because both of these transcription factors are abundant in the primitive myocardium and are limited in their extra-cardiac expression in the early embryo, interactions between SRF and Nkx-2.5 suggest a general mechanism that can control cell-type-specific expression and differentiation of cardiac tissues.

Examination of amino acid groupings within the NK-related homeodomains (Lints *et al.*, 1993) illustrated a close evolutionary relationship among the NK2, NK3, and NK4 genes. One amino acid residue, the tyrosine at position 54, is unique among the *Drosophila* NK2, NK3, and NK4 proteins and their closest vertebrate relatives. Position 54 has previously been suggested to be an influential site for DNA-binding site determination (Otting *et al.*, 1990; Wolberger *et al.*, 1991). The similar conservation of amino acids between NK class homeodomains at positions 4, 42, and 52 (within the N-terminal arm and helix 3 of the homeodomain) may also be significant as they are also implicated in sequence-specific DNA binding (Linn and McGinnis, 1992).

The amino acid sequence across the entire Nkx-2.5 homeodomain is closely related to the mammalian Nkx-2.3 (93%), Nkx-2.6 (82%), and Nkx-2.1 (82%) proteins, and it is therefore likely that all NK family members recognize the same atypical homeodomain DNA-binding core motif. Thus, it will be important to determine how the MSH-2/NK4 factors contribute to differential target gene regulation. One possibility is that variations in the relative abundance of the multiple NK proteins, each bringing in a different regulatory domain, could be competing for the same DNA-binding sites, with the "winner" determining the subsequent transcriptional events. Alternately, it may be that the NK factors contribute to tissue specificity by being tightly restricted, spatially and temporally, in their own expression.

FIG. 4. Results of repeated selection of random oligonucleotide sequences for the ability to bind to Nkx-2.5 protein *in vitro*. Nucleotide sequences of binding sites selected by a bacterially expressed Nkx-2.5 maltose-binding protein fusion protein in gel retardation assays are shown. Nucleotides in bold capital letters represent the apparent core-binding sequence from the selected oligonucleotides; nucleotides underlined were contributed by the PCR primers. Binding sites were optimally aligned by the presence of CAAG or TAAT-like sequences; relative binding activities are indicated as plus signs and the consensus sequence is illustrated. This figure was contributed by Ching Yi Chen, T. C. MC Quinn, and R. J. Schwartz (manuscript submitted).

4. Miscellaneous Homeodomain Genes in Vascular Smooth Muscle

Several other homeodomain mRNAs have been identified as having expression extending into vascular smooth muscle tissue (Table IV). These include *Gax*, a vascular smooth muscle-expressed homeodomain protein that has been suggested to play a part in cell cycle regulation (Gorski *et al.*, 1993). It is probable that more members of this transcription factor superfamily have been identified in vascular smooth muscle to this point than has been generally appreciated. Their known potential for involvement in cell-type determination as well as skeletal and central nervous system pattern development in vertebrates makes them an important group of transcription factors to consider for possible contribution to the process of vascular smooth muscle tissue differentiation.

E. Summary

We have attempted to provide some background for considering the possible importance of several transcription factor families which we would currently implicate as likely to play a role in vascular smooth muscle differentiation. However, at this time there is no compelling data to demonstrate that any of them are important for the generation of the vascular smooth muscle phenotype. More fundamentally, the ability to define a vascular smooth muscle cell according to its distinctive cellular protein/mRNA species and to distinguish it unambiguously from a myofibroblast has yet to be clearly established. This may suggest that an appropriate avenue of investigation could be to analyze gene products held in common in the phenotypic spectrum between smooth muscle cells and myofibroblasts; critical differentiation steps along this transcriptional pathway may be shared by both these cell types. Clearly, much experimental work remains to be done before it will be possible to provide specific discussion of the transcriptional regulation of the vascular smooth muscle phenotype.

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Molecular Controls of Smooth Muscle Hypertrophy and Hyperplasia: Overview

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I. Introduction

Smooth muscle migration, hyperplasia, and hypertrophy are key events in the development of the lesions responsible for cardiovascular disease and its complications. Understanding the genesis of these activities and the roles they play in the different disease entities, including the medial hypertrophy that occurs during hypertension and the intimal hyperplasia that occurs during atherosclerosis and restenosis postangioplasty, will be important if we are to develop approaches to control and prevent these disease processes.

In the chapter on regulation of arterial smooth muscle growth by Reidy, a great deal of information has been generated that has increased our understanding of the basis of smooth muscle migration and proliferation as it occurs in the rat carotid injury model that Reidy and his colleagues have so elegantly studied. In their two chapters, Reidy and Jackson (pharmacology of smooth muscle cell proliferation) have indicated that there are three waves of cellular responses that occur postballoon injury to the rat carotid artery (see also review in chapter by Schwartz *et al*,). Fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) appear to be involved in the first wave of medial replication. Both, particularly PDGF, appear to play important roles in the migration of smooth muscle cells from the media into the intima that defines the second wave. Numerous elements, some still to be identified, appear to be relevant to the third wave, which involves intimal smooth muscle replication and the depo-

sition of large amounts of extracellular connective tissue matrix. Thus the studies of injury to the normal rat carotid artery have provided valuable information concerning these smooth muscle activities and many of the agents responsible for them. This is discussed in depth by Reidy, Schwartz, and Jackson in their respective chapters. Ultimately, however, one must raise the issue of how to build on the data obtained from the rat model of balloon injury and how to relate these observations to questions concerning the phenomenon of intimal hyperplasia in which smooth muscle cells accumulate in the lesions of atherosclerosis or to the changes that occur in humans who suffer from restenosis postangioplasty of these lesions. When advanced lesions of atherosclerosis are treated by angioplasty, the cellular involvement and the status of the tissue complicate our understanding of this process as compared with the response of a normal artery to balloon dilatation.

In their discussion of smooth muscle replication and its relevancy to vascular disease, Schwartz and colleagues discuss the question of the state of differentiation of the smooth muscle cells that are stimulated to replicate. They point out that smooth muscle cells can replicate before losing any α-actin, as determined immunocytochemically, and that in the arterial tree these cells may be derived embryologically from different sources. As indicated by Schwartz *et al.*, the observations of Rosenquist and co-workers demonstrated that smooth muscle cells in the head and neck are neural crest derived, whereas those in the rest of the animal come from mesoderm. Thus there may be heterogeneity among smooth muscle cells in different parts of the arterial tree, which may possibly help to explain their different responsivity to agonists in different anatomic segments.

Schwartz *et al.* also discuss the issue of monoclonality of the lesions of atherosclerosis and assume that the lesions have been shown to be monoclonal, noting the debate over this issue. The monoclonal hypothesis is just that, a hypothesis, not a fact. Published data demonstrate that many lesions of atherosclerosis contain a single isozyme of glucose-6-phosphate-1-dehydrogenase. However, the presence of a single isozyme does not prove monoclonality. Some lesions may be derived from single cells (monoclonal) whereas others may be derived from a population of genetically identical cells (oligoclonal or polyclonal). Our understanding of this issue has been heightened but not yet resolved. Thus the genesis of the lesions as specific inflammatory–proliferative responses and their relation to numerous different etiologic agents require further study and clarification.

Schwartz and colleagues have pointed to the potential importance of bradykinin in the neointimal accumulation of smooth muscle cells

postangioplasty. They note that angiotensin-converting enzyme (ACE) inhibitors may elevate bradykinin levels by preventing its degradation. This interesting observation may explain in part the effectiveness of ACE inhibitors in suppressing neointimal formation in the rat, but not in humans. On the other hand, the cellular composition of human lesions treated by angioplasty is so different from the normal rat artery that the responses of these cells, including lesion smooth muscle, macrophages, lymphocytes, and microvascular endothelium, must still be taken into account. The effects of angiotensin II and the interaction between ACE and bradykinin on these human lesion cells present an interesting opportunity for future investigations of neointimal formation. More data on the advanced lesions of atherosclerosis and, in particular, of the turnover of macrophages, lymphocytes, and smooth muscle need to be obtained.

The use of atherectomy catheters in sampling is difficult to control. With a larger sample base of lesions that truly covers the spectrum of lesions that form and progress to advanced lesions, it may be possible to resolve the issue of just how much turnover of smooth muscle cells occurs in the advanced lesions and whether there are spurts of cell replication or, as Schwartz and colleagues have suggested, a relatively low level of constant replication. The interesting review in the chapter by Schwartz *et al.* of plaque-specific genes expressed by smooth muscle cells reminds us to look for the potential interactions among the different cells of the lesions, how these interactions lead to expression of new genes, and the important question of lineage of the cells in the lesions.

The chapter by Jackson examines the role of endogenous smooth muscle cell mitogens and inhibitors of mitogenesis. Jackson emphasizes that understanding the interactions among macrophages, T lymphocytes, smooth muscle, and endothelium will be important in unraveling the pathogenesis of the lesions of atherosclerosis. The determination of which genes are expressed in which cells at which points in time will be critical if we are to consider modifying lesion evolution and how the process can be interrupted.

Examination of the cellular interactions of atherogenesis (thus far studied extensively) and restenosis postangioplasty (thus far poorly understood) will be important in understanding the roles played by each of the different cells. Both processes contain elements of chronic and possibly immune inflammation, both of which are followed or accompanied by a fibroproliferative response. If we could devise approaches that would optimize the inflammatory–fibroproliferative process so that it retained its protective characteristics but prevent the excessive nature of this response so that lesions do not progress,

we should be able to provide greater benefit for patients who suffer from these problems.

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Smooth Muscle Cell Function from the Clinical Perspective: Overview

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A central concern among clinicians engaged in the care of patients with arteriosclerosis and other forms of obstructive cardiovascular disease is the rate at which arterial reconstructions develop luminal occlusion and lose the capacity to act as conduits for blood. This loss of function occurs regardless of the approach used to restore circulation; over a period of 5 years, a vein bypass graft is just as likely to fail as a small artery treated with balloon angioplasty. In large measure, the loss of function of the graft or the angioplastied artery is the consequence of altered smooth muscle cell mass, function, or both. These changes must be understood in detail if we are to prolong the patency of these vessels after mechanical intervention.

The link between smooth muscle cell mass and function in normal and disease states is suggested in the chapters of this section. Certain conclusions are worth emphasizing and others not previously considered are added. For example, smooth muscle cell mass increases rapidly in acutely damaged or hypertensive vessels. The molecular mechanisms controlling these processes are being discovered. But are these observations relevant for our understanding of vascular smooth muscle cell growth during development and in slowly progressing atherosclerotic lesions as well as in traumatized arteries? We know that fibroblast growth factor (FGF), at least in the rat, is critical for replication of medial smooth muscle. However, we do not know whether basic FGF is a major player in pathologic circumstances. If it is, then is it also a good target for pharmacological blockade? If we are successful at blocking basic FGF and decreasing smooth muscle cell mass, will we then affect luminal diameter and prevent occlusion (stenosis or restenosis)?

Perhaps our approach has been too limited. Although it makes

sense to focus on the treatment of the acute response to injury, perhaps what we really need to do is to suppress the injury response and then seek to restore the normal biological relationships that control wall mass and lumen diameter. Inevitably such thinking leads us back to a consideration of how blood vessels are built in the first place (vasculogenesis and angiogenesis). Although not much is known about vasculogenesis, it is clear that endothelial cells assemble on an extracellular framework and are surrounded shortly thereafter by an investing layer of smooth muscle cells. From these studies, we gain the distinct impression that endothelial cells somehow are able to recruit the smooth muscle cells to the wall and then get them to take up an extraluminal location.

How the number of smooth muscle cells is controlled is not evident. Certain observations indicate that smooth muscle cells investing a vessel might be regulated by signals from the endothelium or from the mechanical forces generated by blood flow and pressure. For example, we know that vascular diameter acutely and chronically is regulated by endothelial cells; it seems likely that changes in diameter are a reflection of an attempt on the part of the endothelium to maintain shear stress at the luminal surface in a tightly controlled range. Vessels dilate as flow increases to growing tissue, and the effect of this dilation is to increase wall stress. It is likely that wall stress is maintained within certain well-defined limits since wall stress is proportional to the ratio of lumen to wall thickness and is quite constant in large arteries of animals ranging in size from mice to elephants. This observation suggests that, directly or indirectly, endothelial cells regulate luminal diameter and wall mass.

It should therefore come as no surprise that endothelial factors affecting vascular tone and diameter might also affect smooth muscle cell growth, and vice versa. For example, nitric oxide is a vasodilator and smooth muscle cell growth inhibitor, and angiotensin II is a vasoconstrictor and smooth muscle cell growth factor. The relative balance of these and many other factors regulated by the endothelium could determine wall mass and diameter. Diseased vessels often become rigid and change their mass for unphysiological reasons (e.g., lipid accumulation, calcification, hemorrhage) without disruption necessarily of the luminal endothelium. From the work of Glagov, we know that atherosclerotic vessels endeavor to accommodate the increased mass by dilating. After a certain time the vessels are unable to dilate and develop luminal narrowing. If the endothelial cell is functioning in a normal way, and it is not clear that it does, it should continue to secrete vasodilating/smooth muscle cell growth-inhibiting factors which in turn might tend to suppress smooth muscle cell growth in the fi-

brous cap. On the other hand, the smooth muscle cells of the cap might be stimulated by the cytokines generated by the macrophages in the underlying lipid core. Caught in this "cross fire," the smooth muscle cells might become dysfunctional and perhaps lose their capacity to replicate or to contract normally. It is of some note that the caps of very advanced lesions are very fibrous and depleted of cells; furthermore, smooth muscle cells cultured from atherosclerotic plaques grow exceedingly poorly in culture.

If the cells in the cap lose their ability to grow and function normally, how then do we understand the reparative process after angioplasty? The available studies indicate that there is very little smooth muscle cell proliferation. Perhaps the answer is that angioplasty achieves what the endothelial cells overlying the stenosis have wanted to do all along, namely, to dilate the vessel. On the other hand, perhaps the artery could be viewed as a biologically burned-out tube which requires an angiogenic response for healing. The microvessels (vasa vasorum) are already there and merely need to be recruited to remodel the thrombus that accumulates at the site of injury. We have been able to demonstrate that such a process could be activated; for example, in inert porous synthetic grafts, capillaries can grow in from the surrounding tissue and provide the cells necessary for repair of the luminal surface. The microvascular endothelium performs a phenotypic flip-flop from sprout to monolayer and the accompanying pericytes become a multilayered neointima of smooth muscle cells and matrix. The net effect is a recapitulation of vascular wall building during development! A similar pattern of repair might take place in calcified, fibrous, rigid atherosclerotic arteries after angioplasty.

When viewed in this way, it becomes clear that we need to know more about the relationship between large and small vessel smooth muscle cells and endothelial cells and the rules of vascular wall assembly. It will be important to understand more clearly what smooth muscle α-actin or myosin expression signifies for smooth muscle function. We need to know more about the natural promoters and inhibitors of smooth muscle cell growth because it is highly likely that their regulation will be of importance for the treatment of the reconstructed vessel.

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Regulation of Arterial Smooth Muscle Growth

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I. Introduction

The proliferation of smooth muscle cells is thought to be a key event in the development of atherosclerotic lesions and is involved in the restenosis of arteries. To identify the factors which control the proliferation of these cells has been the "holy grail" of vascular biology for many years, and while significant advances have been made, this goal has still not been reached.

To understand the approach that has been taken since 1980, it is necessary to reflect that the "reaction to injury theory" as initially proposed by Ross and collaborators(1–3) focused on the platelet-derived growth factor (PDGF) and the interaction of platelets with the denuded artery. The key concepts of this hypothesis were loss of endothelium followed by platelet adherence to the exposed subendothelial matrix. This released platelet PDGF would then interact with the underlying smooth muscle cells and so stimulate their replication. This growth of smooth muscle cells would be the start of intimal lesion formation. Strong support for this theory came from numerous *in vivo* studies in which the endothelium of arteries was deliberately denuded using a variety of mechanical devices(4–8). Almost invariably, these procedures led to the development of smooth muscle cell intimal lesions. With hindsight, it is now clear that these results were interpreted in a too simplistic manner since we now know that injury to the arterial wall involves a complex interaction of several growth factors with the smooth muscle cells. Regardless of

these early misconceptions, the process of mechanical injury to an artery *in vivo* is still widely used to study the process of arterial lesion development, and balloon catheter injury of the rat carotid has now become the standard experimental procedure in many laboratories.

Our early studies of vascular injury showed that loss of endothelium was accompanied by platelet adherence and no increase in smooth muscle cell replication nor was the development of intimal lesions observed(7,9,10). One interesting facet of these studies was that the endothelium was denuded in the artery with fine nylon wire which, when passed into a rat artery, only removed a thin line of endothelial cells. This injury was rapidly repopulated with endothelium and at that time we attributed the lack of response by the smooth muscle cells to this fact. For example, a row of denuded endothelium, approximately five cells wide, was repopulated with endothelium by 48 hr(7), which coincidentally is the approximate time when smooth muscle cells start to divide. In other studies, we noticed that as the width of the denuded zone was increased, total regrowth of the endothelium was delayed, but, more importantly, we were able to detect small increases in smooth muscle cell replication in these arteries. Despite the increased replication, no intimal lesions formed(10). Since the process of cell denudation and of platelet adhesion appeared to be identical after any denudation procedure, we argued that the main reason our injuries did not initiate lesion growth was because of the relatively rapid regrowth of endothelium which suppressed the proliferation of smooth muscle cells. Indeed, several groups had shown that heparan sulfate made by endothelial cells would in fact suppress smooth muscle cell growth(11,12). As discussed later, we now know that the process of mechanically removing the endothelium has a strong influence on smooth muscle cell replication in arteries and that it is likely that the injury inflicted to arteries in these studies did not traumatize the underlying smooth muscle cells and so did not cause the release of basic fibroblast growth factor (bFGF). Thus, the injuries inflicted to arteries in our studies were in all probability different from other denudation procedures in that there was limited trauma to the underlying smooth muscle cells as well as rapid regrowth of the endothelium. At that time, however, we were unable to discern why our results were different from others, but these studies did show that platelet interaction with the denuded artery does not necessarily promote smooth muscle cell replication. This is important since it suggested that factors other than PDGF were important for smooth muscle cell proliferation.

We next turned our attention to trying to understand why balloon catheter injury of arteries would stimulate intimal lesion growth. A va-

riety of devices have been used over the years to damage the endothelium, ranging from diamond-tipped probes to small catheters made of fishing line(4–8). In the early 1970s, investigators started to use balloon catheters which were passed into arteries and then inflated(13,14). This inflated balloon was then dragged along the artery and, within several weeks, invariably caused a significant intimal lesion formation. With the availability of such balloon catheters, it was possible for different laboratories to carry out similar denuding procedures and valid comparisons could be made between species. Indeed, these catheters have been used to stimulate significant intimal lesions in rats, rabbits, dogs, pigs, and primates. We opted to use a 2F balloon catheter in rat carotid artery as our experimental model system to try to understand the process of vascular injury and smooth muscle cell proliferation(4). The carotid artery was chosen because it is a relatively long artery with no side branches, thereby limiting endothelial cell regrowth to the ends of the artery (Fig. 1). This allowed us to

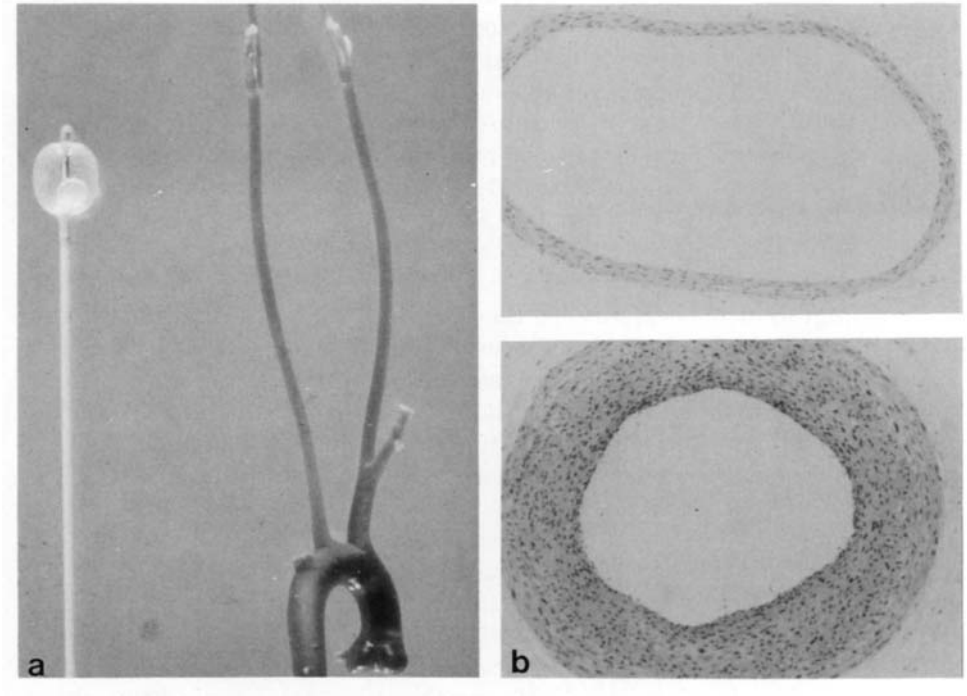

FIG. 1. (a) Model of rat carotid artery with a 2F inflated balloon alongside. (b) An uninjured rat common carotid artery (top) and the pronounced intimal lesion which is formed after use of the 2F balloon catheter (6 weeks after injury) (bottom).

study intimal lesion growth in the absence of endothelium(15,16). The topic of endothelial cell regrowth is not discussed in this chapter; however, we and others have found that the regrowth of endothelium has a profound effect on the growth of smooth muscle cells, but that in most animals this regrowth is limited(15,16). In the rat carotid artery, endothelial cell regrowth occurs on the denuded common carotid from the internal carotid bifurcation and from its junction with the aorta. In practice, this means that approximately 7–10 mm of the distal and proximal ends will be recovered with endothelium, but that the center 10 mm of the artery is never reendothelialized(16). A further advantage of the rat carotid artery is that these arteries respond in a uniform manner to the balloon catheter and few leukocytes are present in these lesions(17). Arteries from many other species, subjected to balloon catheter injury, show similar responses with only a few minor variations. We believe the rat artery is a valid model in which to study smooth muscle cell proliferation, but caution must be used when extrapolating the results from these arteries to the human artery. Apart from the fact that most animal studies examine the effects of injury to normal healthy arteries, which contrasts very markedly from the effect of balloon catheter angioplasty to human arteries, an important difference is that normal rat arteries do not have a smooth muscle cell-rich intima. Therefore, in order to develop intimal lesions in rat arteries, smooth muscle cells have to migrate from the media into the intima. This means that cell migration is essential for lesion growth in rats. This contrasts with humans, in whom most arteries possess a developed intima, and so it is possible that intimal lesions can develop solely via replicaton of intimal cells. It is therefore unclear whether cell migration is necessary for the growth of these intimal lesions. In reality, this could mean that rat lesions could be suppressed by agents that affect smooth muscle cell migration, whereas the same agent might have little or no effect on human lesions. This fact could possibly explain why the angiotensin-converting enzyme (ACE) inhibitors, while proving very effective in blocking lesion growth in rat arteries, had no effect in humans. In our laboratory, we determined that these compounds had no significant effect on smooth muscle cell replication, but markedly affected smooth muscle cell migration. Without any prior knowledge as to the importance of smooth muscle cell migration in human lesions, it may be foolish to believe that this class of drugs could influence lesion growth in humans. It is still our belief that a great deal can be learned about smooth muscle cell growth using this animal model, but it can be misleading to directly apply these findings to other species without fully understanding the process of lesion development.

II. Response of Artery to Injury

A. Replication of Medial Smooth Muscle Cells

We have divided the response of an artery to injury into various stages or waves. These stages are based on the temporal sequence of events which take place in the artery after a widespread denuding injury and coincidentally also correspond with the involvement of different stimuli(18).

Within 24 hr following balloon catheter injury to rat arteries, medial smooth muscle cells start to replicate, and by day 4 these cells migrate into the intima(4,19). We refer to these events as the first and second waves of the smooth muscle cell response. Once smooth muscle cells have reached the intima, these cells then undergo a sustained period of replication which we call the third wave(16). This process of events is outlined in Fig. 2.

The first wave response by the smooth muscle cells has perhaps received the most attention in recent years, and the results from a number of studies have allowed us to draw some firm conclusions as to factors that control this process. After balloon injury, an increase in medial smooth muscle cell replication can be detected within the first 24 hr after injury; it reaches its peak between 48 and 72 hr, is reduced by day 7, and is undetectable by day 14 (Fig. 3). Initially, platelet-released PDGF was thought to be responsible for this medial cell replication, but a group of studies discussed next have now shown that this is not the case. In fact, it appears that another growth factor, bFGF, is the important mitogen for this cell replication.

One key result came from a study in which rats were made thrombocytopenic and then subjected to balloon catheter injury(20). The goal of this study was to determine if platelets or PDGF were important for medial smooth muscle cell replication following injury. We observed that the absence of platelets and presumably platelet factors made no difference to the rate of smooth muscle cell replication, and in fact, smooth muscle cell proliferation in the arteries of thrombocytopenic animals was identical to that observed in the control animals (Fig. 4). The role of PDGF in smooth muscle cell replication has been addressed in a more direct manner either by adding recombinant PDGF to animals or by blocking PDGF with a neutralizing antibody. Neither of these treatments had any effect on the medial smooth muscle cell replication in these balloon catheter-injured rat arteries(21,22). These results suggest that another factor(s) is important for the stimulation of smooth muscle cell replication *in vivo* and that PDGF is not an important mitogen for arterial smooth muscle cells following injury.

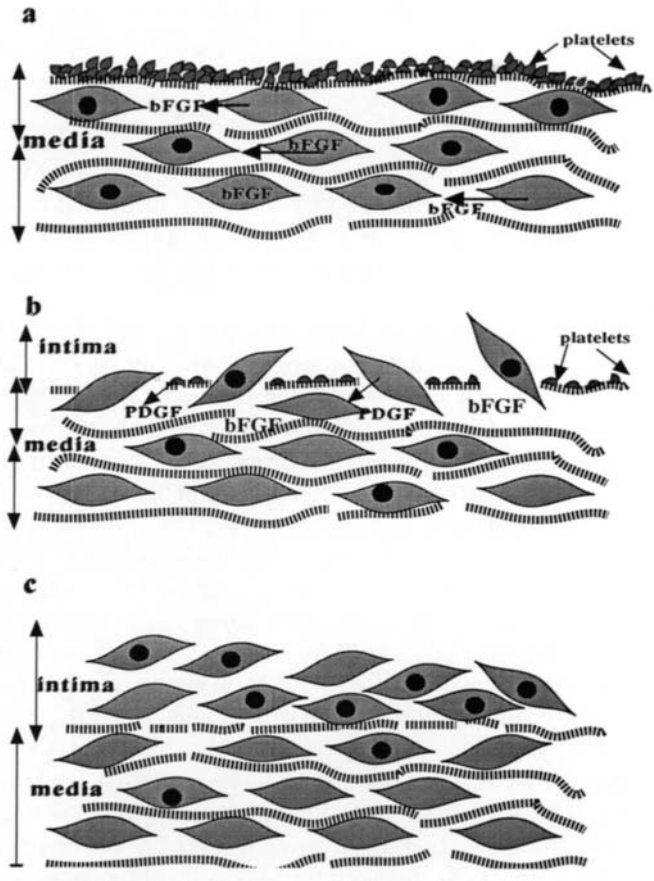

FIG. 2. The various stages of intimal lesion development in injured rat arteries. Details of each stage are discussed in the text in detail. (a) Medial smooth muscle cell replication. This starts between 24 and 48 hr after balloon injury. The injured artery has a layer of platelets on the luminal surface of the denuded artery. An average smooth muscle cell replication rate at this time would be between 9 and 16% (see Fig. 3). Dividing cells are denoted by a dark circle in the cell. The main growth factor important for this cell replication is basic FGF (FGF2) which is released from traumatized cells. Our studies show that in the uninjured arteries, FGF2 is localized in an intracellular pool whereas after injury it is found to be mainly in an extracellular site. (b) Smooth muscle cell migration. Approximately 4 days after balloon catheter injury, smooth muscle cells start to migrate from the media into the intimal space. Thus migrating cells can be quiescent, although most often are replicating. PDGF, presumably from platelets and from smooth muscle cells, has been shown to stimulate migration of these cells without any change in replication. Likewise, bFGF stimulates the movement of smooth muscle cells into the intima. Since there is no marker of migration at present, it is unknown when migration stops. (c) Intimal smooth muscle cell replication. Intimal smooth muscle cells exhibit a high replication for up to 2 weeks after injury (5–70%). Those smooth muscle cells which line the lumen of the still denuded artery continue to have an increased rate of replication even after 1 year (see Fig. 3). Medial smooth muscle cell replication is minimal in these arteries.

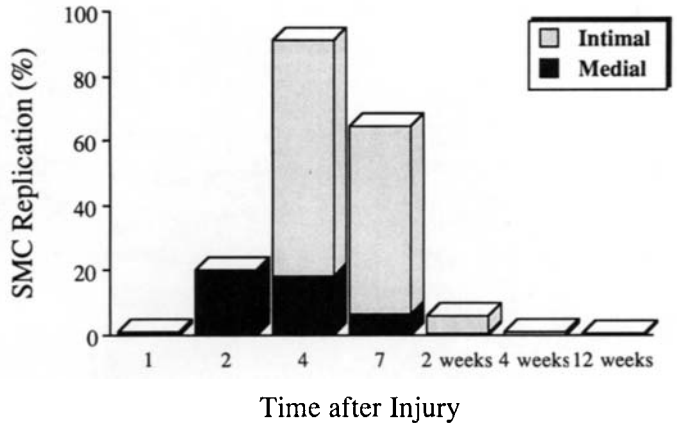

FIG. 3. Time course of medial (■) and intimal (□) smooth muscle cell replication in rat common carotid arteries after balloon catheter injury.

We now believe that bFGF is critical for stimulating the first wave of smooth muscle cell replication. Our first indication that bFGF might be involved in this process came from an experiment in which rat arteries were subjected to two different denuding injuries(19). In particular, we compared the smooth muscle cell replication induced by balloon injury with that observed after a gentle denuding injury. This latter injury is made with a loop of nylon wire (5/0) which is passed into the carotid artery and rotated repeatedly in the artery. This has the effect of totally denuding the artery of endothelium while causing minimal trauma to the underlying medial smooth muscle cells, but still stimulating arterial lesion growth. In many respects, this arterial injury is similar to balloon injury in that total endothelial denudation is achieved and the exposed subendothelium is covered with platelets(19). The response of rat smooth muscle cells in arteries subjected to these two injuries, however, was very different in that the gentle injury caused only an approximate 1–2% of medial smooth muscle cells to replicate, whereas a replication rate of 13% was observed after balloon catheter injury (Fig. 5). This finding suggested to us that the denudation procedure used was just as important as the fact that denudation had occurred. In evaluating factors which might be responsible for this finding, we noted that there were few differences in these injured arteries. Apart from similarities with respect to endothelial cell loss, similar changes were also observed in expression of PDGF-A chain and in transforming growth factor β (TGF-β)(23,24). One marked difference, however, was in the finding

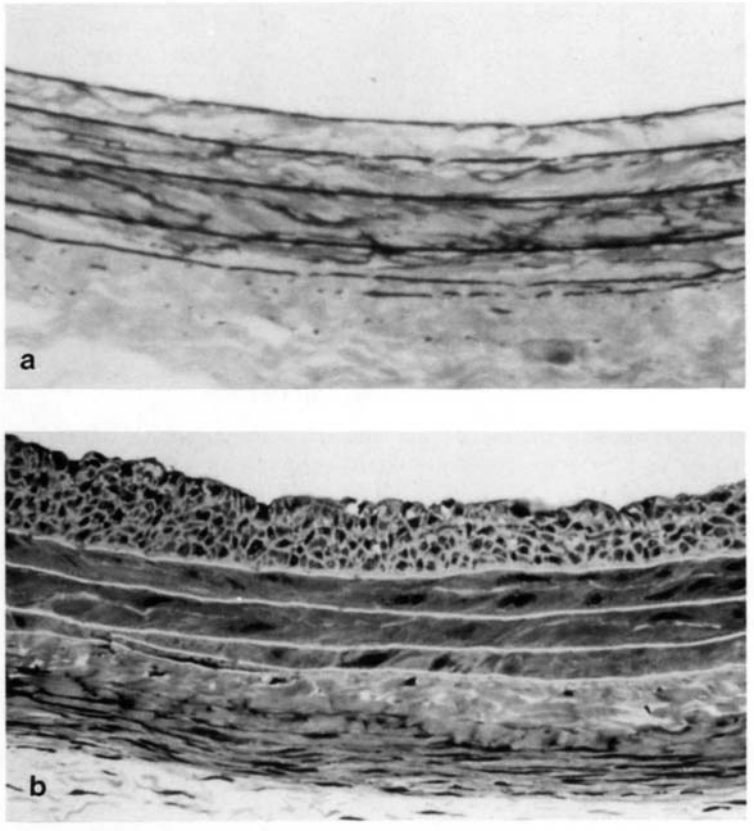

FIG. 4. Sections of rat arteries 7 days after balloon catheter injury. (a) Rat was depleted of platelets throughout the entire experiment and no intima formed. (b) Control rat with pronounced intimal thickening.

that after balloon catheter injury, 20–25% of DNA was lost from the artery, which presumably reflects medial cell damage. After gentle injury, no medial cell loss could be detected. It therefore appeared that injury to the artery was perhaps correlated with smooth muscle cell replication seen in injured arteries.

In trying to relate the importance of cell injury to a specific cellular mechanism, we considered that bFGF may be important in this process. One key fact that influenced our selection of bFGF was that this protein has no signal sequence(25) and therefore cannot be secreted in the normal manner. Indeed, cell injury has been postulated to be one mechanism by which it is released(26). Therefore cell injury, caused by the use of the balloon catheter, may be responsible for the

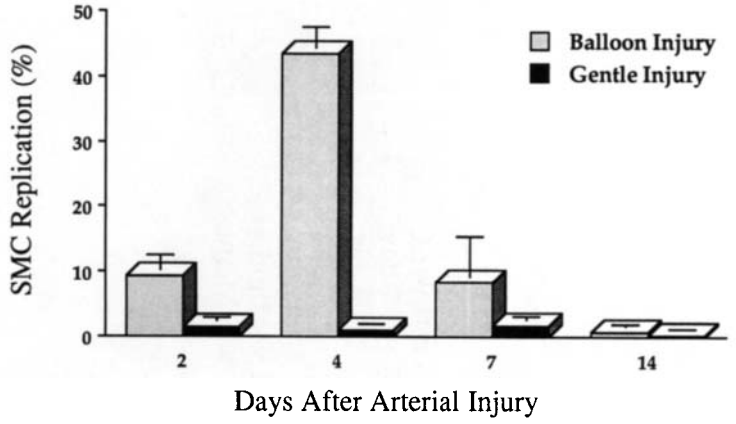

FIG. 5. Replication of medial smooth muscle cells of rat carotid arteries after balloon catheter injury (□) and gentle injury (■). Both these injuries totally denuded the endothelium and caused platelets to adhere to the exposed subendothelium. Despite these similarities in the injury process, the replication rates in these arteries are markedly different. As discussed in the text, the release of bFGF is associated with balloon injury and not with gentle injury.

release of bFGF from the injured smooth muscle cells and thereby for the increased cell replication. After gentle injury, however, little or no cell death was detected and therefore presumably a minimal release of bFGF from the arterial smooth muscle cells occurred. Thus, the degree of injury and the release of bFGF from injured cells may be the molecular trigger for the rapid stimulation of arterial smooth muscle cells following a mechanical injury. This fact would also explain the results referred to earlier in which small zones of endothelium were removed with nylon wire and no smooth muscle cell replication was observed(7,10). In these experiments, we had attributed this lack of any response by the smooth muscle cells to the rapid regrowth of endothelium. In all probability, this form of denuding injury did not liberate bFGF and so no smooth muscle cell replication occurred.

We next turned our attention to validating our hypothesis that the release of FGF2 from smooth muscle cells was a key event for smooth muscle cell replication in these injured arteries. One obvious but important issue was to validate that smooth muscle cells in rat arteries do contain bFGF. Smooth muscle cells *in vitro* have been shown to express FGF2(27), but no data were available for these cells *in vivo*. Our Northern blots of normal rat arteries showed that smooth muscle cells express FGF2 and, interestingly, that a decrease in expression was observed after injury(28) (Fig. 6). Immunolocalization studies also re-

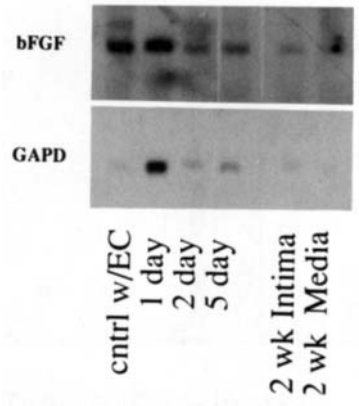

FIG. 6. Expression of bFGF mRNA in balloon-injured rat carotids. Expression is strongest in uninjured arteries and the intimal smooth muscle cells show diminished expression.

vealed that FGF2 was readily detected in the cells of normal uninjured arteries and that following injury this pattern of cellular staining was lost. This observation was confirmed by immunoblots which also showed a fall in bFGF protein levels after injury. That the quiescent normal artery was rich in bFGF was something of a puzzle since it might be expected that the presence of the mitogen would be associated with an increase in cell division. Smooth muscle cell replication in these arteries, however, is in the order of approximately 0.1%. One explanation of why FGF2 in the artery does not initiate replication is that the mitogen is located in a compartment where it is inaccessible to the high-affinity receptors. Presumably, after injury there must be a shift in the distribution of FGF2 which is associated with entry into the cell cycle. To try to document this process, we utilized the fact that FGF2 has a strong affinity for heparin(26). The experiment was based on the supposition that exogenous heparin would bind any available extracellular bFGF and, if given in sufficient concentration, would promote the egress of the mitogen out from the arterial wall(29–31) since this heparin–FGF2 complex would be lost from the artery into the circulation(32). Studies in our laboratory and by others have shown that FGF2 is released into the circulation by an infusion of heparin(32,33). When heparin was injected into normal uninjured animals, however, quantitative loss of FGF2 from these arteries was not observed (Fig. 7). This datum suggests that in the normal artery, which has a very low replication rate, FGF2 is not accessible to the added heparin. Indeed, our immunocytochemical data do lend support to the fact that FGF2 is localized to the smooth muscle cells, and several reports have found that FGF2 is frequently localized to the nuclei of quiescent cells. A very

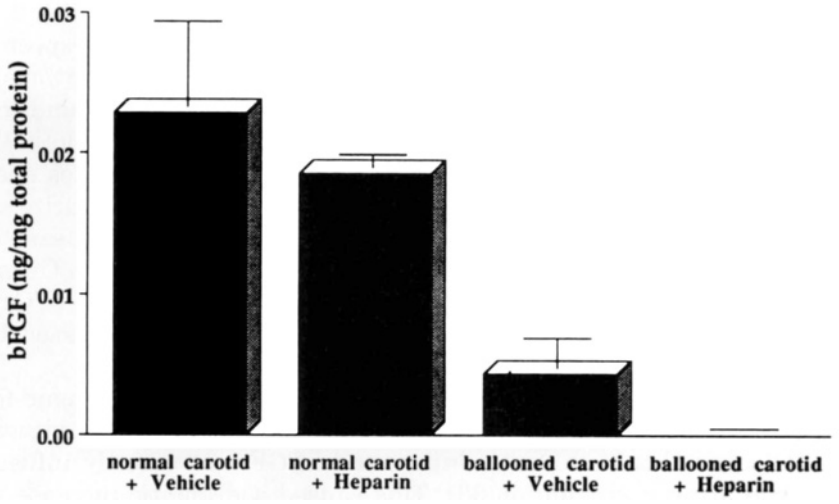

FIG. 7. Quantitation of bFGF content of rat carotid arteries 9 hr after balloon injury and injection of vehicle or heparin (888 USP/kg before injury). Heparin had no effect on uninjured arteries, but we were unable to detect bFGF in injured arteries.

different result was obtained after rat arteries were subjected to balloon injury and an immediate infusion of heparin. In the absence of heparin, a significant drop in the FGF2 content of these arteries was observed after injury, but after heparin we were unable to detect any FGF2 in these arteries (Fig. 7). Immunocytochemistry also showed a dramatic loss of FGF2 from the medial cells following injury and heparin. We believe that these data suggest that balloon catheter injury causes smooth muscle cell injury and release of FGF2 to an extracellular pool where it can bind to both low-affinity and high-affinity receptors. Exogenous heparin is a low-affinity receptor for FGF(26) and so will compete with these arterial receptors for the binding of FGF2(29–31). Once bound to heparin, this FGF2 complex could then diffuse out of the artery and be cleared into the blood pool. We believe that this result supports our hypothesis that FGF2 is normally stored in an intracellular compartment, but after injury it is displaced to an extracellular compartment and so is able to interact with the appropriate receptors. Indeed, we and others have shown that after administration of bFGF, a bolus infusion of heparin caused a marked increase in systemic FGF(32,34).

One interesting fact which arose from this study is that we were unable to detect any bFGF in the injured artery after heparin administration. This is puzzling since balloon injury is thought to kill only

approximately 20–25% of the smooth muscle cells. Thus, if FGF2 is only released from dead or dying cells, then we might have expected only 25% of total FGF2 to be lost from the artery. This is obviously not the case. One explanation comes from the work of McNeil and collaborators(35–37), who have noted that it is possible to nonlethally traumatize cells such that they release intracellular molecules such as bFGF. Therefore, balloon catheter injury may in fact traumatize all the medial cells to some degree and so permit widespread release of FGF2, but only a small percentage of cells are actually killed. Of importance to our studies is that it is possible that most of the FGF2 stored in the smooth muscle cells of the artery may be released by vascular injury.

The next step was to establish that FGF2 is indeed mitogenic for arterial smooth muscle cells. Accordingly, rat arteries were subjected to balloon catheter or gentle injury and bFGF was directly infused into the arterial circulation(38). This caused a dramatic increase in smooth muscle cell replication (Fig. 8), and daily infusion of FGF2 led to a striking increase in intimal lesion size. In fact, the highest rates of smooth muscle cell replication thus far observed in rat arteries were achieved by the addition of FGF2. We therefore concluded that *in vivo* FGF2 is a potent mitogen for arterial smooth muscle cells, and

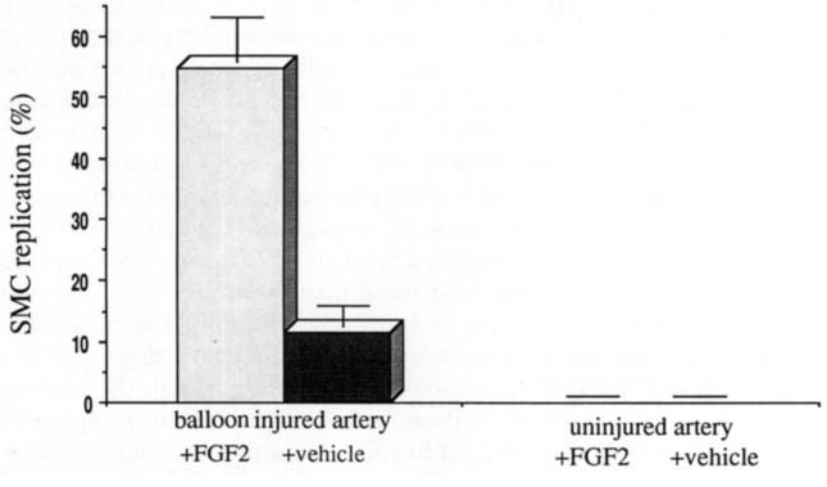

FIG. 8. Effect of added bFGF to smooth muscle cell replication in rat carotid arteries. After balloon injury a single bolus of bFGF (120 μg/rat) caused a significant increase in the replication of medial smooth muscle cells. In uninjured arteries, which have low rates of replication, the addition of bFGF had no effect.

if release of endogenous FGF2 was the critical mitogen for medial smooth muscle cell replication, then any disruption of this process should have a marked effect on smooth muscle cell replication in balloon-injured arteries. To this end, we made an antibody to bFGF which was shown to be effective in blocking the mitogenic action of bFGF and infused it into animals immediately prior to balloon catheter injury. A single injection of this antibody (10 μg/rat) caused a highly significant reduction in smooth muscle cell replication (13 to 1.5%) when measured 24–48 hr after balloon catheter injury(39). We believe this result strongly supports our hypothesis that bFGF is the critical mitogen involved in the stimulation of arterial medial smooth muscle cells after mechanical injury (first wave). Interestingly, this single injection of FGF2 antibody did not block the growth of intimal lesions seen after 7 days, which would suggest that other pathways, independent of medial replication, are important for the growth of arterial lesions.

III. Smooth Muscle Cell Migration

No intimal smooth muscle cells exist in the arteries of rats; this is also true of many other small animals, and consequently smooth muscle cells must migrate from the media into the intima for intimal lesions to develop (see Fig. 2). Little attention has been paid to the process of smooth muscle cell migration in arterial lesion development, but data have shown that interruption of this process has a profound effect on lesion development(20,22). We first became aware of migration as a separate and differentially controlled process in studies using arteries of thrombocytopenic rats. After balloon catheter injury, we noted no change in smooth muscle cell replication, but the absence of platelets markedly suppressed lesion growth(20) (see Fig. 8). We therefore concluded that platelet factors and possibly PDGF were important for the movement of cells into the intima. This issue was addressed directly in a collaborative study with Gordon Ferns, where it was noted that antibodies to PDGF had no effect on intimal smooth muscle cell replication of balloon-injured rat arteries, but that daily administration of the antibody for 8 days markedly suppressed the size of the intimal lesion(22). This result also suggested that PDGF played a more significant role with respect to smooth muscle cell migration rather than to smooth muscle cell replication. The same conclusion was reached in a study in which PDGF was given to rats whose arteries had been injured with a balloon catheter(21). Chronic infusion of PDGF did not change the replication of either intimal or medial smooth muscle cells, but did significantly

increase the size of the intimal lesions. Taken together, the results from these studies strongly support the notion that in an injured artery, PDGF acts by stimulating smooth muscle cell migration into the intima and not by stimulating cell replication. Further, they emphasize that cell migration is a critical component for the growth of an intimal lesion.

In recent years we have become interested in the cellular and molecular events necessary for the migration of smooth muscle cells into the intima. One starting place for these studies was to follow the approach taken by workers in the field of cancer and to determine if smooth muscle cells from injured arteries synthesize proteases which may act to digest the surrounding extracellular matrix and so facilitate cell movement(40,41). We found that, following balloon catheter injury, rat arteries express both uPA and tPA and that a marked increase in plasmin activity was detected 3 days after injury and was sustained for approximately another 10 days(42) (Fig. 9). This time sequence is interesting because the earliest time smooth muscle cells are found in the intima is 4 days after injury. In order to try to link the presence of plasminogen activators with smooth muscle cell

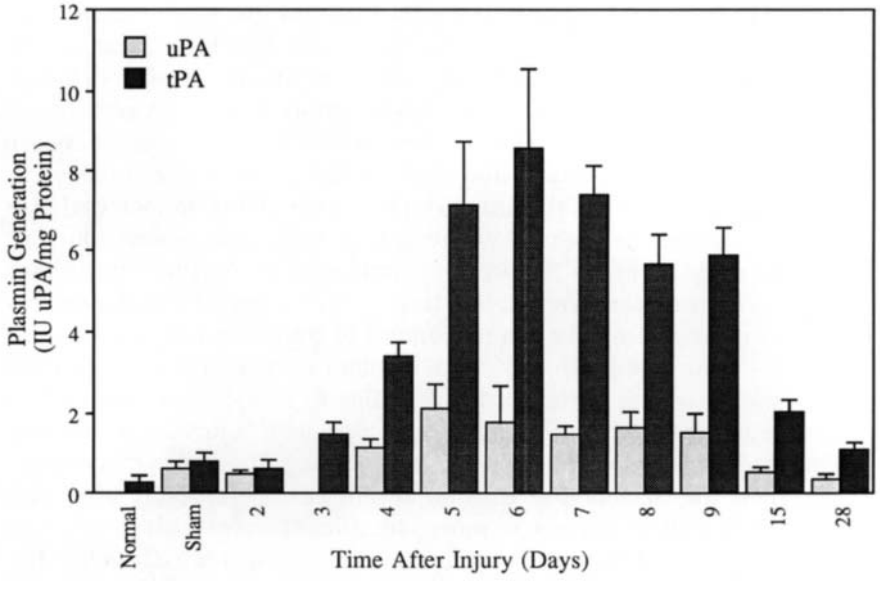

FIG. 9. Plasmin activity of rat carotid arteries after balloon catheter injury. Total plasmin activity was measured in extracts of arterial wall using a chromogenic substrate for plasmin (Spectrozyme). Activity that was quenched by ameloride was determined to be uPA (□) activity and the remaining activity was tPA (■).

migration, we inhibited plasmin and the plasminogen activator with transexamic acid(43,44). This compound binds to the lysine-binding site of plasminogen and plasmin and so blocks the binding of plasminogen activators to plasminogen and also blocks plasmin activity. In these studies we noted a significant reduction in the rate of smooth muscle cell migration in ballooned arteries when rats were treated with this drug with no change in cell replication (Fig. 10). We believe that these data support the concept that plasmin is important for the migration of smooth muscle cells into the intima and presumably does so by degrading various matrix components of the arterial wall.

In other studies we investigated the role of growth factors on the process of cell migration. An antibody against PDGF was found not only to block smooth muscle cell migration but also to inhibit plasmin activity in these ballooned arteries(42) (Fig. 11). We believe these data suggest that chemoattractants such as PDGF are able to induce expression of these proteases. These data may well explain how PDGF is able to promote smooth muscle cell migration. FGF2 is also linked to cell migration and has been shown to promote the migration of endothelial cells and also to stimulate plasminogen activator activity(45). Since FGF2 is released following injury by a balloon catheter,

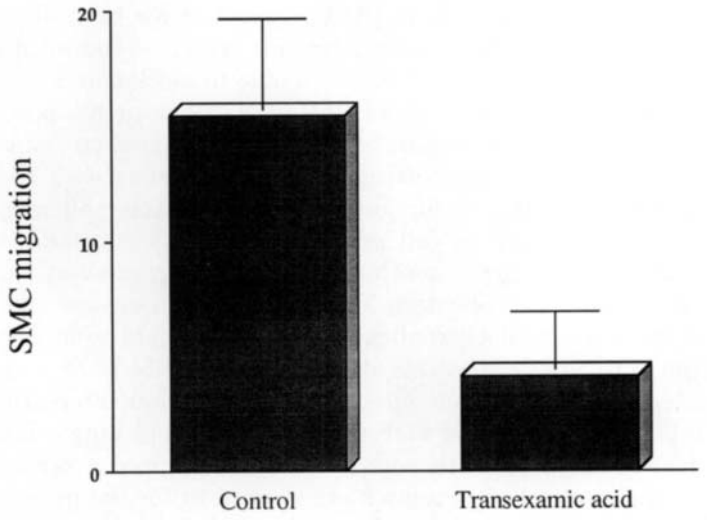

FIG. 10. Effect of plasmin inhibitor, transexamic acid, on smooth muscle cell migration in balloon-injured rat carotid artery. The drug was given daily for 4 days (2.73 g/kg) and the number of smooth muscle cells which had migrated to the intimal surface was quantitated by scanning electron microscopy.

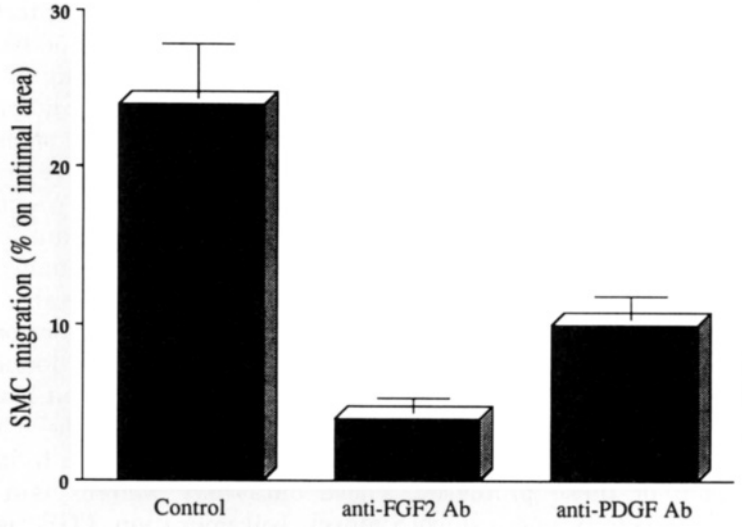

FIG. 11. Influence of FGF2 and PDGF on smooth muscle cell migration in rat balloon-injured carotid arteries. The area of intimal surface covered by smooth muscle cells was quantitated by smooth muscle cells. Antibodies to FGF2 or PDGF were given immediately before injury and the experiment was terminated 4 days later.

we examined the action of bFGF on smooth muscle cell migration in the injured artery. Administering bFGF to rats with injured arteries stimulated the movement of smooth muscle cells into the intima and, as with PDGF, antibodies to FGF2 were able to block this process(46) (Fig. 11). One concern with using FGF2 is that it is such a potent mitogen that it is difficult to separate its effect on migration from its effect on cell replication. Interestingly, in these studies with the FGF antibody, we obtained a reduction in smooth muscle cell migration with no apparent effect on cell proliferation. Thus, it would appear that FGF2 can influence smooth muscle cell migration in isolation from its effect on cell replication. Currently we are unsure as to why the antibody did not block replication, but this could reflect the low titer of the antibody used in this study or that proliferation measured 4 days after injury is not controlled by FGF2. Another interesting feature of this study is that the FGF antibody, while blocking cell migration, did not reduce plasmin activity in these injured arteries. This might suggest that other proteases are important for the migration of smooth muscle cells in the injured arterial wall.

Yet another agent which influences plasmin activity in arteries is heparin. When heparin is continuously administered to rats after balloon injury to their carotid arteries, we observed a significant de-

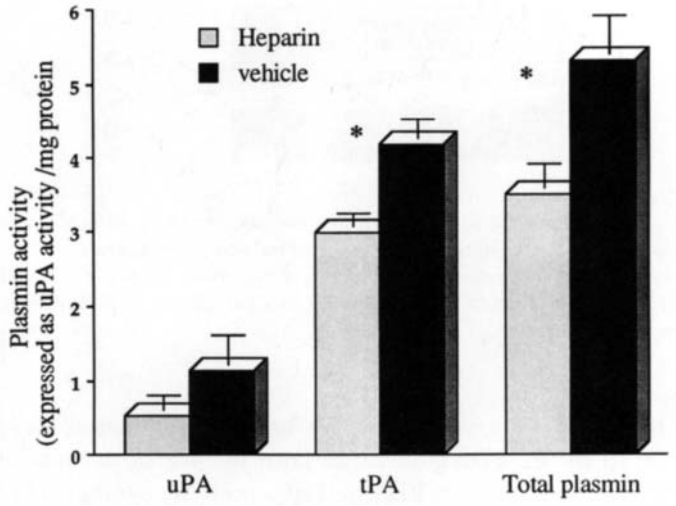

FIG. 12. Plasmin activity in balloon-injured rat carotid arteries. Animal received either heparin (□) (1.0 mg/kg iv per hr) or vehicle (■) for the duration of the experiment. Five days after injury the plasmin activity of these arteries was determined. Data are expressed as international plasminogen activator (uPA) activity per milligram of protein. uPA activity is the amount of total plasmin activity generated inhibited by amiloride.

crease in the total plasmin activity of these arteries (Fig. 12). By using the uPA inhibitor, ameloride, most of the plasmin activity could be shown to be tPA derived. As mentioned earlier, heparin is known to influence the replication of smooth muscle cells, but these data suggest that heparin can also inhibit intimal lesion growth by blocking the process of smooth muscle cell migration(47).

Plasmin is known to be involved in the activation of other proteases which are important in the degradation of extracellular matrix. Matrix metalloproteinases are enzymes found in both normal tissues and in metastatic tumors which have the ability to degrade a variety of matrix molecules(48–52). We have started to examine the role of these enzymes in smooth muscle cell migration. Our work thus far has concentrated on the type IV collagenases or, more precisely, the 72- and 92-kDa gelantinases, which have the ability to degrade type IV collagen, collagen fragments, and gelatin. We have observed that, within 6 hr after balloon injury to rat carotid arteries, a significant increase in an 88-kDa gelatinase was observed and remained elevated for approximately 6 days(53) (Fig. 13). This activity was not seen in the intima, nor was it correlated to smooth muscle cell replication. The 72-kDa activity was expressed in normal arteries and

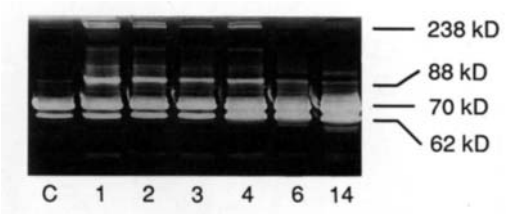

FIG. 13. Zymogram showing metalloproteinase activity in extracts of balloon-injured rat arteries. One day after injury, a new band of substrate lysis was seen at 88 and 238 kDa. These activities were still present at day 4 but were markedly reduced by day 6. Prominant activity at 70 and 62 kDa was seen in all arteries and at days 6 and 14 their activities were increased.

showed little change after injury. We have also detected an increased expression in rat 92-kDa gelatinase both by Northern blots and by *in situ* hybridization. The 88-kDa activity seen in zymograms is in all probability the active form of the 95-kDa correlation which is MMP-9. Thus, there is a correlation in balloon-injured arteries between the expression of MMP-9 and smooth muscle cell migration. The importance of these proteases for the development of arterial lesions is not currently well understood, but using a metalloprotease inhibitor(54), we were able to block almost totally the migration of smooth muscle cells into the intima while having no effect on cell replication. The fact that inhibitors were able to have such potent effects on smooth muscle cell migration suggests a strong link between gelatinases and smooth muscle cell migration.

All metalloproteases are secreted as inactive zymograms and it is necessary to activate these enzymes before their activity can be realized. Interestingly, the 72-kDa gelatinases can be activated by plasmin, but not the 92-kDa gelatinase. We are therefore unclear as to how MMP-9 is activated in the arterial wall, but one possibility is that it can be activated by stromelysin which in turn is activated by plasmin. Evidence for such a connection is not currently available in the injured artery.

One final aspect relating to cell migration is whether the process of cell movement from the media into the intima is necessary or important for lesion growth in arteries which already possess intimal smooth muscle cells. In such arteries, one possibility is that intimal lesion growth is solely due to replication of the existing intimal cells. Since currently there are no reliable markers for cell migration, we are not able to answer this question. In fact, we can detect cell migration reliably by quantitating the numbers of smooth muscle cells in the intima when they first start to move. This means that after bal-

loon injury of rat arteries, cell migration can only be detected by counting the number of cells that arrive in the intima 4 days after injury. This is because there are almost no intimal cells by day 3, and yet a significant number of cells are detected in the intima by day 4. Beyond that time, any change in the intimal cell number could be attributed to intimal replication or cell migration. Thus, in arteries with an existing intima, counting the number of intimal cells would not permit any conclusions to be formed regarding migration, which implies that our ability to recognize smooth muscle cell migration is not very sophisticated. It will be important to obtain these data because the successful inhibition of intimal lesions in the rat thus far is only achieved by agents that inhibit smooth muscle cell migration(53,55). Both PDGF antibodies and the angiotensin-converting inhibitors fall into that category, and these treatments were found to have no effect on intimal cell replication and yet successfully inhibit lesion growth in the rat. The process of smooth muscle cell migration thus far has not been shown to be important in humans, and studies to examine cell migration in large primates will be required before the use of migratory inhibitors could be considered a sensible strategy to block intimal lesion growth.

IV. Intimal Smooth Muscle Cell Replication

If the smooth muscle cell replication in an injured artery is examined at a variety of times after injury, the highest rates of replication are found in the intima, not the media, and this intimal replication, unlike that of the media, is sustained for several weeks(4,16) (see Fig. 3). From these data it is reasonable to conclude that the size of an intimal lesion could be due mainly to intimal cell proliferation. Relatively little is known about the control of intimal smooth muscle cell replication, in part because the experiments on intimal cell replication have failed to discern a clear pathway to their growth control. In this laboratory, our approach has been to extend our knowledge gained with medial smooth muscle cell replication and ask if it will help us understand intimal cell proliferation.

One group of experiments, therefore, was designed to examine the role of bFGF on intimal smooth muscle cells, since one possibility was that FGF synthesized by intimal smooth muscle cells was responsible for this continued replication. Northern blot analysis showed no upregulation of bFGF mRNA by intimal smooth muscle cells; in fact, a decrease was observed(28) (see Fig. 6). Further examination of Western blots of rat arteries showed a decrease in bFGF protein after injury. We have also tried to inhibit intimal smooth muscle cell replica-

tion with our bFGF antibody and, even though it was able to block medial smooth muscle cell replication, no reduction in intimal smooth muscle cell replication was achieved. Collectively, these data would suggest that perhaps bFGF has little to do with intimal replication. In a recent study, however, the expression of bFGF in intimal lesions was examined. Using a new *en face* approach, we were able to detect a bFGF message in those smooth muscle cells which line the lumen of the injured arteries(56). Of particular interest was that bFGF expression was high at early times after injury and almost undetectable at times (6 weeks after injury) when smooth muscle cell replication is low. This association of bFGF expression and cell replication is intriguing and could suggest that bFGF is important for the proliferation of intimal smooth muscle cells. Unlike a freshly balloon-injured artery, however, we have no data of widespread cell death at these late times after injury and so the mechanism for bFGF release is unclear. One possible explanation may be found in the work done by McNeil and colleagues(35,37,57) in which they demonstrated that non-lethal trauma to cells may cause the release of intracellular molecules. In their studies, they suggested that blood flow may initiate this event in the endothelium(36). In our studies, the smooth muscle cells which express bFGF line the luminal surface of the artery and so are in contact with flowing blood. A working hypothesis, therefore, is that bFGF can be released from these cells via non-lethal trauma and so stimulate surrounding smooth muscle cells. Detailed studies on the presence of FGF receptors and the mode of their activation need to be carried out before we can conclude that bFGF is important for intimal smooth muscle cell replication.

Studies have addressed whether intimal smooth muscle cells respond to PDGF. Data from Jawien(21) showed that giving PDGF to animals did not initiate intimal smooth muscle cell replication and infusion of PDGF antibodies when delivered at a sufficient concentration to block smooth muscle cell migration; it likewise had no effect on intimal smooth muscle cell replication(22). These data would suggest that PDGF is not an important mitogen for intimal smooth muscle cells; however, when PDGF was given to animals with quiescent intimal lesions, a small but significant increase in intimal smooth muscle cell replication was detected(21). The actual replication rate was approximately 2%, which is far less than we have observed after the addition of bFGF. It should be remembered, however, that the replication rate of normal uninjured arteries is approximately 0.04%, and therefore this represents a significant increase above normal replication rates.

If PDGF does play a role in intimal cell proliferation, then we need to identify the source of this mitogen. In animal studies, platelet ad-

hesion to the denuded artery is complete within the first 24 hr, and once smooth muscle cells form a luminal surface (approximately 4 days), almost no adherent platelets are present(58). Therefore, if PDGF were to play any role in the chronic replication of smooth muscle cells, the source is not from platelets. The obvious source would be the smooth muscle cells themselves, and we showed that intimal smooth muscle cells when grown in culture were able to synthesize PDGF B-chain; in contrast, medial smooth muscle cells did not(59). Somewhat surprisingly, we were unable to detect PDGF-B chain synthesis *in vivo*, although the intimal smooth muscle cells did express PDGF-A chain(23). The role of PDGF-A chain in the injured artery is puzzling because smooth muscle cells predominantly express the β-receptor, which does not bind PDGF-A chain, and thus the cells of the injured artery express a form of PDGF to which they are unable to respond. One possibility is that the results from the Northern blots cannot recognize if individual cells express PDGF α-receptor, and so a small population may be able to bind PDGF-A chain. Another part of the puzzle concerning PDGF expression in injured arteries is what response PDGF-A chain might elicit from smooth muscle cells. Some interesting new data suggest that PDGF-A chain might inhibit the migration of these cells(60).

The role of PDGF as an endogenously synthesized mitogen has received renewed interest with the finding that intimal smooth muscle cells of rat arterial lesions do express PDGF-B chain(61). These data were obtained using an *en face* approach to *in situ* hybridization, a technique which appears to be more sensitive than conventional *in situ* hybridization on histological cross-sections(56). Approximately 10% of intimal smooth muscle cells were found to express PDGF-B chain, and these cells were scattered throughout the lesion. This finding could suggest an autocrine or paracrine pathway for the stimulation of intimal cells since intimal smooth muscle cells express the β-receptor. Thus, endogenous synthesis of PDGF by smooth muscle cells may be responsible for the small but chronic increase in intimal replication. It has been suggested that, after angioplasty, the replication rate of cells in human lesions rarely exceeds more than 1–2%. Such low rates of replication are often dismissed as having little significance, but it should be remembered that a low rate of replication for several months would lead to a significant increase in lesion size, and it would be interesting to see if PDGF-B chain was expressed in these lesions. This pathway is only speculative since the current data from animal studies show that PDGF plays a role in smooth muscle cell migration and not replication.

In summary, we can now divide the response of an artery to balloon

catheter injury into three or more distinct stages. The first wave is medial smooth muscle cell replication, which is primarily controlled by bFGF. Within 3–4 days after injury, cells begin to migrate to the intima; this is the second stage. Blocking antibodies to both PDGF and bFGF strongly inhibit the migration of smooth muscle cells. The pathways necessary for migration are protease-dependent, and we have been able to block smooth muscle cell migration with inhibitors of plasmin and metalloproteinases. The third wave of response to balloon injury is the replication of intimal smooth muscle cells. It has been difficult to show that these cells respond significantly to bFGF since their response is far less than that observed after acute injury. Only a small change in replication is triggered by PDGF. Of interest is that data have shown that a selected population of intimal smooth muscle cells express both bFGF and PDGF. It is possible, however, that the synthesis of these factors is responsible for the low but chronic smooth muscle cell replication seen for several weeks in these injured arteries. Finally, it is also important to realize that a number of other smooth muscle mitogens, e.g., molecules of the renin/angiotensin system, are present in the vessel wall, but have been explained in less depth. A discussion of the broad range of molecules able to stimulate or inhibit smooth muscle cell replication is included in the chapters by Jackson and by Schwartz and colleagues.

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Pharmacology of Smooth Muscle Cell Proliferation

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I. Introduction

Vascular smooth muscle cell proliferation is believed to contribute to the pathophysiology of atherosclerosis and hypertension, and also to underlie the failure of restorative surgical procedures such as angioplasty and bypass grafting (Ross, 1986; Schwartz *et al.,* 1990; Austin *et al.,* 1985; Fuster and Chesebro, 1986). Achieving pharmacological control of smooth muscle cell proliferation is likely to produce major benefits in terms of decreased morbidity and mortality. Consequently, many studies have been designed to identify factors that may stimulate proliferation and to detect synthetic agents that may inhibit it.

The usual protocol for determining the capacity of a factor to promote smooth muscle cell growth *in vitro* involves rendering cells quiescent by incubating them in a medium that is deficient in growth-

supporting substances. The factor to be tested is then added, and growth is measured either by determining the increase in cell number or by measuring the incorporation of a labeled nucleotide such as [^{3}H]thymidine or 2′-bromodeoxyuridine. These metabolic precursors can be used to identify cells that are synthesizing DNA, with the rate of proliferation being expressed as the proportion of the cells in the culture that are positively labeled. An alternative method applicable with [^{3}H]thymidine is to measure the amount of tritium incorporated into the acid-insoluble fraction of the cell layer, which contains the DNA. These protocols may be used to identify factors that can stimulate quiescent smooth muscle cells to proliferate, and also to identify factors that are not mitogenic themselves but which are necessary for other substances to express their full mitogenic activity. Assays like these are also suitable for the investigation of potential inhibitors of smooth muscle cell growth, which can be stimulated either by the reintroduction of serum or by the addition to the culture medium of a discrete mitogen.

One potential problem with studies *in vitro* is that they usually employ cells that are the progeny of a small proportion of the cells in the tissue of origin. For example, after enzymatic dispersion of the rat thoracic aorta, approximately 9% of the smooth muscle cells survive in primary culture (Jackson, 1989). The cells tested *in vitro* may therefore be derived from a specialized subpopulation with unrepresentative properties, and it is necessary to exercise some caution in extrapolating data obtained from cell culture systems to the situation *in vivo*.

Smooth muscle cell proliferation in experimental animals can also be quantified by the use of labeled nucleotides. Cross sections of arteries can be used to determine the proportion of labeled cells, or the total arterial DNA can be extracted for the measurement of the specific incorporation of [^{3}H]thymidine. Another commonly used technique for investigating mitogens and antiproliferative agents *in vivo* is to measure intimal expansion in terms of intimal depth or area on arterial cross sections. Unless it is combined with direct measurements of DNA synthesis, this is a flawed technique; although smooth muscle cell proliferation contributes to intimal expansion (Clowes *et al.,* 1983b), a number of other processes are also involved, reviewed elsewhere in this book by Michael Reidy. These include the migration of smooth muscle cells from the media to the intima (Clowes and Schwartz, 1985) and the secretion of extracellular matrix (Clowes *et al.,* 1983a). Stimulation or inhibition of these processes by substances under test may also result in changes in the rate of intimal expansion.

So far, most of the evidence implicating specific smooth muscle mitogens has come from investigations that are structured much like Koch's

postulates, which state that a suspected disease-causing microorganism must be observed in every case of the disease; that it must be isolated and grown in pure culture; that inoculation of the pure culture into a healthy animal should reproduce the disease; and that the microorganism must be detected in the experimentally diseased animal. Translating the postulates into the language of vascular biology, they suggest that the suspected mitogen must be detected in tissue containing proliferating smooth muscle cells; that it must be purified and identified; and that injection of the purified mitogen into healthy animals should provoke smooth muscle cell proliferation. Another element could be added; that administration of an antagonist specific for the suspected mitogen should inhibit smooth muscle cell proliferation. Many drugs have been investigated for their effects on smooth muscle cell proliferation, a field reviewed by Jackson and Schwartz (1992), and this chapter concentrates on the conclusions that can be reached from pharmacological studies about endogenous smooth muscle mitogens and endogenous inhibitors of smooth muscle cell proliferation.

One important conclusion is that arterial smooth muscle cells are potentially influenced by many mitogens. Serotonin, histamine, and norepinephrine are all present in plasma; platelets can deliver platelet-derived growth factor (PDGF), transforming growth factor β, thrombin, and a variety of eicosanoids; and cellular damage, as probably occurs in necrosing atherosclerotic lesions and after balloon angioplasty, causes release of basic fibroblast growth factor (bFGF). Of these factors, only bFGF has been firmly established as a smooth muscle cell mitogen *in vivo,* as discussed elsewhere in this book by Michael Reidy. However, it is still not clear whether bFGF acts alone to stimulate replication or whether it requires cofactors to express its full mitogenic potential. Also, bFGF does not appear to be mitogenic for intimal smooth muscle cells. The studies reviewed in this chapter have enabled us to add to the expanding list of potential smooth muscle mitogens, but it remains for us to determine the relative contributions of each of these factors to smooth muscle cell replication in spontaneous and iatrogenic arterial injury.

II. Pharmacological Evidence for Potential Endogenous Smooth Muscle Cell Mitogens

A. Renin–Angiotensin System

Renin is a proteolytic enzyme that converts the plasma protein angiotensinogen to angiotensin I, an inactive decapeptide. Two amino acid residues are cleaved from angiotensin I by angiotensin-convert-

ing enzyme (ACE), isoforms of which are found in tissues and circulating in the plasma. This synthetic pathway, and its relationship to kinin metabolism, is shown in Fig. 1.

Canine aortic smooth muscle cells have been shown to synthesize renin *in vitro* (Re *et al.,* 1982), and renin messenger RNA has been detected in rat aorta (Samani *et al.,* 1987; Holycross *et al.,* 1992). Angiotensinogen messenger RNA has also been detected in rat aorta (Campbell and Habener, 1986; Cassis *et al.,* 1988; Naftilan *et al.,* 1991). Rat and rabbit aortic tissues can generate angiotensin II from angiotensin I, suggesting that they contain ACE (Saye *et al.,* 1984; Egléme *et al.,* 1990). It therefore appears that all components of the renin–angiotensin system are present in vascular tissue.

Exogenous angiotensin II significantly stimulates human smooth muscle cell growth *in vitro* at nanomolar concentrations (Campbell-Boswell and Robertson, 1981). Lyall and associates (1988a,b), in studies on the proliferation of rat mesenteric arterial smooth muscle cells in culture, found that concentrations as low as 1 n*M* stimulated an increase in cell number. Stimulation of replication was blocked by the angiotensin II structural analog saralasin, which competes for the angiotensin II receptor. This suggests both that the proteolytic degrada-

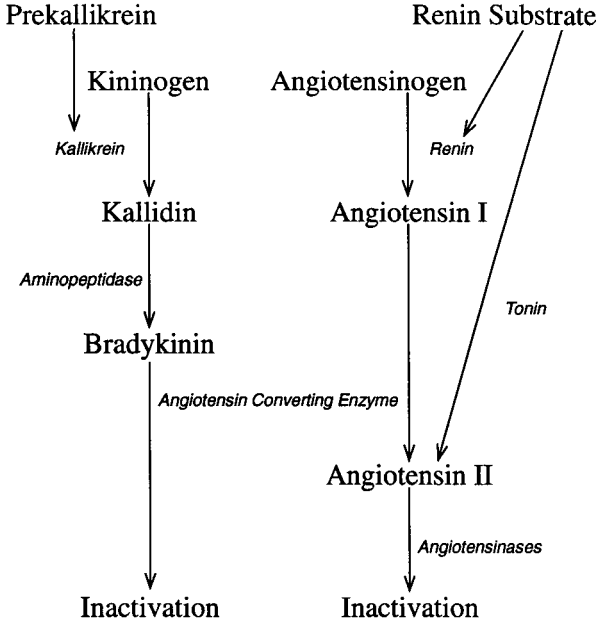

FIG. 1. Biosynthetic pathways within the renin–angiotensin and kinin systems. Enzyme names are in italics.

tion of angiotensin II is not involved in its mitogenic effect and that angiotensin II acts directly on the smooth muscle cell rather than interacting with a serum component. The involvement of angiotensin II in the growth of arterial smooth muscle cells *in vitro* was highlighted by Emmett and Harris-Hooker (1986), who found that saralasin significantly, dose dependently, and reversibly inhibited smooth muscle cell proliferation in the presence of fetal calf serum.

Geisterfer *et al.* (1988) were unable to detect any hyperplastic effect of angiotensin II at a concentration of 1 μM using rat thoracic aortic smooth muscle cells growing in fetal calf serum or in a defined serum-free medium containing partially purified PDGF. However, at concentrations of 1 nM and above, significant increases in the mean cellular protein content were observed, indicating hypertrophy. The hypertrophic effect was blocked by saralasin. The dose–response curve for the hypertrophic effect of angiotensin II was extremely flat, spanning 5 orders of magnitude between the lowest effective concentration and the maximally effective concentration. This may have been the consequence of breakdown of angiotensin II, which was added in fresh culture medium every 2 to 3 days. Lyall and co-workers (1988a,b) showed by radioimmunoassay that incubation for 3.5 hr in medium containing 10% fetal calf serum degraded 99.9% of exogenous angiotensin II.

Berk and colleagues (1989) also found no effect of angiotensin II on the replication of rat thoracic aortic smooth muscle cells in culture, although DNA synthesis was significantly increased at concentrations of 100 nM and above. This suggests that polyploid cells were being formed. Hypertrophic effects were observed at angiotensin II concentrations as low as 0.1 nM.

It is interesting to note that the effects of angiotensin II on smooth muscle cell proliferation seem to be related to exposure of the cells to serum. Angiotensin II appears to cause hyperplasia in smooth muscle cells maintained in growth-supporting concentrations of serum throughout the experiment (Campbell-Boswell and Robertson, 1981; Lyall *et al.,* 1988a,b; Scott-Burden *et al.,* 1991a). In contrast, in studies in which cells are rendered quiescent by incubation in medium containing little or no serum, hypertrophic effects are observed when angiotensin II is administered at the same time as reintroduction of serum (Geisterfer *et al.,* 1988; Berk *et al.,* 1989). Hyperplastic effects of angiotensin II have not been observed under these conditions (Geisterfer *et al.,* 1988; Berk *et al.,* 1989; Kato *et al.,* 1991; Scott-Burden *et al.,* 1991a). A possible exception is the study by Hamada and associates (1990) in which rat thoracic aortic smooth muscle cells were incubated in serum-free medium for 1 day prior to exposure to

angiotensin II. The incorporation of [^{3}H]thymidine into DNA was significantly stimulated at low nanomolar concentrations. However, cell numbers were not determined so it is not clear whether angiotensin II stimulated cellular replication or simply caused an increase in ploidy. Campbell-Boswell and Robertson (1981) examined the effects of angiotensin II on smooth muscle cells cultured in 1% fetal calf serum for 2 days prior to introduction of the experimental medium. Under these conditions, the stimulation of cell replication by angiotensin II was approximately 75% of that found in cells maintained in 10% serum throughout and exposed to the same concentration of angiotensin II. However, the induction of quiescence by culturing the cells in 5% platelet-poor plasma-derived serum for 2 days did not inhibit the subsequent mitogenic effect of angiotensin II. Similar findings were reported by Scott-Burden *et al.* (1991a), who were able to detect a very week mitogenic effect of 50 n*M* angiotensin II in rat vascular smooth muscle cells growing in 1% plasma-derived serum. This suggests that the hyperplastic response of smooth muscle cells is dependent on continuous exposure to serum, and that in the presence of low concentrations of serum the mitogenic response gradually disappears. It is clear that cellular quiescence is not an issue because quiescence induced by platelet-poor plasma-derived serum did not inhibit the mitogenic response to angiotensin II. It therefore appears that one or more factors not derived from platelets are critical for the mitogenic response and that absence of the factors for 2 or more days inhibits the response. Whether they operate by inducing a state of smooth muscle cell growth that is particularly sensitive to the mitogenic effects of angiotensin II, or alternatively by inducing an intracellular signal that is necessary for the angiotensin II mitogenic signal to be fully transduced, is unknown.

It is not clear what the cofactors for angiotensin II-induced mitogenesis might be. Campbell-Boswell and Robertson (1981) showed that the removal of cationic substances from platelet-poor plasma-derived serum with carboxymethyl–Sephadex abolished its ability to support the mitogenic effect of angiotensin II. During this procedure the serum was also concentrated by ultrafiltration through a filter with a 10,000 Da cutoff. Therefore, cofactor activity binds to carboxymethyl–Sephadex and/or contains elements with molecular weights below 10,000.

The mitogenic activity of angiotensin II under appropriate experimental conditions *in vitro* suggests that smooth muscle cells may respond similarly *in vivo*. This possibility was addressed in a study by Daemen and colleagues (1991) in which angiotensin II was administered to rats by continuous subcutaneous infusion. The labeling index

of smooth muscle cells in the carotid arterial media was significantly increased both in unmanipulated vessels and in those subjected to balloon catheter injury. In the latter vessels, in which a neointima had been allowed to develop, angiotensin II infusion significantly increased the intimal smooth muscle cell labeling index also.

The relative lack of potency of angiotensin I as a vasoconstrictive hormone has prompted drug companies to develop synthetic inhibitors of ACE as treatments for hypertension. Interest has focused on the use of ACE inhibitors to inhibit smooth muscle cell proliferation. Kuriyama and co-workers (1988) found that the ACE inhibitor captopril had no effect on the growth of the A7r5 embryonic rat thoracic aortic smooth muscle cell line in serum, and Sachinidis *et al.* (1992) found no effect with another ACE inhibitor, enalapril, in rat aortic smooth muscle cells. These results may be the consequence of a restricted supply of angiotensin I because Andre *et al.* (1990) have shown that rat aortic smooth muscle cells are able to convert exogenous angiotensin I to angiotensin II.

Beneficial effects have been obtained with the ACE inhibitor captopril in hypercholesterolemic animal models, including the cholesterol-fed cynomolgus monkey (Aberg and Ferrer, 1990) and the Watanabe heritable hyperlipidemic rabbit (Chobanian *et al.,* 1990).

Powell *et al.* (1989) investigated the effect of oral administration of the ACE inhibitor cilazapril to rats in which the left common carotid artery was injured with a balloon catheter. Cilazapril significantly inhibited the accumulation of smooth muscle cells in the intima and also decreased the proportion of the arterial circumference adjacent to neointima. This may not necessarily have been caused by inhibition of smooth muscle cell proliferation because it could have been the consequence of decreased smooth muscle cell migration. The reduced circumferential extent of the neointima in treated animals supports the concept that cilazapril inhibits smooth muscle cell migration in the rat balloon injury model: if cilazapril only inhibited proliferation, smooth muscle cells would still be expected to be distributed across the intimal surface because nonproliferating smooth muscle cells migrate to the intima *in vivo* (Clowes and Schwartz, 1985). However, large portions of the arterial circumference were free of neointima, suggesting that cilazapril inhibits smooth muscle cell migration in the balloon-injured rat carotid artery. This conclusion is supported by similar findings with the ACE inhibitors benazepril (Prescott *et al.,* 1991), ramipril (Capron *et al.,* 1991), and lisinopril (Jackson and Reidy, 1992).

It is not known whether the effects of ACE inhibitors are mediated through the decreased formation of angiotensin II or are the indirect

consequence of inhibition of ACE. The autocoid bradykinin is inactivated by proteolytic degradation by ACE, and ACE inhibitors therefore raise its circulating levels (Mersey *et al.,* 1977; see Fig. 1). Bradykinin is mitogenic for smooth muscle cells growing in culture (Paquet *et al.,* 1989), and the inhibitory effects of ramipril on intimal thickening *in vivo* are potentiated by the bradykinin receptor antagonist Hoe 140 (Farhy *et al.,* 1992). These data suggest that ACE inhibitors act partially through a kinin axis. However, the inhibitory effects of cilazapril on injury-induced intimal thickening are surmounted by a concurrent administration of angiotensin II (Osterrieder *et al.,* 1991). Also, angiotensin II receptor antagonists inhibit intimal thickening after balloon catheter injury (Prescott *et al.,* 1991; Kauffman *et al.,* 1991; Osterrieder *et al.,* 1991; Pan *et al.,* 1992; Farhy *et al.,* 1992; Azuma *et al.,* 1992), indicating that some of the activity of ACE inhibitors is also mediated through the angiotensin system. Because angiotensin II causes a release of aldosterone from the adrenal zona glomerulosa, ACE inhibitors may reduce circulating levels of this hormone (Tobian, 1977). Angiotensin II induces smooth muscle cells to secrete endothelin (Scott-Burden *et al.,* 1991b), which is a mitogen in its own right (Hirata *et al.,* 1989; Nakaki *et al.,* 1991). Also, angiotensin II causes increased sympathetic tone and increases vasopressin release through an effect on the central nervous system (Severs and Daniels-Severs, 1973), and it facilitates the release of catecholamines from peripheral adrenergic nerve terminals (Zimmerman, 1978). A link between the renin–angiotensin system and the sympathetic nervous system in the control of smooth muscle cell proliferation was shown by van Kleef *et al.* (1992) in a study of the effects of the α_1-adrenergic receptor antagonist, prazosin, on proliferation induced by angiotensin II in rat arteries. This study showed that the mitogenic effect of angiotensin II *in vivo* was mediated via adrenergic stimulation rather than through an increase in blood pressure.

B. Catecholamines

Epinephrine and norepinephrine are synthesized ultimately from the amino acid phenylalanine. They are the main transmitters in the sympathetic nervous system, and their major sites of synthesis are postganglionic sympathetic nerve fibers and the adrenal medulla. The concentrations of epinephrine and norepinephrine in plasma are about 0.2 to 4 μM (Bauch *et al.,* 1987).

Sympathetic agonists stimulate the growth of arterial smooth muscle cells in culture. Blaes and Boissel (1983) showed that epinephrine dose dependently stimulates the proliferation of rat thoracic aortic

smooth muscle cells; the minimum effective concentration is 1 n*M*. In this study, norepinephrine and the β-adrenergic receptor agonist isoproterenol were also found to stimulate cellular proliferation. The role of β-adrenergic receptors in the stimulation of proliferation was confirmed by showing that the selective β-adrenergic receptor antagonist propranolol significantly inhibited the response to epinephrine. However, there was no effect of propranolol or of the selective α-adrenergic receptor antagonist phentolamine, both at 10 μ*M*, on the proliferation of smooth muscle cells in serum. Bell and Madri (1989) showed that norepinephrine, 1 μ*M*, stimulates the proliferation of bovine aortic smooth muscle cells in culture. Epinephrine and norepinephrine stimulate with equal potency the proliferation of rat thoracic aortic smooth muscle cells growing in fetal calf serum, but epinephrine is more efficacious (Bauch *et al.*, 1987). Peak effects were produced at 1 μ*M*. The proliferation of the A7r5 smooth muscle cell line is stimulated by 10 μ*M* norepinephrine and is inhibited by the α-adrenergic receptor antagonist bunazocine and by the α- and β-adrenergic receptor antagonist labetalol (Kuriyama *et al.*, 1988). Similar findings were reported by Nakaki and associates (1990), who investigated the effects of catecholamines on the restimulation of DNA synthesis in quiescent rat aortic smooth muscle cells in serum-free medium. In subconfluent cultures, norepinephrine stimulated DNA synthesis at concentrations of 100 p*M* and above. In confluent cultures the stimulatory effect was only seen at concentrations 1000-fold greater. These effects were inhibited by the nonselective α-adrenergic receptor antagonist phentolamine and by the α_1-adrenergic receptor antagonist prazosin. The α_1-adrenergic receptor agonist phenylephrine also stimulated DNA synthesis, but with lower potency than norepinephrine. Norepinephrine was found, paradoxically, to inhibit the restimulation of DNA synthesis in cloned cells of high passage number, an effect apparently caused by β_2-adrenergic receptor stimulation since it was blocked by the selective antagonist butoxamine. This compound also augmented the stimulatory effects of norepinephrine on DNA synthesis in cells of lower passage number. These results led the authors to conclude that stimulation of α_1-adrenergic receptors stimulates arterial smooth muscle cell growth, but that stimulation of β_2-adrenergic receptors has an inhibitory effect. This is opposite to the conclusion reached by Blaes and Boissel (1983), who found that isoproterenol stimulates smooth muscle cell proliferation, but the discrepancy may be related to the different assay conditions employed in the two studies. One point seems clear, that stimulation of the α_1-adrenergic receptor induces or augments the proliferation of smooth muscle cells *in vitro*.

This also appears to be true *in vivo*. Epinephrine stimulates DNA syn-

thesis in the cells of the aortic media in rabbits fed a cholesterol-enriched diet (Cavallero *et al.,* 1973). Bevan (1975) found that sympathetic denervation of the ear artery by removal of the superior cervical ganglion in young rabbits causes a significant reduction in smooth muscle cell DNA synthesis in the artery. Chemical destruction of sympathetic nerve terminals by the administration of 6-hydroxydopamine causes a reduction in the number of smooth muscle cells in the rabbit aortic media (Fronek *et al.,* 1978). In rats sympathectomized by a combination of immunological and chemical means, there is a significant reduction in the number of medial smooth muscle cell layers in large and small mesenteric arteries, although not in the superior mesenteric artery (Lee *et al.,* 1987). The medial cross-sectional area was also reduced in large mesenteric arteries. Administration of the α_1-adrenergic receptor agonist methoxamine to chickens results in the formation of thoracic aortic intimal accumulations of smooth muscle cells (Majesky *et al.,* 1985). Another α_1-adrenergic receptor agonist, phenylephrine, does not stimulate DNA synthesis in the thoracic aorta of normal rats (Majesky *et al.,* 1990). These conflicting results may be related to differences in aortic morphology between chickens and rats: in chickens, occasional smooth muscle cells are found in the subendothelial intima (Majesky *et al.,* 1985), but these are not found in normal rats (Reidy and Schwartz, 1981). Perhaps in the uninjured vessel, increased α_1-adrenergic receptor stimulation by administration of pharmacological doses of exogenous agonists provokes the proliferation of intimal but not of medial smooth muscle cells. Since sympathectomy inhibits medial smooth muscle cell proliferation, endogenous catecholamines may be involved in normal cell turnover processes in the vessel wall during development and maturity.

The role of α_1-adrenergic receptor stimulation in the control of the proliferative response of smooth muscle cells in situations of arterial injury has been established in studies in which the balloon catheter has been used. Prazosin inhibits DNA synthesis in rat thoracic aortic smooth muscle cells, but not in other proliferating tissues (Jackson *et al.,* 1988), and reduces intimal hyperplasia in the rabbit abdominal aorta (O'Malley *et al.,* 1989). Fingerle *et al.* (1991) showed that both prazosin and urapidil, another α_1-adrenergic receptor antagonist, reduced the DNA content of balloon-injured rat carotid arteries but only urapidil inhibited intimal thickening.

C. Eicosanoids

The eicosanoids are a family of products derived from the oxidation of fatty acids and include the prostaglandins, prostacyclins, thromboxanes, and leukotrienes. The eicosanoid biosynthetic pathway is shown in Fig. 2.

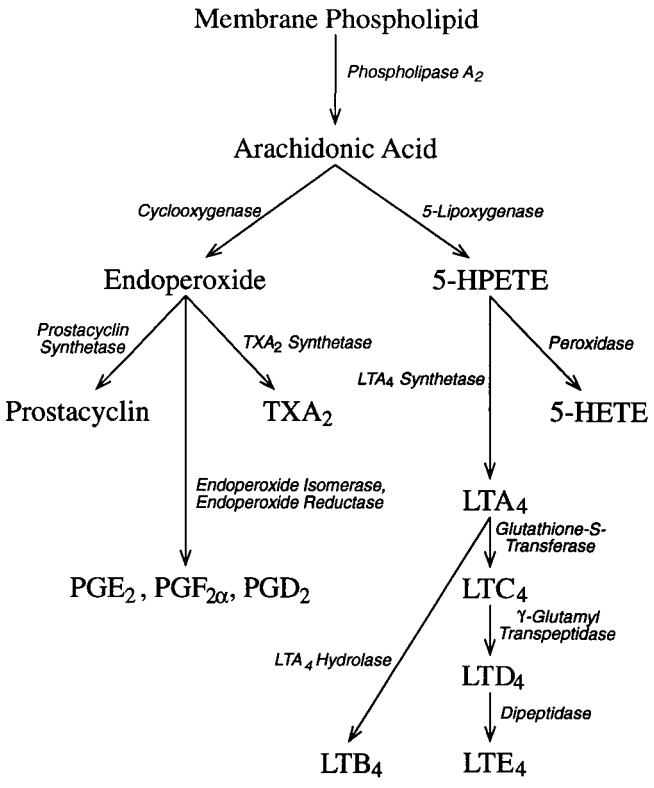

FIG. 2. Biosynthetic pathways for eicosanoids. Enzyme names are in italics.

An appreciable amount of evidence now exists to suggest that arterial smooth muscle cells can synthesize growth stimulatory eicosanoids. Using punch samples of bovine pulmonary arterial media, Menconi and colleagues (1984) detected synthesis of thromboxane A_2 (TXA_2). TXA_2 production by rat thoracic aortic smooth muscle cells was reported by Ishimitsu and colleagues (1988a,b). It is also synthesized by excised thoracic aorta, but it is not possible to attribute this exclusively to smooth muscle cells since endothelial cells and fibroblasts can also synthesize TXA_2 (Menconi *et al.,* 1984).

The stable, 9,11-epithio-11,12-methano- analog of TXA_2 dose dependently stimulates the incorporation of [^{3}H]thymidine into DNA of rat thoracic aortic smooth muscle cells, with a maximal effect at a concentration of 10 μM (Ishimitsu *et al.,* 1988a,b). Similar results were reported by Akopov and associates (1988) and by Hanasaki *et al.* (1990), using the stable TXA_2 analog U46619. These results suggest that any endogenous TXA_2 tone that may have been present in the

culture system was at best submaximal. On the face of it, the failures of two thromboxane synthase inhibitors, sodium 5-(3-pyridinylmethyl)benzofuran-2-carboxylate and dazoxiben, to inhibit the proliferation of canine carotid arterial smooth muscle cells growing in serum (Lindblad *et al.,* 1988) support this hypothesis. However, these agents may cause prostaglandin endoperoxides to accumulate and these substances have agonist activity at the TXA_2 receptor. One way of reducing the availability of TXA_2 receptor agonists is to expose cells to a cyclooxygenase inhibitor such as aspirin, although this will also inhibit any production of growth inhibitory prostaglandins such as prostaglandin E and prostacyclin (see Section III,C). There is no consensus on the effects of cyclooxygenase inhibitors on smooth muscle cell growth *in vitro.* Antiproliferative effects have been reported with aspirin (Hauss *et al.,* 1979) and ibuprofen (Lindblad *et al.,* 1988). Both aspirin (Lindblad *et al.,* 1988) and indomethacin (Cornwell *et al.,* 1979; Nemecek *et al.,* 1986; Hirosumi *et al.,* 1987; Palmberg *et al.,* 1987; Brinkman *et al.,* 1990) have been reported to be ineffective. Indomethacin has also been shown to stimulate replication (Morisaki *et al.,* 1988a,b).

Daltroban, a TXA_2 receptor antagonist, does not inhibit intimal thickening in rat carotid arteries injured by air-drying when administered by continuous intravenous infusion at a dose of 100 μg/kg/hr (Levitt *et al.,* 1991). This is strong evidence against a mitogenic role for TXA_2 in this experimental model. The analysis of effects of cyclooxygenase inhibitors on smooth muscle cell proliferation *in vivo* is complicated by their profound effects on platelet aggregation and is beyond the scope of this chapter. The field has been reviewed by Jackson and Schwartz (1992).

Canine and human coronary arteries synthesize lipoxygenase products, including leukotrienes C_4, D_4, and E_4 (LTC_4, LTD_4, and LTE_4), when incubated with arachidonic acid and the calcium ionophore A23187 (Piomelli *et al.,* 1987). Cultured rabbit aortic smooth muscle cells also generate lipoxygenase products from exogenous arachidonic acid (Larrue *et al.,* 1984). However, no evidence was found for the processing of exogenous arachidonic acid via the lipoxygenase pathway in human umbilical arterial smooth muscle cells (Brinkman *et al.,* 1990). LTB_4 was initially found to inhibit rabbit aortic smooth muscle cell growth in culture, the half-maximal effect being produced at the very high concentration of 28 μ*M* (Smith *et al.,* 1984). Later, Palmberg and colleagues (1987, 1989) showed that LTB_4, LTC_4, and LTD_4 all significantly stimulate proliferation in rat thoracic aortic smooth muscle cells, with maximal effects being produced at 10 p*M*. LTE_4 was without effect, as was an inactive isomer of LTD_4. LTB_4, LTC_4,

and LTD_4 stimulated DNA synthesis in smooth muscle cells in serum-free medium at concentrations as low as 10 f*M*. These cells were not really quiescent because the [^{3}H]thymidine index was 15%, even in the absence of leukotrienes, and the capacity of leukotrienes to propel quiescent cells into the cell cycle remains unresolved. The growth stimulatory effect of LTB_4, but not of LTC_4, was inhibited by indomethacin and by aspirin, suggesting that this effect is modulated via a cyclooxygenase product (Palmberg *et al.,* 1987). Brinkman *et al.* (1990) investigated the effects of inhibitors of lipoxygenase on the proliferation of human umbilical arterial smooth muscle cells *in vitro.* Both nordihydroguiaretic acid and caffeic acid inhibited proliferation, suggesting an involvement of lipoxygenase products. Because there was no evidence in these cells of metabolism of exogenous arachidonic acid via the lipoxygenase pathway, the authors suggested that lipoxygenase metabolism of endogenous fatty acids other than arachidonate may play a role in the control of smooth muscle cell proliferation.

D. Serotonin

Serotonin is found in platelets and also in the mast cells of rodents and cattle. It is not found in human mast cells (Douglas, 1985). The concentration of serotonin in human blood is approximately 40 n*M*, and the concentration in human platelets is approximately 2.6 pmol/million platelets (Frattini *et al.*, 1979).

Serotonin initiates DNA synthesis in quiescent bovine aortic smooth muscle cells, with a maximal effect at a concentration of 1 μ*M* (Nemecek *et al.,* 1986), although Kavanaugh and colleagues (1988) found maximum effects at the extremely high concentration of 100 μ*M*. At nanomolar concentrations, serotonin also stimulates DNA synthesis in rat and porcine thoracic aortic smooth muscle cells (Paquet *et al.,* 1989; Parrott *et al.,* 1991). Similar effects were noted at this concentration by Bell and Madri (1989) using bovine aortic smooth muscle cells.

The mitogenic effects of serotonin on smooth muscle cells are blocked by 5-HT_2 receptor antagonists (Nemecek *et al.,* 1986; Parrott *et al.,* 1991). Stimulation of this receptor also stimulates the synthesis of prostacyclin by bovine aortic smooth muscle cells (Coughlin *et al.,* 1981, 1984; Nemecek *et al.,* 1986; Kavanaugh *et al.,* 1988), although prostacyclin production in response to serotonin by explants of bovine aortic media is mediated via the 5-HT_1 receptor (Demolle *et al.,* 1989), and it has been suggested that the mitogenicity of serotonin may be mediated in part by metabolites of arachidonic acid (Nemecek *et al.,* 1986). It seems unlikely that prostacyclin itself could be responsible

for the mitogenic effect since it generally inhibits smooth muscle cell proliferation (Orekhov *et al.,* 1983, 1986; Akopov *et al.,* 1988). In a test system in which serotonin stimulates smooth muscle cell proliferation, prostacyclin is without effect (Nemecek *et al.*, 1986). Since serotonin stimulates the liberation of arachidonic acid from smooth muscle cell membrane lipids (Kavanaugh *et al.,* 1988), it is possible that the increased levels of prostacyclin reflect increased activity in the arachidonic acid metabolic pathway and that TXA_2 or a leukotriene is responsible for the increased proliferation.

E. Thrombin

Thrombin, factor IIa, is a serine protease that catalyzes the conversion of fibrinogen to fibrin and activates factors V, VIII, and XIII. It is derived from prothrombin, factor II, by the coordinated actions of factor V, factor Xa, phospholipid, and Ca^{2+} and is therefore formed at sites of vascular injury. Physiological inhibitors of thrombin include antithrombin III, α_2-macroglobulin, and α_1-antitrypsin, but thrombin can be protected from inhibition by antithrombin III by deposition on the subendothelial extracellular matrix (Bar-Shavit *et al.,* 1989). The approximate maximum concentration of thrombin achieved during blood coagulation is 140 n*M* (Walz *et al.,* 1985).

There is conflicting evidence for mitogenic effects of thrombin on vascular smooth muscle cells. Ishida and Tanaka (1982) grew rabbit aortic smooth muscle cells in 10% fetal calf serum, then added thrombin at the same time as complete serum withdrawal. Concentrations up to 10 n*M* were without effect, and cell numbers decreased at 100 n*M*. Significant mitogenic effects of 10 n*M* thrombin were observed in neonatal rat vascular smooth muscle cells by Huang and Ives (1987) and in bovine aortic smooth muscle cells by Graham and Alexander (1990). Berk and associates (1990) did not detect any effect of α-thrombin on the proliferation of quiescent rat vascular smooth muscle cells at concentrations up to 100 n*M*, despite marked increases in protein synthesis, intracellular Ca^{2+} concentration, and intracellular pH. Inhibition of the proteolytic activity of α-thrombin with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone prevented all of these effects. γ-Thrombin, a form with enzymatic activity but which does not convert fibrinogen to fibrin, caused increases in protein synthesis and in intracellular pH but did not affect intracellular Ca^{2+} concentration. A synthetic peptide, corresponding to the cryptic amino terminus of the thrombin receptor that is revealed after proteolysis by thrombin, stimulates rabbit aortic smooth muscle cell growth in culture (Herbert *et al.,* 1992). These results suggest that the proteolytic activity of thrombin is an important determinant of its mitogenicity

in vascular smooth muscle cells. This is in agreement with the proposed proteolytic activation mechanism of the thrombin receptor (Vu *et al.,* 1991). However, bovine aortic smooth muscle cells have been shown to respond mitogenically to nanomolar concentrations of both α-thrombin and diisopropylfluorophosphate-conjugated, enzymatically inactive α-thrombin (Bar-Shavit *et al.,* 1990). One possible explanation for these discrepant results is that smooth muscle cells express both the proteolytically activated thrombin receptor and an as yet uncharacterized thrombin receptor that is not dependent on proteolytic degradation.

Hirudin, a leech polypeptide that specifically inhibits thrombin, blocks its mitogenic effect (Bar-Shavit *et al.,* 1990). The authors suggest that hirudin interacts with a part of the thrombin molecule, distinct from its active proteolytic site, that is responsible for conferring mitogenic activity. Warfarin is a congener of bishydroxycoumarin that interferes with the hepatic synthesis of prothrombin and other vitamin K-dependent clotting factors. It therefore induces hypoprothrombinemia and reduces the availability of thrombin. Oral administration of warfarin to rats with air-drying injury to the common carotid artery significantly reduces neointimal thickening measured 4 weeks after injury (August and Tilson, 1980).

III. Pharmacological Evidence for Potential Endogenous Inhibitors of Smooth Muscle Cell Proliferation

A. Adenosine

Adenosine is a nucleoside that is formed from 5′-adenosine monophosphate (AMP) by the action of 5′-nucleotidase (Berne, 1980). The concentration of adenosine in canine arterial blood plasma is 0.26 μ*M*, but it can rise substantially in some vascular beds after nerve stimulation (Fredholm and Sollevi, 1981). Cultured arterial smooth muscle cells express both the A1 and A2 subtypes of the adenosine receptor (Anand-Srivastava *et al.,* 1982; Mills and Gewirtz, 1990). Occupation of the A1 receptor by adenosine inhibits adenylate cyclase activity, and thus reduces the intracellular concentration of cyclic AMP, while occupation of the A2 receptor has the opposite effect (Mills and Gewirtz, 1990). The effects of adenosine receptor stimulation on the rate of DNA synthesis in rat aortic smooth muscle cells exposed to PDGF have been investigated by Jonzon *et al.* (1985) and by Querol-Ferrer *et al.* (1992). Exposure of quiescent cells to human platelet PDGF resulted in an increase in the [^{3}H]thymidine labeling index, which was significantly inhibited by adenosine at concentrations above 10 μ*M*. The inhibition of PDGF-stimulated DNA synthesis was

also observed with the adenosine analogs 5′-*N*-ethylcarboxamidoadenosine and L-phenylisopropyladenosine. The latter was approximately equipotent with adenosine, but 5′-*N*-ethylcarboxamidoadenosine, which has a greater affinity for A2 receptors, was 10 times as potent. These data suggest that adenosine may play a role in the negative control of vascular smooth muscle cell proliferation, an effect mediated through the A2 receptor. It is not clear which of the two subtypes of this receptor is involved.

B. Adrenocorticosteroid Hormones

The biosynthesis and the release of adrenocorticosteroid hormones from the adrenal cortex are under the control of the adrenocorticotropic hormone. The release of this hormone from the anterior pituitary gland is in turn controlled by the corticotropin releasing factor, which is present in high concentration in neurosecretory cells in the paraventricular nucleus of the hypothalamus. The predominantly secreted adrenocorticosteroid hormone in man is cortisol, or hydrocortisone, which is present in plasma at a total concentration of 0.14 to 0.55 μ*M* under normal conditions (Schimmer and George, 1989).

Hydrocortisone (1 μ*M*) significantly inhibits [3H]thymidine incorporation in human fetal aortic smooth muscle cells, and DNA content is significantly reduced at 100 n*M* (Järveläinen *et al.,* 1982). Similar results were found by Berk *et al.* (1988) with rat thoracic aortic smooth muscle cells. The addition of hydrocortisone (2 μ*M*) significantly inhibited cell replication and the incorporation of [^{3}H]thymidine into DNA. Inhibition of replication was also seen with the synthetic glucocorticoid dexamethasone (1 μ*M*), but not with the sex hormones testosterone (2 μ*M*) or progesterone (2 μ*M*). Inhibition of cell growth by hydrocortisone was associated with a change in cell morphology, with the cells appearing larger, flatter, and more polygonal. The authors suggest that this morphologic change could cause a density-
dependent inhibition of cell growth at lower cell densities, which is consistent with the growth profile of hydrocortisone-treated cells. The rate of growth during the logarithmic phase was equal to that in control cultures, but the plateau phase was achieved at a lower cell density. Longenecker and associates (1982, 1984) also examined the effects of glucocorticoids on smooth muscle cell growth *in vitro*. Using bovine aortic smooth muscle cells, they found significant inhibition by dexamethasone at a concentration of 1 n*M*. This inhibition was counteracted by growing the cells on dishes coated with extracellular

matrix, an effect possibly caused by growth factors binding to the matrix and overcoming the inhibition. Hydrocortisone (0.1 μM) and corticosterone (0.1 μM) were also growth inhibitory. Interestingly, the growth of primary cultures of bovine aortic smooth muscle cells was markedly more sensitive to dexamethasone than the growth of secondary cultures. In their 1984 study, Longenecker and colleagues found that dexamethasone reduced both the rate of growth and the final saturation density of smooth muscle cells, which is consistent with the lack of effect on growth rate found by Berk *et al.* (1988). However, the earlier report from Longenecker *et al.* (1982) showed a significant effect only on saturation density. Hauss and co-workers (1979) examined the inhibitory effects of the synthetic glucocorticoid prednisolone on the proliferation of rat and porcine thoracic aortic smooth muscle cells in culture. Significant inhibition of proliferation of porcine cells was observed at the high concentration of 56 μM. Parenteral administration of prednisolone to rats at a dose of 2 mg/animal/day for 4 days produced a slight inhibition of the growth of smooth muscle cells subsequently isolated from the aortas. The development of neointimal hyperplasia in the ear artery of rabbits in response to a crushing injury is significantly inhibited by intravenous treatment with 0.2 mg prednisolone/animal/day (Voss *et al.*, 1988).

Dexamethasone, at concentrations as low as 10 nM, inhibits the proliferation of rat thoracic aortic smooth muscle cells in culture (Hirosumi *et al.*, 1987). Inhibitory effects on smooth muscle cell migration were noted at concentrations above 100 pM. This study also investigated the effect of intramuscular administration of dexamethasone on the development of the neointima in response to investment of the rabbit carotid artery with a polythene cuff for 21 days. Intimal thickening was dose dependently inhibited by dexamethasone, the lowest effective dose was 1 mg/kg/day. Presumably this effect was the result of inhibition both of smooth muscle cell proliferation and of smooth muscle cell migration to the intima. Dexamethasone (0.05 mg/kg/day) has also been shown to inhibit intimal thickening in the balloon-injured rabbit carotid artery (Chervu *et al.*, 1989; Colburn *et al.*, 1992) and at 0.2 mg/kg/day in a rat vasculitis model (Prescott *et al.*, 1989).

The main action of the glucocorticoids is the induction of lipocortin, a protein that inhibits phospholipase A_2 (Peers and Flower, 1990). This enzyme catalyzes the liberation of arachidonic acid from cellular phospholipids, and its inhibition therefore reduces eicosanoid biosynthesis. The inhibitory effects of glucocorticoids on arterial smooth muscle cell proliferation may thus be the consequence of the reduced

availability of growth-stimulatory eicosanoids such as TXA_2 and leukotrienes. The effects of these agents on arterial smooth muscle cell growth are reviewed in Section I,C.

If glucocorticoids were an important growth-inhibitory influence on smooth muscle cells in the arterial wall, hypophysectomy would be expected to cause an increase in smooth muscle cell proliferation in response to vascular injury. This is because removal of the pituitary reduces the circulating levels of hydrocortisone by approximately 90% (Tiell *et al.,* 1978). However, the development of intimal thickening in the balloon-catheterized rat aorta is significantly inhibited by hypophysectomy (Tiell *et al.,* 1978; Bettmann *et al.,* 1981; Khorsandi *et al.,* 1992). Bettmann *et al.* (1981) and Khorsandi *et al.* (1992) also found that hypophysectomy accelerated the rate of regrowth of endothelium in balloon-denuded rat aorta, suggesting that the inhibition of intimal thickening in hypophysectomized rats could be the consequence of suppression by the enothelium. In contrast, Tiell *et al.* (1978) did not observe any difference in endothelial regrowth between control and hypophysectomized animals, indicating that the inhibition of neointimal development was not the indirect consequence of rapid recoverage by endothelium. This hypothesis is supported by the finding that smooth muscle cell proliferation in hypophysectomized balloon-injured rats, measured at a time when there is no endothelial regrowth, is virtually abolished (Faulmüller *et al.,* 1992). This leads to the conclusion that the pituitary is necessary for the elaboration of an essential cofactor for smooth muscle cell proliferation. Bettmann *et al.* (1981) could not reverse the inhibitory effect of hypophysectomy by coadministering thyroxin, hydrocortisone, deoxycorticosterone acetate, and growth hormone. The pituitary is also a source of prolactin, follicle-stimulating hormone, luteinizing hormone, thyro-tropin, adrenocorticotropic hormone, lipotropin, endorphins, enkepha-lins, and melanocyte-stimulating hormone (Murad and Haynes, 1985). These hormones themselves modulate the synthesis, secretion, and activity of a wide range of hormones and neurotransmitters in the body. Dissection of the pathways involved in hypophysectomy-induced suppression of smooth muscle cell proliferation will be a formidably difficult task, but determination of the mitogenic activity for smooth muscle cells of plasma-derived serum prepared from normal and hypophysectomized animals may provide some useful clues. This approach was utilized by Stiles *et al.* (1979), who determined the effects on the growth of BALB/c 3T3 cells of plasma prepared from hypophysectomized rats. This material had 5% of the potency of normal rat plasma in promoting DNA synthesis in cells briefly exposed to PDGF. The activity of hypophysectomized rat plasma was restored to normal by the addition of somatomedin C.

C. *Eicosanoids*

Some of the products of arachidonate metabolism, notably TXA_2 and the leukotrienes, are growth stimulants for smooth muscle cells. However, a number of prostaglandins have been found to inhibit smooth muscle cell proliferation. Pathways of eicosanoid metabolism are shown in Fig. 2.

An appreciable amount of evidence now suggests that arterial smooth muscle cells can synthesize prostaglandins. Huttner *et al.* (1977) showed that guinea pig aortic smooth muscle cells secrete prostaglandins of the E and I series into the culture medium when supplied with appropriate fatty acid precursors. Cornwell and co-workers (1979) were able to attribute this to the synthesis of prostaglandins rather than release from preformed stores by showing that the cyclooxygenase inhibitor indomethacin reduced the concentrations of prostaglandin (PG) E and PGI_2 in conditioned medium. PGE_2, $PGF_{2\alpha}$, and PGI_2 (prostacyclin) are synthesized by porcine aortic smooth muscle cells supplied with exogenous arachidonic acid (Ody *et al.,* 1982). Using bovine pulmonary arterial smooth muscle cells, Menconi and colleagues (1984) detected the synthesis of $PGF_{2\alpha}$ and prostacyclin which was augmented by exogenous arachidonic acid. There was no detectable synthesis of PGE_2. However, punch samples of bovine pulmonary arterial media synthesized PGE_2 and prostacyclin. Larrue *et al.* (1984) also compared the prostaglandin synthetic activity of rabbit aortic smooth muscle cells in intact artery and in culture. PGE_2, prostacyclin, and $PGF_{2\alpha}$ were all synthesized in response to the addition of arachidonic acid. The major product, both *ex vivo* and *in vitro,* was prostacyclin. Human umbilical arterial smooth muscle cells generate PGE_2, prostacyclin, and $PGF_{2\alpha}$ from exogenous arachidonate *in vitro* (Brinkman *et al.,* 1990). Prostaglandin synthesis by smooth muscle cells has been shown to be stimulated by a variety of factors, including interleukin-1 (Albrightson *et al.,* 1985; Rossi *et al.,* 1985; Libby *et al.,* 1988), PDGF (Sinzinger *et al.,* 1988), vasopressin (Blay and Hollenberg, 1989), bradykinin (Menconi *et al.,* 1984), angiotensins I and II but not angiotensin III (Menconi *et al.,* 1984), and serotonin (Coughlin *et al.,* 1984).

The ability of prostaglandins to modulate the proliferation of arterial smooth muscle cells was first demonstrated by Huttner and associates (1977). The growth of guinea pig aortic smooth muscle cells was inhibited by PGE_1 and PGE_2 at 2 and 20 μM, respectively. Pietilä and co-workers (1980) confirmed and extended these results, reporting significant inhibition of the incorporation of [^{3}H]thymidine in rabbit aortic smooth muscle cells by PGE_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$.

Although the inhibitory actions of prostaglandins of the E series on arterial smooth muscle cell proliferation *in vitro* have subsequently been reported by several groups, it has become clear that the conditions of the study are critical determinants of the observed effect. In the studies of Huttner *et al.* (1977), Pietilä *et al.* (1980), Sjölund *et al.* (1984), Smith *et al.* (1984), and Orekhov *et al.* (1986), E-series prostaglandins inhibited the proliferation of arterial smooth muscle cells growing continuously in serum. Loesberg and colleagues (1985) restimulated cell growth in quiescent human aortic smooth muscle cells by adding 1% serum supplemented with PDGF and adding PGE_1 (25 n*M*) at various time intervals later. Addition up to 12 hr after restimulation inhibited [^{3}H]thymidine incorporation, but addition 14 to 18 hr after restimulation was without effect. Nilsson and Olsson (1984) used a similar protocol to examine the effects of PGE_1 on rat thoracic aortic smooth muscle cells. Restimulation of quiescent cells by the addition of PDGF was inhibited by PGE_1 (140 n*M*), but only if it was given within 6 hr. It therefore appears that the inhibitory effect of PGE_1 is produced either by administration to proliferating cells or by administration to quiescent cells within a few hours of restimulation. The concentration of PGE_1 required to inhibit the proliferation of growing cells was on average 100 times greater than that required to inhibit the restimulation of quiescent cells.

Inhibitory effects on arterial smooth muscle cell proliferation have also been noted with PGD_2 (Nilsson and Olsson, 1984; Smith *et al.*, 1984; Orekhov *et al.*, 1986) and PGE_2 (Huttner *et al.*, 1977; Pietilä *et al.*, 1980; Nilsson and Olsson, 1984; Smith *et al.*, 1984; Loesberg *et al.*, 1985). Effects with F-series prostaglandins have been contradictory. Pietilä and co-workers (1980) found significant inhibitory actions with $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (both 10 μ*M*), but Cornwell *et al.* (1979) reported significant stimulation. No effect was found with $PGF_{2\alpha}$ (0.6 μ*M*) by Nilsson and Olsson (1984) or at unspecified doses by Smith and colleagues (1984) and Loesberg *et al.* (1985).

Prostacyclin, in the form of its relatively stable analog carbacyclin, has been found to inhibit at micromolar concentrations the incorporation of [^{3}H]thymidine in human aortic intimal smooth muscle cells growing in culture (Orekhov *et al.*, 1983, 1986; Akopov *et al.*, 1988). Similar effects were noted with authentic prostacyclin by Morisaki and co-workers (1988a) using rabbit aortic smooth muscle cells. Prostacyclin was without effect, at concentrations up to 100 μ*M*, on the restimulation of growth in quiescent bovine aortic smooth muscle cells (Nemecek *et al.*, 1986). Cicletanine, a diuretic that stimulates the synthesis of prostacyclin in rat thoracic aortic smooth muscle cells (Dorian *et al.*, 1988), inhibits the restimulation of quiescent rat

mesenteric arterial smooth muscle cells in response to serum or to PDGF (Sato *et al.,* 1989).

D. Heparinoids

Heparin is a glycosaminoglycan that is particularly abundant in lung, liver, and intestinal mucosa. It is found in mast cells, which accumulate in most organs around small blood vessels (Tharp, 1989). Heparan sulfate is a structurally related compound, which is present in extracellular matrix and at cell surfaces, and has been shown to be present in the aorta (Radhakrishnamurthy *et al.,* 1977; Clowes *et al.,* 1984).

Hoover and colleagues (1980) showed that heparin dose dependently inhibits the serum-stimulated proliferation of rat aortic smooth muscle cells in culture, with 50% inhibition of growth occurring at a concentration of approximately 10 μg/ml. This corresponds to 670 n*M*, assuming a molecular weight of 15,000. No difference in growth inhibitory activity between anticoagulant and nonanticoagulant fractions of heparin exists. Since the latter species has low affinity for antithrombin III, it is unlikely that the growth inhibitory properties of heparin are related to inactivation of thrombin. Reilly *et al.* (1989) went on to show that the inhibition of bovine aortic smooth muscle cell proliferation is maximized by preincubation of quiescent cells with heparin for 48 hr prior to reintroduction of serum.

The effects of heparin on smooth muscle cell growth have also been investigated in animal models of arterial injury. Clowes and Karnovsky (1977) administered heparin by intravenous infusion to rats commencing 24 hr after dessicating injury to the common carotid artery. There was no difference in neointimal thickening between control and heparin-treated rats 10 days after injury. During the next 4 days there was considerable expansion of the intima which was inhibited by heparin, with the result that at 14 days after injury the neointimas of injured carotid arteries were significantly smaller in the heparin-treated group. This difference was attributed to inhibition of smooth muscle cell proliferation. Similar results were reported by Guyton *et al.* (1980), who also showed a dose-related inhibitory action of heparin on neointimal thickening. This effect was observed with both anticoagulant and nonanticoagulant fractions of heparin. Reduction of neointimal thickening was also observed in the balloon-injured rabbit aorta after a continuous intravenous infusion of heparin (Berk *et al.,* 1991). Local release of heparin from a gel enclosed in a periadventitial cuff around the rat common carotid artery significantly inhibits balloon catheter-induced neointimal thickening in this vessel (Okada *et al.,*

1989). The intravenous administration of heparin to rats for 14 days significantly inhibits the increase in the DNA content of the common carotid artery caused by balloon catheter injury (Clowes and Clowes, 1985, 1986). Further direct evidence of an effect of heparin on arterial smooth muscle cell proliferation *in vivo* was obtained by Majesky *et al.* (1987), who measured the [^{3}H]thymidine labeling index of smooth muscle cells in the rat common carotid artery. An intravenous infusion of heparin significantly inhibited proliferation measured 33 hr after balloon catheter injury. Delay of commencement of administration of heparin until 18 hr after injury did not prevent an inhibitory effect, but delay until 27 hours abolished the inhibition. This is rather surprising in view of the finding by Clowes and Karnovsky (1977) that neointimal thickening in the air-drying model of arterial injury is inhibited by heparin through a mechanism that becomes manifest 10 days after injury. These results suggest that heparin inhibits both medial smooth muscle cell proliferation and neointimal expansion. This latter effect could be the result of inhibition of neointimal smooth muscle cell proliferation, of inhibition of synthesis of extracellular matrix in the neointima, or of inhibition of migration of smooth muscle cells from the media to the intima. There is experimental support for all of these alternatives (Clowes and Clowes, 1985, 1986).

Several hypotheses have been advanced to account for the antiproliferative activity of heparin. It suppresses the elevation of the levels of messenger RNA for c-*myb,* a protooncogene that is involved in progression through the cell cycle (Reilly *et al.,* 1989). Heparin also inhibits intracellular Ca^{2+} mobilization, through an effect on the inositol-1,4,5-trisphosphate receptor (Kobayashi *et al.,* 1989; Yamamoto *et al.,* 1990). Another possibility is that heparin binds and sequesters polypeptide growth factors such as bFGF or PDGF (Shing *et al.,* 1984). Finally, Majack and colleagues (1985, 1986) have shown that heparin disrupts the deposition of thrombospondin in the extracellular matrix of smooth muscle cells. Thrombospondin appears to be essential for smooth muscle cell proliferation *in vitro* (Majack *et al.,* 1988).

E. Somatostatin

Somatostatin is a tetradecapeptide hormone that inhibits the release of growth hormone from the pituitary gland (Moreau and DeFeudis, 1987). It is found in the hypothalamus and in sympathetic ganglia. Nanomolar concentrations of an octapeptide derivative of somatostatin, angiopeptin, inhibit DNA synthesis in explants of rat common carotid artery (Vargas *et al.,* 1989), suggesting that this material has an effect on vascular smooth muscle cell proliferation that is not mediated by growth hormone. The subcutaneous administration of an-

giopeptin inhibits DNA synthesis and neointimal thickening in the rat common carotid artery after air-drying injury (Lundergan *et al.,* 1989), but this property is not shared by some other analogs of somatostatin that also inhibit the release of growth hormone. Angiopeptin also inhibits DNA synthesis (Asotra *et al.,* 1989) and neointimal thickening (Conte *et al.,* 1989) in balloon-injured rabbit arteries.

IV. Conclusions

This chapter reviews the pharmacological evidence that supports consideration of various endogenous substances as smooth muscle cell growth control factors. Among the potential growth stimulants are angiotensin II, norepinephrine, epinephrine, thromboxane A_2, leukotrienes, serotonin, and thrombin. The list of growth inhibitors includes adenosine, adrenocorticosteroid hormones, prostaglandins, heparin, and somatostatin. These are not exhaustive lists because they do not include potential mediators without an associated pharmacology; neither do they include growth factors such as bFGF and PDGF, which are now being investigated by means of inhibitory antibodies.

Some of the growth stimulants just listed can be considered to be wound hormones, released at sites of vascular damage in order to minimize blood loss. This is achieved by restriction of blood flow, through vasoconstriction; plugging of the wound, by platelet aggregation; and restoration of vessel integrity, by smooth muscle cell proliferation. Thromboxane A_2, leukotrienes, serotonin, and thrombin can all be placed in this category. However, angiotensin II and the catecholamines do not fit so easily because the involvement of these substances in the control of blood pressure means that smooth muscle cells are exposed to them under normal physiological conditions. Is the very low rate of smooth muscle cell replication in the normal vessel (Schwartz *et al.,* 1990) the result of a balance between growth inhibitors, such as adrenocorticosteroid hormones or vessel wall heparan sulfate, and these omnipresent growth stimuli? An alternative possibility is that, *in vivo,* angiotensin II and catecholamines are not sufficient to induce replication. As yet no single agent, including polypeptide growth factors, has been shown to stimulate quiescent smooth muscle cells to proliferate *in vivo,* suggesting that a combination of stimuli is necessary for the induction of replication. This is an issue that has not been addressed experimentally. However, profound inhibition of smooth muscle cell proliferation is produced in injured rat arteries both by inhibitors of bFGF (Lindner and Reidy, 1991) and by antagonists of the α_1-adrenergic receptor (Jackson *et al.,* 1988). This may be evidence that both bFGF and norepinephrine or epinephrine is nec-

essary for the induction of growth in this system and that deletion of either component is inhibitory. The nature of the interplay between polypeptide and nonpolypeptide growth factors, and the influence of growth inhibitors, are important challenges in the pharmacological investigation of the control of smooth muscle cell proliferation.

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Smooth Muscle Cell and Fibroblast Biological and Functional Features: Similarities and Differences

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I. Introduction

Fibroblasts and smooth muscle cells share several functions such as collagen, elastin, and proteoglycan synthesis or contractile activities (Wissler, 1984; Komuro, 1990). In the normal adult, some of these functions are specifically exerted by the fibroblast (e.g., collagen synthesis) or the smooth muscle cell (e.g., contractility), but during development or pathological conditions this can change. Collagen synthesis becomes proeminent in smooth muscle cells during development and during the formation of an atheromatous plaque (Wagner, 1990), whereas contractility may be exerted by fibroblasts during the healing of a wound, resulting in the phenomenon of wound contraction (Gabbiani *et al.*, 1971, 1972). In this respect, it is noteworthy that the actin isoform typical of smooth muscle cells (α-smooth muscle actin,

see later) is expressed in fibroblasts during wound contraction and fibrocontractive diseases, suggesting that fibroblasts can acquire smooth muscle-like features in particular situations.

The molecular structure of the cytoskeleton and its relationship to the specialized contractile apparatus of the smooth muscle cell are discussed at length in the chapter by Small and North. For our purposes, however, cytoskeletal proteins represent useful markers of differentiation, and the study of their expression allows the characterization of phenotypic modifications corresponding to functional changes which take place during physiological and pathological processes (Osborn and Weber, 1982; Rungger-Brändle and Gabbiani, 1983; Skalli *et al.*, 1987, 1989). The purpose of this chapter is to review the cytoskeletal features of fibroblastic and smooth muscle cells and to relate them to adaptive situations in which an overlapping of functional activities of the two cell types is present.

The cytoskeleton of eukaryotic cells is composed of three filamentous systems (Bershadsky and Vasiliev, 1988): (1) microfilaments, made up mainly of actin and myosin; (2) intermediate filaments formed by at least six distinct classes of proteins (Albers and Fuchs, 1992); in mesenchymal cells, intermediate filaments are generally homopolymers of vimentin or desmin (Raats and Bloemendal, 1992); lamins are present, as in other cells with different embryological origin, in nuclear membranes; and (3) microtubules consisting mainly of tubulin. By two-dimensional polyacrylamide gel electrophoresis (PAGE; Garrels and Gibson, 1976; Whalen *et al.*, 1976) and amino acid sequence analysis (Vandekerckhove and Weber, 1978, 1979, 1981), six isoforms of actin (Rubenstein, 1990; McHugh *et al.*, 1991), produced by different genes, have been described in mammalian tissues: the β- and γ-cytoplasmic isoforms, which are expressed by all cells, and the α-cardiac, α-skeletal, and α- and γ-smooth muscle isoforms, which are limited to specific cell types. Myosin is a polymeric molecule composed of two heavy chains and four light chains, with each chain displaying multiple isoformic variations (Bandman, 1985). The analysis of cytoskeletal elements has been facilitated by the production of specific antibodies for smooth muscle and nonmuscle myosin heavy chain isoforms (Benzonana *et al.*, 1988; Eddy *et al.*, 1988; Giuriato *et al.*, 1992), for the intermediate filament proteins vimentin and desmin (Franke *et al.*, 1978; Gard *et al.*, 1979; Osborn *et al.*, 1981; Kocher *et al.*, 1984), and for actin isoforms (Gown *et al.*, 1985; Skalli *et al.*, 1986b, 1988; Tsukada *et al.*, 1987). A monoclonal antibody against the α-smooth muscle isoform of actin (Skalli *et al.*, 1986b) which, as previously mentioned, is predominant in vascular smooth muscle cells (Gabbiani *et al.*, 1981) has been particularly use-

ful for the characterization of smooth muscle cell phenotypic features. The regulation of specific actin mRNA expression in smooth muscle cells and fibroblasts has been studied by means of specific molecular probes (Barret and Benditt, 1987; Kocher and Gabbiani, 1987; Bochaton-Piallat *et al.*, 1992).

II. Cytoskeletal Features of Smooth Muscle Cells

A. Normal Conditions

In the normal fetal and newborn (Fig. 1a) rat aortic media, smooth muscle cells show a fibroblast-like ultrastructural morphology with an extensive rough endoplasmic reticulum, a prominent Golgi complex, and only a few myofilaments (Gerrity and Cliff, 1975; Kocher *et al.*, 1985; Nakamura, 1988). They proliferate and secrete extracellular matrix components such as collagen and elastin. In adult rat aortic media (Fig. 1b), smooth muscle cells develop an important microfilamentous network, distributed throughout the cytoplasm, whereas the endoplasmic reticulum and the Golgi apparatus become confined to the perinuclear space; the synthetic and proliferative activities de-

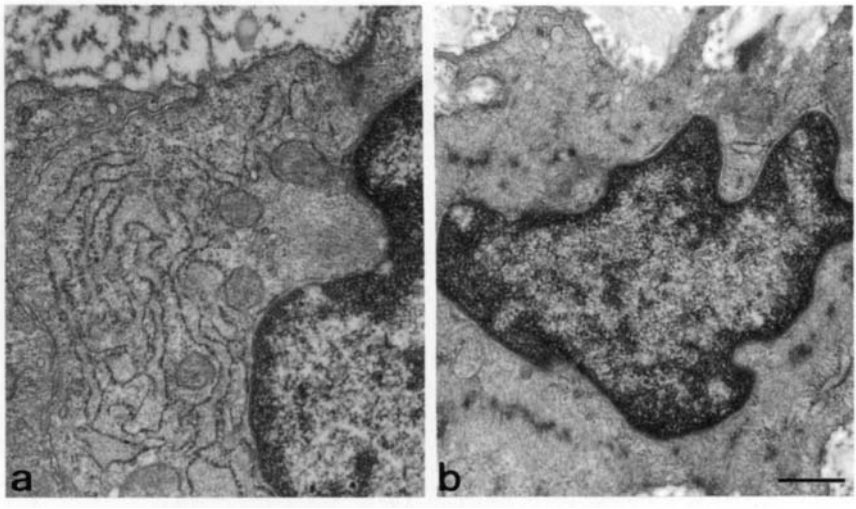

Fig. 1. Morphologic features of aortic smooth muscle cells in a newborn (a) and a 5-week-old (b) rat. The cytoplasm of the smooth muscle cells in the newborn rat (a) contains a well-developed rough endoplasmic reticulum and Golgi apparatus, and many mitochondria. Microfilaments are barely visible, whereas they become the major cytoplasmic component in smooth muscle cells of a 5-week-old animal (b). Bar, 0.5 μm. From Kocher *et al.* (1985), with the kind permission of the publisher.

crease and the ability to contract is established, becoming the prevalent smooth muscle cell function. Each cell is encircled by a basement membrane composed of collagen type IV, laminin, and heparan sulfate proteoglycan (Carey, 1991). Cytoskeletal features of arterial smooth muscle cells vary characteristically during development (Kocher *et al.*, 1985; Skalli and Gabbiani, 1985; Owens and Thompson, 1986; Kuro-o *et al.*, 1989; Giuriato *et al.*, 1992). The actin, vimentin, desmin, and tropomyosin (a protein involved in muscle contraction within the myosin–actin interaction) content of smooth muscle cells increases gradually from fetal to adult animals (Berner *et al.*, 1981; Schmid *et al.*, 1982; Kocher *et al.*, 1985; Skalli and Gabbiani, 1985). The number of desmin-containing cells also increases from 13% in fetal rat aorta to 51% in adult rat aorta (Skalli and Gabbiani, 1985). The β-actin isoform is predominant in fetal and newborn animals, but gradually the α-smooth muscle actin isoform becomes quantitatively the most important, as seen by two-dimensional PAGE (Gabbiani *et al.*, 1981; Kocher *et al.*, 1985; Kocher and Gabbiani, 1986; Owens and Thompson, 1986). In the normal adult rat aortic media, despite a homogeneous morphology, smooth muscle cells are heterogeneous as far as intermediate filament pattern is concerned: practically all smooth muscle cells contain vimentin, but, as described earlier, about 51% of them contain, in addition, desmin (Kocher *et al.*, 1984; Skalli and Gabbiani, 1985).

B. Adaptive and Pathological Modulations

In the atheromatous plaque, the cytoskeletal features of smooth muscle cells are modified compared to resident medial smooth muscle cells (Kocher and Gabbiani, 1986) and become somehow similar to those of fetal cells; thus atheromatous smooth muscle cells acquire a "dedifferentiated" phenotype as far as the cytoskeleton is concerned (Gabbiani *et al.*, 1984). Remarkably, these changes are practically the reverse of what has been described for developing smooth muscle cells (Kocher *et al.*, 1985): (1) smooth muscle cells lose microfilaments and acquire a prominent rough endoplasmic reticulum and Golgi apparatus; (2) the actin content per cell decreases and there is a switch from the predominance of the α-isoform to the predominance of the β-isoform (Fig. 2); (3) the number of cells which express only vimentin, as well as the content of vimentin per cell, increases; and (4) the number of desmin-positive cells decreases. In rats, however, about 2 months after balloon catheter-induced endothelial lesion, when the vessel wall is reendothelialized, the expression of α-smooth muscle actin in the intimal thickening reaches normal levels and most cells become desmin positive (Kocher *et al.*, 1984). In human advanced atheroma-

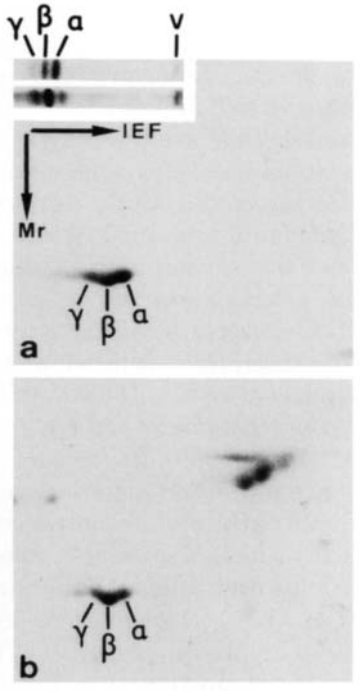

FIG. 2. Isoelectric focusing (IEF) and two-dimensional gel electrophoresis of human aortic media (a) and fibrous plaque (b) total protein extracts. In the media, the α-isoform of actin is predominant. In the fibrous plaque, the β-isoform of actin becomes predominant. V, vimentin; Mr, molecular weight ratio. From Kocher and Gabbiani (1986), with the kind permission of the publisher.

tous lesions, smooth muscle cells positive for desmin are also observed (Kocher and Gabbiani, 1986). What remains to be defined is how a dedifferentiated smooth muscle cell again acquires a contractile phenotype.

During the atheromatous process, the macromolecular network of the basement membrane, which contributes to maintain the homeostasis of smooth muscle cells (Carey, 1991), is probably modified by locally liberated factors with proteolytic activities (Campbell *et al.*, 1992). Furthermore, cytokines (Casscells, 1991) and extracellular matrix components (Madri and Basson, 1992), which are known to influence the shape, the replication, and the migration of smooth muscle cells, can induce cellular modifications, leading to the modulation of smooth muscle cell phenotype. Developmental changes in smooth muscle cell are discussed in the chapter by Glukhova and Koteliansky.

Many studies have described the action of mitogenic agents on smooth muscle cells of the aortic media and their possible role in the evolution of the atheromatous plaque. Platelet-derived growth factor (PDGF; Thyberg *et al.*, 1983; Ross *et al.*, 1990), secreted by both macrophages and smooth muscle cells, and fibroblast growth factor

(Burgess and Maciag, 1989), secreted by macrophages as well as injured smooth muscle cells (see chapter by Reidy), are potent chemotactic and mitogenic substances, which can also influence smooth muscle cell differentiation (Casscells, 1991). When cultured smooth muscle cells are treated with PDGF or fibroblast growth factor, proliferation increases and α-smooth muscle actin expression decreases (Corjay *et al.*, 1990; Holycross *et al.*, 1992; A. Desmoulière and G. Gabbiani, unpublished observations). Björkerud (1991) has shown that transforming growth factor β1 (TGF-β1) induces α-smooth muscle actin expression in cultured smooth muscle cells. Interferon-γ (IFNγ), secreted by activated T helper lymphocytes and present in experimentally injured arteries and human atheromatous plaques (Hansson *et al.*, 1988), inhibits both proliferation and expression of α-smooth muscle actin in cultured smooth muscle cells (Hansson *et al.*, 1988, 1989). It has been suggested that smooth muscle cell quiescence in normal arterial vessels is actively maintained by locally produced extracellular matrix components such as proteoglycans and heparin, which also inhibit smooth muscle cell proliferation in different *in vivo* and *in vitro* situations (Clowes and Karnovsky, 1977; Guyton *et al.*, 1980; Hoover *et al.*, 1980). After a balloon catheter-induced endothelial lesion, the switch from α- to β-actin mRNA production at 24 hr is not prevented in heparin-treated animals compared to saline-treated controls, but injured arteries, in which smooth muscle cell proliferation is inhibited by heparin, exhibit a reexpression of the differentiated phenotype rich in α-smooth muscle actin as early as 5 days after injury (Clowes *et al.*, 1988). Myointimal smooth muscle cell proliferation produced by balloon catheterization of rat carotid artery is inhibited by nonanticoagulant, acylated heparin derivatives (Pukac *et al.*, 1991). These compounds may be capable of exerting a pharmacologic action preventing myointimal proliferative lesions that are responsible for the failure of percutaneous angioplasty. *In vitro*, heparin decreases smooth muscle cell growth and increases α-smooth muscle actin protein and mRNA (Fig. 3) expression in primary and passaged smooth muscle cells (Desmoulière *et al.*, 1991). Nonanticoagulant, acylated heparin derivatives produce similar effects (Bârzu *et al.*, 1992). Generally, the action of heparin on α-smooth muscle actin expression is related to its antiproliferative activity. However, in some culture conditions (e.g., rat aortic smooth muscle cells cultured in the presence of whole blood serum), heparin affects proliferation without affecting α-smooth muscle actin expression (Desmoulière *et al.*, 1991). Furthermore, although 10% fetal bovine serum is as mitogenic as 20 ng/ml PDGF, it does not alter the expression of α-smooth muscle actin mRNA, whereas PDGF suppresses the expression of α-smooth muscle

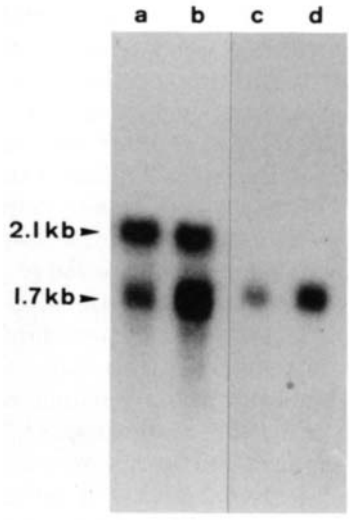

FIG. 3. Effect of heparin on actin isoform mRNA expression in rat cultured smooth muscle cells. Hybridization of Northern blots with a total actin probe (a, b) results in two bands: the 2.1-kb band corresponds to cytoplasmic actin mRNAs, and the 1.7-kb band corresponds to α-smooth muscle actin mRNA. Hybridization with a specific oligonucleotide (c, d) shows a unique 1.7-kb band, corresponding to α-smooth muscle actin mRNA. In heparin-treated cells (200 μg/ml; b, d), α-smooth muscle actin mRNA expression is increased compared with control smooth muscle cells (a, c). From Desmoulière *et al.* (1991), with the kind permission of the publisher.

actin by 70–80% (Corjay *et al.*, 1989). Continuous exposure of smooth muscle cells to PDGF maintains an 80% inhibition of α-smooth muscle actin synthesis but does not maintain cells in a proliferative state, as measured by the thymidine labeling index (Blank and Owens, 1990). These findings indicate that the expression of α-smooth muscle actin is not always related to proliferative activity.

Smooth muscle cell modifications observed in pathological processes involve cell-to-cell and cell-to-extracellular matrix interactions mediated by growth factors, cytokines, and extracellular matrix components. It is not known whether all of the smooth muscle cells of the arterial media have the same potential to migrate, grow, and change their phenotype. Cloning experiments could help in the study of smooth muscle cell heterogeneity.

C. Cultured Populations and Clones of Smooth Muscle Cells

When smooth muscle cells isolated from adult rat media are cultured in the presence of fetal calf serum, they proliferate and show decreased expression of α-smooth muscle actin, desmin, and smooth muscle myosin heavy chains; these cells acquire cytoskeletal features of fetal or atheromatous smooth muscle cells (Gabbiani *et al.*, 1984; Skalli *et al.*, 1986a). On this basis, it is generally assumed that cultured arterial smooth muscle cells are a reliable model to study smooth muscle cell phenotypic modifications. Furthermore, compared

to freshly isolated cells, primary cultures of smooth muscle cells from newborn animals show no change in the number of α-smooth muscle actin containing cells and a less important decrease in the number of desmin and smooth muscle myosin heavy chains containing cells than that seen in primary cultures of smooth muscle cells from adult animals (Bochaton-Piallat *et al.*, 1992). These results are in agreement with previous work showing that newborn aortic smooth muscle cells in culture develop properties that occur *in vivo* during normal postnatal development (Gordon *et al.*, 1986; Majesky *et al.*, 1988). It is noteworthy that populations of smooth muscle cells cultured from newborn rats express preferentially certain mRNAs and proteins (e.g., osteopontin and elastin) compared to similar populations cultured from adult animals (Stone *et al.*, 1987; Giachelli *et al.*, 1991; Majesky *et al.*, 1992; Gadeau *et al.*, 1993; see chapter by Schwartz and colleagues). Furthermore, with increasing age of the donor animal, the replicative activity of cultured smooth muscle cell populations increases and differentiation features decrease (Stemerman *et al.*, 1982; Hariri *et al.*, 1986; McCaffrey *et al.*, 1988; Bochaton-Piallat *et al.*, 1993), contrary to what is classically observed in fibroblasts, which show a decrease of replicative activity with age (Macieira-Coelho, 1988; Bayreuther *et al.*, 1992). Although these observations have been convincingly made only in rats, they suggest that cells of different origins do not react stereotypically to aging by decreasing their replicative activity *in vitro*.

To evaluate the potential of individual smooth muscle cells to keep differentiated features *in vitro*, smooth muscle cells have been isolated from media of animals from different ages and cloned by limiting dilution during primary culture (Bochaton-Piallat *et al.*, 1992, 1993). Clones were studied at the fifth passage for cytoskeletal protein expression. Smooth muscle cell clones showed different cytoskeletal features with different levels of differentiation: (1) α-smooth muscle actin, smooth muscle myosin heavy chains, and desmin positive (Figs. 4a, 4b, and 4e); (2) α-smooth muscle actin and smooth muscle myosin heavy chains positive, desmin negative (Figs. 4c–4e); (3) α-smooth muscle actin and desmin positive, smooth muscle myosin heavy chains negative; (4) α-smooth muscle actin positive, smooth muscle myosin heavy chains and desmin negative; and (5) α-smooth muscle actin, smooth muscle myosin heavy chains and desmin negative. In clones obtained from newborn animal smooth muscle cells, 50% belonged to type one and 50% to type two. In the parental population, the disappearance of desmin positive cells, the persistence of α-smooth muscle actin in about 90% of cells, and the persistence of smooth muscle myosin heavy chains in about 40% of cells were ob-

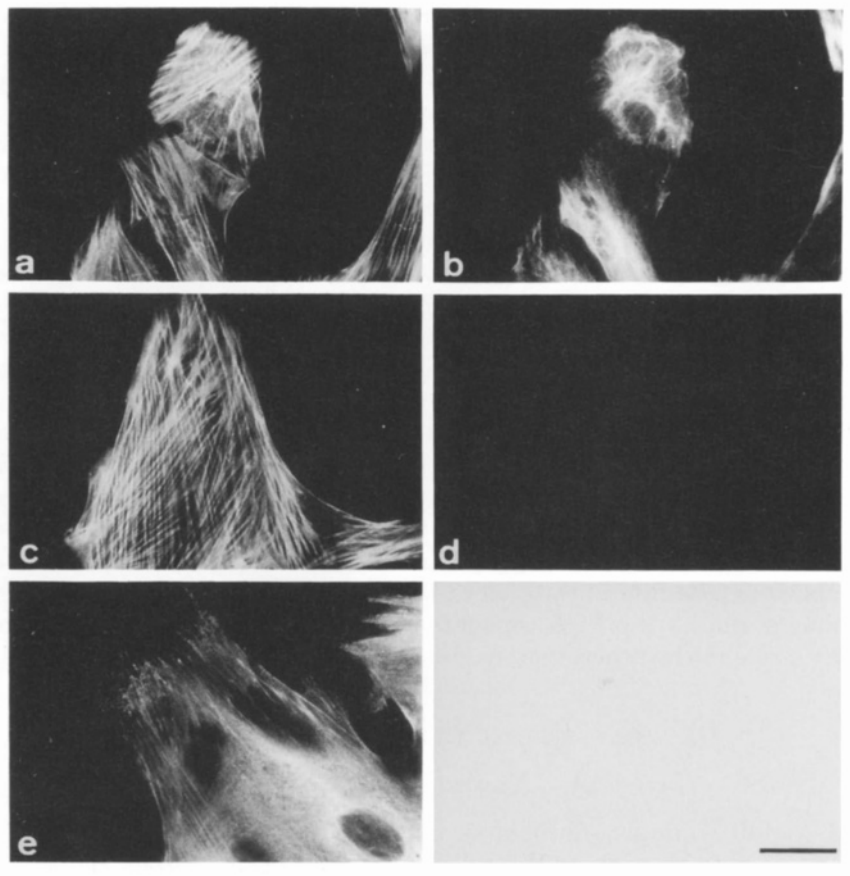

FIG. 4. Double immunofluorescence staining of cloned newborn rat aortic smooth muscle cells at the fifth passage for α-smooth muscle actin (a, c) and desmin (b, d). One clone (type one) is positive for both stainings (a, b) and the other (type two) is positive only for α-smooth muscle actin (c, d). The typical interrupted distribution of smooth muscle myosin, present in all clones derived from newborn rat aortic smooth muscle cells (type one and two), is seen in (e). Bar, 10 μm. From Bochaton-Piallat *et al.* (1992), with the kind permission of the publisher.

served. In adult animals, the clones obtained still were of type one and two and were much more differentiated compared to their parental populations. Finally, clones from old (18 months and more) animals showed an heterogeneity of phenotypic features and generally a low degree of differentiation. No clone was positive to smooth muscle myosin heavy chains (indicating that smooth muscle myosin heavy chain expression is a marker for terminal smooth muscle dif-

ferentiation). Most were of type three and four and some of type five, i.e., had lost all the typical smooth muscle cytoskeletal markers. These results are consistent with other reports demonstrating the heterogeneity of the aortic media smooth muscle cells (Kocher *et al.*, 1984; Larson *et al.*, 1984; Haudenschild and Grünwald, 1985; Babaev *et al.*, 1990). The fact that after a balloon catheter-induced endothelial lesion some smooth muscle cells show only a migrating activity toward the lumen while others also proliferate (Clowes and Schwartz, 1985) illustrates clearly that smooth muscle cells do not possess the same capacities in response to injury. Furthermore, clones display different features compared to parental populations. These observations are of interest in view of the suggestion that the development of the atheromatous plaque involves the replication of one or few smooth muscle cells (Benditt and Benditt, 1973; Majesky *et al.*, 1985; see chapter by Schwartz and colleagues). Cloning experiments show that a certain proportion of cells are able to undergo clonal growth *in vitro* and to express different patterns of differentiation according to the age of the donor animal. Smooth muscle cells derived from old animals, which dedifferentiate more importantly than those from young animals and have a high replicative capacity, are good candidates to play a role in the atheromatous process.

III. Cytoskeletal Features of Fibroblasts

A. Normal Conditions

In adult animals, fibroblasts generally have a slender fusiform shape, a smooth contoured nucleus, and contain numerous cisternae of rough endoplasmic reticulum and mitochondria (Fig. 5a); they display few microfilaments and intermediate filaments and do not establish specialized contacts among them (Gabbiani and Rungger-Brändle, 1981). In fetal animals, fibroblasts can exhibit cytoplasmic bundles and gap junctions (Ross and Greenle, 1966).

The identification of cytoskeletal differentiation markers in fibroblasts has led to the recognition of a phenotypic heterogeneity among these cells (Table I), which was underestimated morphologically and which might be related to different biological behaviors (for a review see Sappino *et al.*, 1990b). All fibroblastic cells are generally thought to express vimentin, although defined subtypes of these cells have been found to express desmin (Table I), e.g., in the uterine mucosa (Glasser and Julian, 1986), lymphatic organs, including the spleen (Toccanier-Pelte *et al.*, 1987), testicular stroma (Skalli *et al.*, 1986b), hepatic perisinusoidal cells (Yokoi *et al.*, 1984; Schmitt-Gräff *et al.*, 1991), and lung septa (Kapanci *et al.*, 1992). Generally, fibroblastic

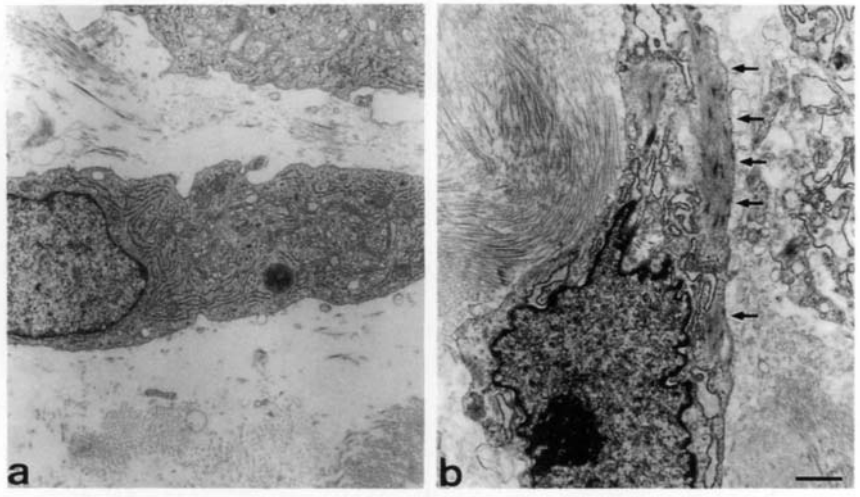

FIG. 5. Transmission electron micrographs of fibroblastic cells from saline solution (a) and TNFα plus heparin derivative (b)-induced capsules. In saline solution-treated tissue (a), fibroblasts are characterized by a smooth nuclear outline and a cytoplasm rich in rough endoplasmic reticulum, Golgi apparatus, and mitochondria. After TNFα plus heparin derivative treatment (b), stress fibers, (arrows) often located beneath the plasmalemma and parallel to the main axis of the cell, are present in a large proportion of fibroblastic cells. Dense bodies are scattered within these bundles. Bar, 1 μm. From Desmoulière *et al.* (1992b), with the kind permission of the publisher.

cells do not contain α-smooth muscle actin, but immunohistochemical studies have revealed that, for example, a category of reticular cells in lymph nodes and spleen (Toccanier-Pelte *et al.*, 1987), testicular (Skalli *et al.*, 1986b) and bone marrow (Schmitt-Gräff *et al.*, 1989; Charbord *et al.*, 1990) stromal cells, and cells of the theca externa of the ovary (Czernobilsky *et al.*, 1989) express this protein (Table I). Similarly, human intestinal pericryptal cells show smooth muscle differentiation features (Table I; Sappino *et al.*, 1989). These findings suggest that some stromal cells are equipped with muscular elements and might participate in visceral contraction. It seems clear now that fibroblasts of many tissues may have distinct cytoskeletal features. Microenvironmental factors probably play an important role in the development of these features. It has been suggested that each organ contains fibroblasts with specific differentiated characters (Sappino *et al.*, 1990b; Shimizu and Yoshizato, 1992). Moreover, Fabra *et al.*, (1992) have shown that specific fibroblasts influence the invasive potential of highly metastatic human colon carcinoma cells. Komuro (1990) has proposed to categorize fibroblasts into subtypes depending

TABLE I

NORMAL AND PATHOLOGICAL FIBROBLASTIC CELLS DISPLAYING ULTRASTRUCTURAL AND/OR IMMUNOCHEMICAL FEATURES OF SMOOTH MUSCLE DIFFERENTIATION

Localization and/or situation	Stress fibers	Desmin	α-smooth muscle actin	Smooth muscle myosin heavy chains	Ref.
Normal tissues					
Uterine submucosa	−	+	−	−	Glasser and Julian (1986)
Reticular cells of lymph nodes and spleen	+	+	+	+	Toccanier-Pelte *et al.* (1987)
Intestinal pericryptal cells	+	+	+	Not known	Sappino *et al.* (1989)
Intestinal villous core	+	+	+	+	Kaye *et al.* (1968)
Testicular stroma	+	+	+	+	Skalli *et al.* (1986b)
Theca externa of the ovary	+	Not known	+	Not known	Czernobilsky *et al.* (1989)
Periodontal ligament	+	Not known	Not known	Not known	Beertsen *et al.* (1974)
Adrenal gland capsule	+	Not known	Not known	Not known	Bressler (1973)
Hepatic perisinusoidal cells	−	+	−	−	Yokoi *et al.* (1984)
Lung septa	+	+	−	−	Kapanci *et al.* (1992)
Bone marrow stroma	+	Not known	+	Not known	Charbord *et al.* (1990)

Pathological situations					
Normal healing granulation tissue	+	−	+[a]	−	Darby *et al.* (1990)
Hyperthrophic scars	+	+[a]	+	+[b]	Baur *et al.* (1975)
Dupuytren's disease (nodule)	+	+[a]	+	+[b]	Skalli *et al.* (1989)
Scleroderma (dermis and esophageal submucosa)	+	+[a]	+	−	Sappino *et al.* (1990a)
Asthma (bronchial mucosa)	+	Not known	+	Not known	Roche (1991)
Anterior capsular cataract	+	Not known	+	Not known	Schmitt-Gräff *et al.* (1990)
Liver cirrhosis	+	+[a]	+	+[a]	Rockey and Friedman (1992)
Lung fibrosis	+	+[a]	+	+[a]	Kapanci *et al.* (1990)
Kidney fibrosis	+	−	+	Not known	Johnson *et al.* (1991)
Stroma reaction to tumors	+	+	+	+[a]	Schürch *et al.* (1981)
Fibroblastic cells of granulation tissue produced by					
GM-CSF	+	−	+	−	Vyalov *et al.* (1993)
TGF-β1	+	−	+	−	Desmoulière *et al.* (1993)
TNFα	+	−	−	−	Desmoulière *et al.* (1992b)
TNFα + heparin	+	−	+	+[a]	Desmoulière *et al.* (1992b)

[a]Only in a proportion of cells.
[b]Only in a proportion of cases.

on their main functions: (1) fibrogenesis, (2) tissue skeleton or barrier, (3) intercellular communication system, (4) gentle contractile machinery, (5) endocrine activity, and (6) vitamin A storing. Among these functions, at least contractility and maintenance of tissue shape are directly related to cytoskeletal activities.

B. Adaptive and Pathological Modulations

The first description of a fibroblastic phenotypic modulation was made by means of electron microscopy in granulation tissue fibroblasts (Gabbiani *et al.*, 1971). In addition, it was observed that strips of granulation tissue from rats and humans, when treated *in vitro* with pharmacological agents, contract or relax in a manner similar to smooth muscle (Majno *et al.*, 1971; Gabbiani *et al.*, 1972; Ryan *et al.*, 1973, 1974). Serotonin, histamine, vasopressin, epinephrine, and norepinephrine stimulate contraction whereas papaverine, prostaglandin E_1 and prostaglandin E_2 induce relaxation in tissues stimulated to contract. These results suggest that the granulation tissue fibroblasts acquire features morphologically and functionally close to those of smooth muscle cells; such modified fibroblasts are called myofibroblasts. Cells very similar morphologically to the myofibroblast (containing stress fibers but not expressing normally α-smooth muscle actin) have been described in normal pulmonary parenchyma (Table I; Kapanci *et al.*, 1974, 1992). It has been suggested that these contractile cells play a role both in normal pulmonary structure and function and in alterations related to development of pulmonary fibrosis (Adler *et al.*, 1989; Leslie *et al.*, 1992).

Myofibroblasts disclose irregular cellular outlines with numerous and long cytoplasmic extensions, bundles of microfilaments (Fig. 5b), and are partly enveloped by a basal lamina similar to smooth muscle cells. They are connected by intermediate or adherens junctions and by gap junctions. Myofibroblasts develop cell-to-stroma attachment sites through the so-called fibronexus, which is a transmembrane complex of intracellular microfilaments in apparent continuity with extracellular fibronectin fibers (Singer, 1979; Singer *et al.*, 1984). Finally, the nucleus displays deep indentations (Fig. 5b).

The cytoskeletal characterization of stromal cells present in a variety of human and experimental soft tissue specimens known to contain myofibroblasts has revealed the presence of four main phenotypes: (1) coexpressing vimentin and cytoplasmic actin isoforms (V cells); (2) coexpressing vimentin, desmin, and cytoplasmic actin isoforms (VD cells); (3) coexpressing vimentin, cytoplasmic actin isoforms, and α-smooth muscle actin (VA cells); and (4) coexpressing vi-

mentin, desmin, cytoplasmic actin isoforms, and α-smooth muscle actin (VAD cells). Among the smooth muscle cell markers expressed by myofibroblastic populations *in vivo*, α-smooth muscle actin is the most common, followed by desmin whereas smooth muscle myosin heavy chains are practically always absent (Eddy *et al.*, 1988; Sappino *et al.*, 1990b; Leslie *et al.*, 1992).

Myofibroblasts have been described in three types of pathological processes (Table I): (1) situations related to tissue injury and inflammation leading to repair phenomena and tissue remodeling, (2) quasi-neoplastic proliferative conditions, and (3) the stromal response to neoplasia (Schürch *et al.*, 1992). The existence of myofibroblastic tumors as bona fide neoplasms remains controversial (Schürch *et al.*, 1992).

In wound contraction and connective tissue remodeling, the temporary or more permanent appearance of specialized contractile proteins in fibroblastic cells (Table I; Skalli *et al.*, 1989; Darby *et al.*, 1990; Kapanci *et al.*, 1990) has allowed the suggestion that these modified cells or myofibroblasts influence connective tissue remodeling at least in part through contractile forces (for review, see Skalli and Gabbiani, 1988).

In normal wound healing, when contraction stops and the wound is fully epithelialized, myofibroblasts containing α-smooth muscle actin disappear, most likely by apoptosis (Darby *et al.*, 1990; Desmoulière *et al.*, 1995); classically the scar becomes less cellular than granulation tissue and is composed of typical fibroblasts with well-developed rough endoplasmic reticulum and no microfilaments or α-smooth muscle actin. In pathological situations characterized by persistence or remodeling, such as hyperthrophic scars (Table I; Baur *et al.*, 1972; Skalli *et al.*, 1989), myofibroblasts express α-smooth muscle actin more permanently. Among quasi-neoplastic proliferative conditions, Dupuytren's disease has been studied extensively (Table I; Skalli *et al.*, 1989; Schürch *et al.*, 1990). Myofibroblasts are allegedly responsible for Dupuytren's contracture (Gabbiani and Majno, 1972). The role of myofibroblasts in the stroma reaction to epithelial tumors is not clearly elucidated (Sappino *et al.*, 1988; Schmitt-Gräff and Gabbiani, 1992). The stroma reaction has been considered either a defensive mechanism to isolate the tumor or to actively participate in the tumor progression by allowing angiogenesis and, in some instances, by facilitating the invasion and metastatization of tumor cells (Schürch *et al.*, 1992).

Little is known about the mechanisms leading to the development of fibroblastic cytoskeletal features similar to those of smooth muscle cells and of their persistence in some pathological conditions. As with

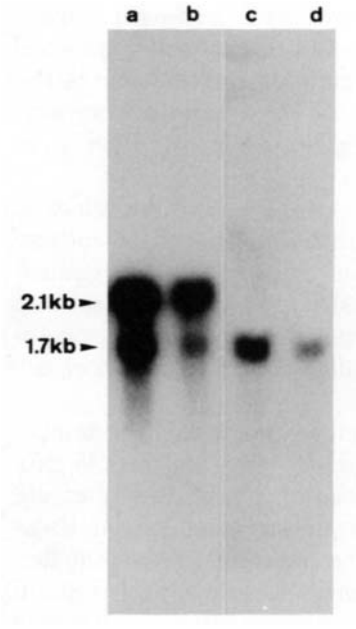

FIG. 6. Effect of IFNγ on actin isoform mRNA expression in rat subcutaneous cultured fibroblasts. Hybridization (see legend of Fig. 3) of Northern blots with the total actin probe (a, b) or with the oligonucleotide specific for α-smooth muscle actin mRNA (c, d) shows that both total actin (a, b) and α-smooth muscle actin (c, d) mRNA expressions are decreased in IFNγ-treated cells (1000 U/ml; b, d) for 72 hr compared with control cells (a, c). From Desmoulière *et al.* (1992a), with the kind permission of the publisher.

smooth muscle cells, cytokines and extracellular matrix components are good candidates for modulating fibroblast phenotype and cytoskeletal protein expression. *In vitro*, IFNγ decreases α-smooth muscle actin protein and mRNA expression (Fig. 6) as well as proliferation (Desmoulière *et al.*, 1992a). IFNγ also decreases collagen production *in vitro* and *in vivo* (Melin *et al.*, 1989; Grandstein *et al.*, 1990). The properties of this cytokine make it a good candidate to exert antifibrotic activity *in vivo*. Preliminary results (Pittet *et al.*, 1994) have shown that IFNγ injection decreases the size of hypertrophic scars; in Dupuytren's disease, after IFNγ treatment, nodules become smaller and the mobility is improved. In both cases, the expression of α-smooth muscle actin in myofibroblasts is decreased.

In cultured fibroblasts, heparin increases the expression of both α-smooth muscle actin protein and mRNA (Desmoulière *et al.*, 1992b). The analysis of [^{3}H]thymidine incorporation in synchronized cells suggests that heparin produces a selection of α-smooth muscle actin expressing cells. For *in vivo* studies, osmotic minipumps filled with saline solution, recombinant murine tumor necrosis factor-α (TNFα) without or with nonanticoagulant heparin, were implanted subcutaneously (Fig. 5; Desmoulière *et al.*, 1992b). After 14 days, TNFα produced a significant fibroblast accumulation but the fibroblastic cells

were positive for α-smooth muscle actin only in the presence of heparin. Heparin probably induces α-smooth muscle actin expression in a subpopulation of fibroblasts stimulated to proliferate by TNFα. Thus proliferation and α-smooth muscle actin synthesis by fibroblasts appear to be distinct phenomena during the formation and progression of granulation tissue.

We have shown that TGF-β1 is able to induce the expression of α-smooth muscle actin in granulation tissue myofibroblasts (Desmoulière *et al.*, 1993). Furthermore, the expression of α-smooth muscle actin by TGF-β1 is induced in growing and quiescent (Fig. 7) cultured fibroblastic populations. The expression of α-smooth muscle actin observed in fibroblasts cultured in the presence of fetal calf serum is partly inhibited by the addition of antibodies against TGF-β1. TGF-β1 represents one of the main regulators of α-smooth muscle actin expression both in smooth muscle cells (Björkerud, 1991) and in fibroblasts (Desmoulière *et al.*, 1993). After granulocyte macrophage–colony-stimulating factor local application, the appearance of α-smooth muscle actin-rich myofibroblasts (Rubbia-Brandt *et al.*, 1991) is preceded by an accumulation of macrophage clusters (Vyalov *et al.*, 1993) which could produce one or more α-smooth muscle actin expression inducing factors, among which TGF-β1 is a likely candidate. Khalil *et al.* (1989) have shown that, in pulmonary fibrosis induced by intratracheal instillation of bleomycin, an accumulation of

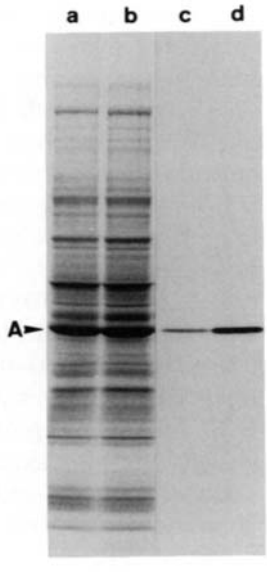

FIG. 7. Effect of TGF-β1 on α-smooth muscle actin expression in quiescent human subcutaneous cultured fibroblasts. Cells were cultured in the presence of 10% plasma-derived serum without (a, b) or with TGF-β1 (5 ng/ml; b, d). Densitometric scanning of Coomassie blue-stained gels (a, b) shows that the percentage of actin per total protein is not significantly modified by TGF-β1 treatment. Immunoblotting (c, d) shows that TGF-β1 increases the α-smooth muscle actin content of nongrowing human fibroblasts. A, actin.

α-smooth muscle actin expressing myofibroblasts is observed around clustered macrophages with a high expression of TGF-β. It is well known that TGF-β increases the accumulation and the deposition of extracellular matrix compounds leading to the development of fibrosis (Border and Ruoslahti, 1992; Sporn and Roberts, 1992). The action of TGF-β on α-smooth muscle actin expression confirms and extends the notion that TGF-β plays an important role in both fibroblast differentiation and fibrosis formation.

C. *Cultured Populations and Clones of Fibroblasts*

The presence of α-smooth muscle actin in primary and passaged cultures of fibroblastic populations has been reported by several laboratories (Vanderkerckhove and Weber, 1981; Leavitt *et al.*, 1985; Skalli *et al.*, 1986b); it has also been shown that the expression of this protein is decreased after transformation (Leavitt *et al.*, 1985; Okamoto-Inoue *et al.*, 1990). However, it has always been controversial whether these α-smooth muscle actin expressing cells derive from smooth muscle cells and/or pericytes present in the tissue from which cultures have been produced or whether they represent a true feature of fibroblastic cultures. We have observed that α-smooth muscle actin is always present in a variable proportion of cells in rat, mouse, and human fibroblastic cultures or clones (see later, Desmoulière *et al.*, 1992a). The expression of desmin is generally low; in several populations, no desmin-positive cells are found. The presence of smooth muscle myosin heavy chains containing cells has been evaluated in some fibroblastic populations (Desmoulière *et al.*, 1992a). Fibroblasts from rat fetuses or adult subcutaneous tissue and from normal human breast dermis or Dupuytren's nodules contain between 5 and 15% smooth muscle myosin heavy chain positive cells (Desmoulière *et al.*, 1992a). In human embryo lung fibroblasts and 3T3 cells, no smooth muscle myosin heavy chain expression is detected.

Using different markers (e.g., proliferative rate, extracellular matrix, or proteinase secretion), clonal heterogeneity has been reported in morphologically homogeneous fibroblastic populations (Brinckerhoff and Nagel, 1981; Korn and Downie, 1989; Goldring *et al.*, 1990; Rodemann and Müller, 1990). To evaluate if a subpopulation of bona fide fibroblasts has the potential to express α-smooth muscle actin, we have cloned and subcloned some of these fibroblastic populations. Even after cloning and subcloning, a certain percentage of cells were positive for α-smooth muscle actin (Table II). It is noteworthy that α-smooth muscle actin can be expressed by a proportion of cells in a population cultured from a single α-smooth muscle actin-positive or α-smooth muscle actin-negative cell. This observation suggests that

TABLE II

EVALUATION OF α-SMOOTH MUSCLE ACTIN-CONTAINING CELLS IN RAT AND HUMAN FIBROBLAST CLONES[a]

Source of clones	No. of clones containing various percentages of α-smooth muscle actin-positive cells				
	<5%[b]	5 to 15%	15 to 30%	30 to 50%	>50%
Rat fetus (31)[c]	0	6	12	13	0
Human embryo lung (ICIG-7) (98)	13	20	25	22	18
Rat dermis (14)	2	4	4	2	2
Mouse embryo (3T3) (33)	0	6	21	6	0
Dupuytren's nodule (22)	9	6	3	3	1
Human mammary gland (23)	5	8	6	2	2

[a]From Desmoulière *et al.* (1992a)
[b]The percentage was always higher than 1%.
[c]Numbers in parentheses represent the total number of clones.

α-smooth muscle actin expression is a feature of fibroblast populations and hence is not confined to smooth muscle cells (see Section IV for further discussions of this point). Moreover, when the expression of α-smooth muscle actin increases during subculture, the proliferative activity decreases. We believe that α-smooth muscle actin expression in cultured fibroblastic populations is a feature of fibroblastic cells, which may be related to functions exerted by fibroblasts under particular environmental conditions *in vivo*. This assumption is corroborated by the finding that α-smooth muscle actin is expressed by fibroblasts cultured from organs where *in situ* fibroblasts do not contain this protein. This has been described in lens cells (Schmitt-Gräff *et al.*, 1990) and in mammary gland stroma (Ronnov-Jessen *et al.*, 1990) and has been correlated with the observation that, under pathological conditions *in vivo*, both lens cells (Schmitt-Gräff *et al.*, 1990) and mammary gland fibroblasts (Sappino *et al.*, 1988) can express α-smooth muscle actin. However, presently, the genetic and environmental factors regulating α-smooth muscle actin expression in fibroblasts are poorly known. The microenvironmental factors described earlier (e.g., cytokines and proteoglycans) may be important in producing the selection of α-smooth muscle actin-positive fibroblasts in a given population.

Our results show that fibroblasts grown *in vitro* can be stimulated to divide and may also be stimulated to leave cell cycle and "differentiate" (i.e., express α-smooth muscle actin) as fibroblast do *in vivo* in the granulation tissue of a wound (Streuli *et al.*, 1993). Fibroblastic cells repeatedly pressed to divide attain a "differentiated" state in which

they are refractory to further mitotic stimulation and to subculturing under our culture conditions. We can assume, as suggested by previous works (Martin *et al.*, 1974; Bell *et al.*, 1978; Macieira-Coelho and Taboury, 1982), that loss of division potential *in vitro* represents differentiation instead of aging. Furthermore, cloning and subcloning experiments show that features of the cultured parental population are representative of a subpopulation present *in vivo* and furnish evidence for selection events in mass culture (Zavala *et al.*, 1978).

IV. Conclusions and Perspectives

It is presently more and more accepted that phenotypic features of smooth muscle cells and fibroblasts are not uniform (Gabbiani *et al.*, 1984; Campbell and Campbell, 1990; Komuro, 1990; Sappino *et al.*, 1990b; Desmoulière and Gabbiani, 1992). This plasticity or diversity is clear during development (Kocher *et al.*, 1985) and, in adults, takes place (1) in normal conditions, resulting in different cytoskeletal features of these cells according to their location; and (2) in pathological situations when smooth muscle cells or fibroblasts react to damaging or inflammatory agents. Here generally the response of each type of cell is stereotyped in that smooth muscle cells assume a synthetic phenotype (for review, see Campbell *et al.*, 1988; Desmoulière and Gabbiani, 1992) whereas fibroblasts become more contractile (for review, see Skalli and Gabbiani, 1988). Both cells tend to proliferate on pathological stimuli. The phenotypic modulation of both cell types corresponds to changes in the expression of cytoskeletal proteins, among which those of intermediate filament proteins (i.e., vimentin and desmin) as well as those of contractile proteins (i.e., actin and myosin heavy chain isoforms) have been, at least partially, characterized. In smooth muscle cells, the reaction to an endothelial lesion includes the decrease of α-smooth muscle actin and smooth muscle myosin heavy chain synthesis (as well as the increase of collagen synthesis), whereas in fibroblasts, tissue injury stimulates α-smooth muscle actin and, albeit exceptionally (see later), smooth muscle myosin heavy chain synthesis (Buoro *et al.*, 1993). Thus, a spectrum of cells may be present in the organism characterized by different phenotypes, going from the typical smooth muscle cell expressing high amounts of α-smooth muscle actin, smooth muscle myosin heavy chains, and desmin to the typical fibroblast expressing cytoplasmic actins, nonmuscle myosin heavy chain isoforms, and vimentin. Moreover, it is possible to observe a full differentiation of fibroblasts into bona fide smooth muscle cells (Buoro *et al.*, 1993; Chiavegato *et al.*, 1995).

Several laboratories have attempted to produce antibodies discrimi-

nating between fibroblasts and smooth muscle cells. The lack of success obtained up to now supports the assumption of a heterogeneity of fibroblast and smooth muscle cell phenotypes. By means of a tenascin cDNA probe and Northern blot analysis, Schwögler *et al.* (1992) have shown alternative splicing products of the tenascin gene which distinguish rat liver fat storing cells from arterial smooth muscle cells and skin fibroblasts. This type of approach could help to define specific differentiation markers for fibroblasts and smooth muscle cells. It should be underlined that normal smooth muscle cells in adult animals appear to be well-differentiated cells in which contractility is the main function. Their evolution on pathological stimuli is toward a less differentiated phenotype. On the other hand, bona fide quiescent fibroblasts are probably poorly differentiated resting cells with the potential of developing several functions such as synthesis of connective tissue components, contractility, and cytokine production.

It has been shown that mesenchymal cells (including fibroblasts and smooth muscle cells) express at least two nonmuscle myosin heavy chain isoforms (Borrione *et al.*, 1990) and it is conceivable that the modulation of their expression participates in the definition of different smooth muscle cell and fibroblast phenotypes, making the transition between the two cell types more complex and elaborated. Expression of smooth muscle myosin heavy chains is very important in some myofibroblasts, particularly in those participating in the stroma reaction to epithelial tumors (Chiavegato *et al.*, 1995). Allegedly phenotypic modifications involving changes in cytoskeletal protein expression correspond to subtle functional adaptations in response to microenvironmental changes. One of the major challenges for future studies will be the correlation between phenotypic and functional changes of smooth muscle cells and fibroblasts during developmental and pathological situations.

The expression of α-smooth muscle actin in myofibroblasts during the healing of an open wound in coincidence with granulation tissue contraction probably corresponds to the acquisition of contractile features by fibroblastic cells (Darby *et al.*, 1990). This assumption is corroborated by the observation that the expression of α-smooth muscle actin disappears in the scar and persists in more permanent pathological settings such as hypertrophic scars, fibromatoses, and/or fibrotic retractions (Skalli *et al.*, 1989; for review, see Sappino *et al.*, 1990b). However, the molecular basis of α-smooth muscle actin action has not yet been elucidated. Studies along this line will not only explain more clearly the function of α-smooth muscle actin, but will possibly allow to interfere with the biochemical processes leading to pathological changes such as scar contracture and organ deformation.

It is noteworthy that α-smooth muscle actin is expressed constitutively in a proportion of fibroblastic cells in culture (Desmoulière *et al.*, 1992a). This expression is modulated by cytokines such as IFNγ and TGF-β1 which reduce and increase it, respectively, and by extracellular matrix components such as heparin, which again increases it. α-smooth muscle actin is present in myoepithelial cells (Skalli *et al.*, 1986b) together with cytokeratin, where it allegedly exerts a contractile activity. Thus, contrary to what has been generally taught, it is possible that α-smooth muscle actin is a protein present in muscular and nonmuscular structures, in relation with contractile activities, and is not a protein typical of smooth muscle cells, which sometimes is expressed in other cell types. In this respect it is noteworthy that a protein expressing the same N-terminal sequence of α-smooth muscle actin has been localized in several lower vertebrates and invertebrates, including the planaria (Pascolini *et al.*, 1992); here it is expressed in regenerating epithelial and mesenchymal cells in coincidence with tissue remodeling, similar to that observed in higher vertebrates. It has also been shown that the expression of α-smooth muscle actin is regulated by repressive mechanisms, which are maximal in nonmuscle cells (Carroll *et al.*, 1988; Stoflet *et al.*, 1992). All these observations support the possibility that α-smooth muscle actin expression has appeared originally in relation to the contractile activities of nonmuscle cells and has then segregated in smooth muscle cells, particularly in vascular smooth muscle cells.

In conclusion, we have shown that the smooth muscle cells and the fibroblasts exhibit several phenotypic variants that are characterized by the expression of different cytoskeletal and contractile proteins. These variants correspond to functional adaptations to developmental, physiological, and pathological situations. It remains to be investigated (1) whether the two cell types can fully modulate one into another; (2) how these biochemical changes regulate functional activities; and (3) how these biochemical changes are influenced by genetic and microenvironmental factors. Research along these lines will undoubtedly help in the understanding of mechanisms regulating vascular development, smooth muscle cell, and fibroblast function and leading to pathological situations such as atherosclerosis and tissue fibrosis. Already at this point, however, it appears that cells which have been called fibroblasts or smooth muscle cells are biologically related and are in some aspects interchangeable. The specialization of these cells is clear in higher vertebrates under normal situations but becomes less clear in lower vertebrates or invertebrates as well as in higher vertebrates during pathological situations.

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Smooth Muscle Cell Proliferation in Hypertension: Possible Contribution to Arterial Remodeling

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I. Introduction

In established hypertension, peripheral vascular resistance is elevated. This is primarily due to structural changes in the arterial tree, most notably to an elevation of the wall thickness to lumen diameter ratio (w/l) in small arteries. This chapter considers whether hyperplasia of arterial smooth muscle cells contributes to the change in wall thickness. Although this chapter emphasizes structural changes in ex-

perimental animal models, we will point out that methods developed in these models resulted in several experimental approaches that can also be applied in humans. For instance, the techniques that were developed to study the structure and contractility of rat small arteries are equally workable in the small arteries that can be dissected from tissues resected during surgery in humans or from skin biopsies taken from human volunteers or patients (Aalkjaer and Mulvany, 1981; Aalkjaer *et al.*, 1987a,b,c; Nielsen *et al.*, 1991; Heagerty *et al.*, 1993; Korsgaard *et al.*, 1993). This now allows for rapid feedback between animal experiments and clinical research. Furthermore, because multiple biopsies can be taken from the same individuals (e.g., before and after drug treatment) (Heagerty *et al.*, 1988), analyses of arterial smooth muscle in human hypertension are no longer exclusively descriptive but may include preplanned testing of hypotheses as well.

II. Experimental Models of Hypertension

A wide range of animal models have been used to study vessel wall changes in the hope that some basic and common mechanism might provide new clues as to the ontogeny of human essential hypertension (Table I). Initially, models concentrated on the role of renal ischemia, resulting in various forms of renal artery clipping and aorta coarctation (Goldblatt *et al.*, 1934; Rytandt, 1938; Wilson and Byrom, 1939). Subsequently, alterations of renal and vascular functions became popular in the form of chronic administration of corticosteroids (in combination with high salt intake) or of vasoconstrictor agonists (Grollman *et al.*, 1940; Dickinson and Yu, 1967; Brown *et al.*, 1981; Griffin *et al.*, 1991). A next pivotal step was the selective inbreeding of animals with high blood pressure and the creation of genetically hypertensive rat strains (Okamoto and Aoki, 1963; Okamoto *et al.*, 1972; Yamori and Swales, 1994). This was accomplished at several centers but the spontaneously hypertensive rat strain (SHR) that was obtained at Kyoto University has been studied most extensively (Okamoto and Aoki, 1963; Okamoto *et al.*, 1972). Most of this chapter is based on these animals.

The time of onset of hypertension is controversial. In some, but not all, colonies, SHR are hypertensive at birth (Gray, 1984; Lee, 1985; Lee and Smeda, 1985; Eccleston-Joyner and Gray, 1988; Smeda *et al.*, 1988; Yang *et al.*, 1989). Although there are claims that structural changes precede hypertension (Eccleston-Joyner and Gray, 1988), the lack of a prehypertensive phase in some colonies has complicated any analysis of the cause and effect relationship.

A second genetic strain in widespread use is the SHR stroke-prone

TABLE I

SOME OF THE WIDELY USED EXPERIMENTAL ANIMAL MODELS OF HYPERTENSION

Introduction	Model	Control[a]	Ref.
1934	One-kidney, one-clip hypertension	Sham	Goldblatt *et al.* (1934)
1938	Aorta coarctation	Sham	Rytandt (1938)
1939	Two-kidney, one-clip hypertension	Sham	Wilson and Byrom (1939)
1940	DOCA salt	DOCA + low salt intake	Grollman *et al.* (1940)
1962	Dahl salt-sensitive (SS) hypertensive rats	SS + low salt intake SR + *high salt intake*	Dahl *et al.* (1962a,b)
1963	Okamoto–Kyoto spontaneously hypertensive rats (SHR)	WKY (?)	Okamoto and Aoki (1963); Okamoto *et al.* (1972); Lezin *et al.* (1992)
1967	Chronic infusion of low doses of angiotension II	e.g., coinfusion with hydralazine	Dickinson and Yu (1967); Griffin *et al.* (1991)
1974	Stroke-prone SHR	WKY (?)	Okamoto *et al.* (1974)
1990	Mouse ren-2 transgenic rats	SD(H)	Mullins *et al.* (1990)

[a]DOCA, deoxycorticosterone acetate; SS, Dahl salt-sensitive rats; SR, Dahl salt-resistant rats; WKY, Wistar–Kyoto rats; SD(H), Hamburg strain of normotensive Sprague–Dawley rats.

rat (SHRSP). Deliberate inbreeding of SHR resulted in a substrain characterized by an even higher level of hypertension and a high incidence of stroke (Okamoto *et al.*, 1974). In addition to hemodynamic and mechanistic analyses, survival studies can easily be performed in SHRSP. A third, and independent, selective inbreeding resulted in the development of a rat strain in which blood pressure is notably sensitive to salt intake [e.g., salt-sensitive Dahl hypertensive rats (Dahl *et al.*, 1962a,b)]. Finally, thanks to growing insights in pathogenic mechanisms and major advances in molecular biology, transgenic hypertensive animals are becoming available as well (Mullins and Ganten, 1990). So far, rats overexpressing the mouse ren-2 gene during some stage of their development (Mullins *et al.*, 1990) have been studied most extensively.

It is very important to consider appropriate normotensive controls for these genetic models. Other than the transgenic models, all of the genetic backgrounds are unclear. It has been established that various locally available WKY substrains are far more variable than SHR (e.g., Lezin *et al.*, 1992; Lindpaintner *et al.*, 1992). Thus, numerous papers associating some "genetic" trait with hypertension as the basis of SHR/WKY comparisons may be misleading. In many cases these traits may exist as secondary traits in any hypertensive animal. Other controls (see Table I) may involve sham-operated animals, members of related strains with secondary hypertension, litter mates maintained on a low salt diet, or litter mates that are continuously treated with antihypertensive drugs. In some cases, it was evaluated whether changes of interest cosegregated with blood pressure in F2 generations obtained through cross-breeding (e.g., Mulvany, 1988; Lindpaintner *et al.*, 1992). Studies of genetically mapped rat models should identify the gene loci associated with hypertension in the near future.

It may be relevant to subdivide the experimental animal models not only in terms of primary (genetic) or secondary, but also on the basis of the degree and of the rate of development of the hypertension. A large and rapid increase in blood pressure (severe hypertension) is more likely to be accompanied by end organ damage and vascular injury than a moderate elevation that evolves over several months (mild hypertension).

III. Elevated Resistance

Mean arterial pressure is determined by the cardiac output and by the resistance to flow in the system. Resistance in turn depends on the viscosity of the blood and on the architecture of the arterial tree.

According to the law of Poiseuille, resistance is affected more markedly by arterial diameter than by the length and number of the arterial segments. Based on the same law, arterial resistance resides primarily in small "resistance-sized" arteries (diameter <300 μm) and in arterioles (precapillary vessels with one layer of smooth muscle cells) (Folkow, 1982, 1986; Mulvany, 1983, 1984, 1986, 1987; Bohlen, 1986, 1989; Mulvany and Aalkjaer, 1990; Korner and Angus, 1992). This has been illustrated by recordings of intravascular pressure at several locations along the arterial tree in several organs. The pressure drop along small resistance arteries upstream from the microcirculation differs, however, between vascular beds. Acute changes in arterial resistance are primarily brought about by vasoconstriction and vasodilatation, resulting from contraction and relaxation of the arterial smooth muscle cells.

A hyperkinetic state of the heart has been observed during the development of some forms of hypertension. During established hypertension, however, cardiac output is normal but resistance is elevated (Folkow, 1982; Mulvany and Aalkjaer, 1990; Korner and Angus, 1992). This has been demonstrated by venous occlusion plethysmography, microsphere techniques, implanted flow probes, and perfusion studies in isolated organs. In both primary and secondary hypertension and in experimental animal models as well as in hypertensive patients, resistance is not only elevated under basal conditions. During maximal vasoconstriction (R_{max}), and during maximal vasodilatation (R_{min}), resistance is also increased compared to normotensive controls (Table II) (Folkow *et al.*, 1958, 1971; Folkow, 1982; Adams *et al.*, 1989; Korner and Angus, 1992). Elevation of R_{min} (ranging between 20 and 100%) indicates that arterial structural changes underlay the increase in resistance. The arterial structural changes that have been observed in hypertension include an elevation of the wall to lumen ratio and a reduction of the number of microvessels (rarefaction) (LeNoble *et al.*, 1990b; Boegehold *et al.*, 1991; Struijker Boudier, 1994). Both can lead to a reduction of the average overall lumen diameter of the arterial system. Because resistance is related to the fourth power of radius, the reduction of lumen diameter that would be required to explain the elevation of R_{min} is actually quite small (5 to 15%, see Table II).

Arterial structural changes are more likely causally related to the development of hypertension than any of the arterial functional changes that have been documented. This is illustrated in Table III in which it is evaluated to what extent arterial hypertrophy (↑ w/l), rarefaction, hyperexcitability of arterial smooth muscle, reduced endothelium-dependent relaxation, and hyperinnervation of blood ves-

TABLE II

DIFFERENCES IN SYSTOLIC BLOOD PRESSURE (SBP), HINDQUARTER VASCULAR RESISTANCE (R), AND OVERALL ARTERIAL LUMEN DIAMETER ($\varnothing_i$) IN EXPERIMENTAL ANIMAL MODELS OF HYPERTENSION COMPARED TO THEIR RESPECTIVE NORMOTENSIVE CONTROLS

Model conditions	Renal hypertensive rabbits			4-week-old SHR			50-week-old SHR		
	Min	Rest	Max	Min	Rest	Max	Min	Rest	Max
ΔSBP (mmHg)		+45			+4			+90	
ΔR (%)	+70	+88	+100	+21		+36	+27		+31
$\Delta\varnothing_i$ (%)	−13	−15	−16	− 5		− 8	− 6		− 6

Note: Mean differences are shown under resting conditions (rest) and during maximal vasodilatation (min) and maximal vasoconstriction (max). Adapted from Korner and Angus (1992) and Adams *et al.* (1989).

sels meet new Koch's postulates of pathogenesis (Korner and Swales, 1991). Changes in arterial structure and innervation meet these postulates more closely than specific functional changes:

1. There is a plausible mechanism by which they can elevate resistance.
2. Structural changes may be present before significant pressure elevation.
3. Structural changes seem to cosegregate with high blood pressure in cross-breeding experiments, although more work is needed in this area.
4. Correction of structural changes reduces blood pressure.

Unfortunately, these are observations that do not support a central, etiologic role for wall structure:

1. Reduction of blood pressure through treatment with β-blockers, vasodilators, or diuretics does not uniformly reverse arterial structural changes.
2. Deliberate induction of wall hypertrophy or rarefaction does not necessarily lead to hypertension (see later discussion). This is also the case for hyperinnervation (Head, 1991; Zettler *et al.*, 1991; Lee *et al.*, 1992a).

Neither arterial hypertrophy nor rarefaction necessarily lead to a reduction of lumen diameter and an elevation of vascular resistance. Vascular resistance depends on the relationship between wall mass and lumen diameter (Baumbach and Heistad, 1989; Mulvany, 1990, 1993; Heistad and Baumbach, 1992; Heagerty et.al., 1993). Furthermore, recording of resistances may be helpful to demonstrate the na-

TABLE III

POSSIBLE PRIMARY PATHOGENIC MECHANISMS IN RESISTANCE ARTERIES OF SPONTANEOUSLY HYPERTENSIVE RATS[a]

Postulate	↑ w/l	Rarefaction	↑ SN	↑ pCa	↑ Em, B_{max}	↓ EDRF
Plausible relationship	True	True	True	True	True	True
Present early	True	True	True	True	?	True
Cosegregation with HBP	True	?	True	False	?	?
Correction of defect corrects HBP	True	?	True	?	(True)	?
Correction of HBP does not correct defect	True	True	True	False	True	False
Induction of defect leads to HBP	(False)	(True)	(False)	True	(True)	True

Note. The extent to which possible pathogenic mechanisms meet the New Koch Postulates, postulates which should be satisfied if the mechanism is to be a cause of hypertension. w/l, wall thickness to lumen diameter ratio; SN, sympathetic nerve density; pCa, intracellular free calcium concentration, refers to abnormal cellular calcium handling; Em, B_{max}, membrane potential and receptor density, refers to hyperexcitability; EDRF, endothelium-derived relaxing factor, refers to endothelium-dependent relaxations in general; information in parentheses means that only limited information is available.

[a]Adapted from Korner and Swales (1991).

ture and extent of the required structural changes. To locate structural changes and to evaluate the contribution of changes in cell volume, cell size, or of remodeling, other approaches are described next. Information with respect to rarefaction can be found elsewhere (Struijker Boudier, 1994).

IV. Wall to Lumen Ratio

Measurements of wall:lumen ratios are complicated. The wall (or media) thickness and the size of the lumen depend on the experimental conditions (e.g., Fig. 1; Lew, 1991; Lew and Angus, 1992). Since water and therefore tissue are incompressible, wall volume is assumed to remain constant over a broad range of conditions. Therefore, and in view of the (visco)elastic properties of the arterial wall, changes in transmural pressure and in segment length will modify wall thickness and lumen diameter in opposite directions. Values for w/l should thus take into account the conditions under which measurements were obtained. For example, there is an ongoing debate as to whether blood vessels of hypertensives and normotensive controls should be compared under identical experimental conditions or under the conditions that operate *in vivo*.

While arterial wall mass and arterial lumen diameter drop markedly along the arterial tree as it branches, the w/l (at a distending pressure of 100 mmHg) remains rather constant throughout the system. Values between 0.05 and 0.08 have been reported for normotensive rat and human large and small arteries. An increase of up to 100% in w/l has been observed in established hypertension. Both a decrease in lumen radius and an increase in medial thickness have often been referred to as "hypertrophy". However, it may be important to discriminate changes in w/l from absolute wall hypertrophy (Mulvany *et al.*, 1978; Baumbach and Heistad, 1989; Heistad and Baumbach, 1992; Heagerty *et al.*, 1993 Mulvany, 1993) because the underlying mechanisms may differ and because relative and absolute hypertrophy may be involved to a different extent in different types of blood vessels.

In established hypertension, not all vascular beds display the same w/l increase. In SHR, for instance, the change is less marked in the kidney than in the mesenteric, cerebral, or hindquarter skeletal muscle vascular bed (Mulvany, 1983; Mulvany and Aalkjaer, 1990; Skov *et al.*, 1992). Furthermore, not all vessels are affected simultaneously during development in SHR. In young "prehypertensive" SHR, w/l has been found to be 50% elevated in small mesenteric and renal arteries, whereas structure was not (yet) modified in superior mesenteric artery

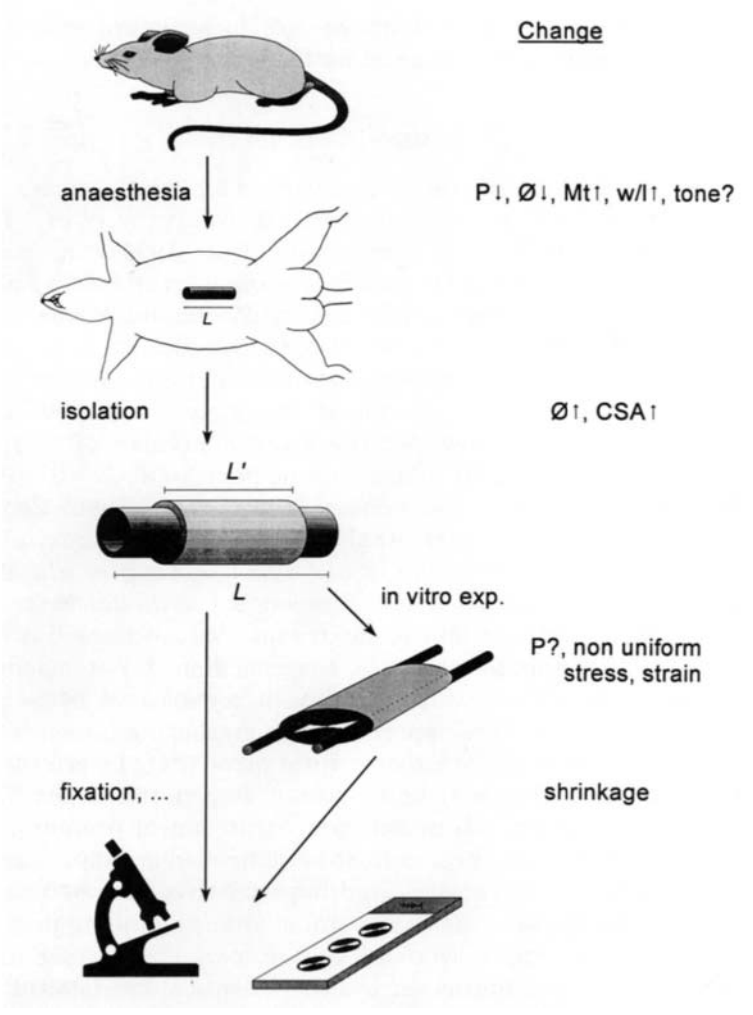

FIG. 1. Measurements and pitfalls of arterial dimensions. Wall and lumen characteristics can only rarely be determined in conscious animals or individuals. The measurements often require anesthesia, isolation, *in vitro* experimentation, fixation, and/or histology. Each of these steps are accompanied by changes that are not necessarily identical for normotensive and hypertensive subjects.

or thoracic aorta (Lee, 1985, 1987; Lee *et al.*, 1988; Smeda *et al.*, 1988). Gray, on the other hand, described a significant increase in the w/l of the carotid arteries of newborn SHR but also found a significant elevation of blood pressure in these animals (Gray, 1984; Eccleston-Joyner and Gray, 1988), Regional differences, differences between SHR sub-

strains, and technical aspects with respect to structural and blood pressure measurements may account for these discrepancies.

V. Lumen Diameter

A smaller lumen diameter has been reported for small arteries from different vascular beds of SHR and SHRSP (Mulvany *et al.*, 1978, 1985; Baumbach and Heistad, 1989; Bund *et al.*, 1991). Smaller lumen has also been described for mesenteric small arteries of renal hypertensive rats (Korsgaard and Mulvany, 1988) and mouse ren-2 transgenic rats (Thybo *et al.*, 1992). Also, in subcutaneous resistance arteries of untreated essentially hypertensive patients, a significant reduction of lumen diameter was noted (Heagerty *et al.*, 1988, 1993; Korsgaard *et al.*, 1993). However, there are a number of technical reservations about these critical measurements.

Ultrasound echo Doppler techniques (Safar *et al.*, 1990; Benetos *et al.*, 1993; Girerd *et al.*, 1994; Kool *et al.*, 1994) and intravital microscopy (Bohlen, 1986; LeNoble *et al.*, 1990a,b; Lash *et al.*, 1991) have been used to measure lumen diameters *in situ* for large and small arteries, respectively. Morphometry on cross sections has been used for vessels fixed under pressure, whereas diameter–tension and diameter–pressure relationships have been constructed in isolated unfixed vessels. Each of these approaches to measuring lumen is susceptible to several sources of experimental error (Fig. 1), relevant to the small differences that may be suspected. In general, lumen diameter is influenced by smooth muscle tone, transmural pressure, and segment length. Often all three cannot be controlled or maintained at the same level for normotensives and hypertensives. Diameter measurements *in vitro* on the other hand suffer from a sampling problem, hysteresis, and from length-related issues. Especially for small arteries and arterioles, it is uncertain whether identical anatomical segments can actually be isolated from normotensives and hypertensives in view of the complexity of the vascular network and of the possibility that the network is altered not only in terms of vessel diameter but also in terms of the number and length of the segments. Diameter–tension and diameter–pressure relationships are characterized by a high degree of hysteresis. They are thus poorly reproducible and are influenced by several specific aspects of the experimental protocol. Upon isolation, unfixed vessels invariably collapse radially and retract longitudinally (Fig. 1). Subsequent pressurization increases diameter and segment length. The actual diameter change is influenced by the lengthening that is allowed. In most experiments, length is maintained constant at a poorly defined preset level. During radial

stretching of isolated vessel segments as in organ chambers or myographs, axial length remains reasonably constant over a broad range of diameters. The axial length that is maintained corresponds to the length of the vessel in the absence of external mechanical forces. If vessels of hypertensives are hypertrophic (i.e., have a larger wall volume), this unstressed length will be a larger fraction of the *in situ* length than in the case of normotensives. Thus apparent differences in lumen diameter may be induced by a difference in longitudinal recoil resulting from a difference in wall volume.

VI. Wall Mass

Measurements of arterial wall mass are more robust than those of diameter or w/l. They can consist of a planimetric measurement of media cross-sectional area (CSA) on cross sections of fixed vessels and only rest on the assumption that density remains constant. Also, segment length is assumed to be constant or to change to the same extent in the experimental groups. The latter assumption is not necessarily valid. When estimates of wall (or media) CSA are derived from measurements of thickness and lumen diameter, as during intravital microscopy of small vessels and ultrasonic determination of large vessel wall properties *in vivo*, the estimates are obviously influenced by all possible experimental errors outlined earlier. The media CSA changes markedly along the arterial tree as it branches. This is due to changes in both the smooth muscle and the extracellular matrix compartment. For instance, in an elastic vessel, such as the thoracic aorta, more than 50% of the media is occupied by elastin and collagen whereas in small muscular arteries these components account for less than 20% of the media (Mulvany and Aalkjaer, 1990; Dobrin and Mrkvicka, 1992; Glagov *et al.*, 1992). In hypertension, increases in media CSA of up to twofold have been observed. This increase, however, is not a universal finding. In resistance arteries of genetically hypertensive rats and of essentially hypertensive patients, an elevated media cross-sectional area was observed in some of the reported studies while in others no statistically significant change could be obtained (Mulvany *et al.*, 1985; Bund *et al.*, 1991; Heagerty *et al.*, 1993; Korsgaard *et al.*, 1993).

Local arterial wall hypertrophy can result from (i) an increase in the size of the extracellular compartment, due to increased water lodging or increased deposition of extracellular matrix material, and (ii) from an increase in the smooth muscle mass, due to increases in the size or number of the arterial smooth muscle cells. Only the latter is substantiated by experimental findings. Whether wall hypertrophy

contributes to elevation of arterial resistance (especially R_{min}) depends on the side of the vessel where it occurs (Heistad and Baumbach, 1992; Korner and Angus, 1992; Korsgaard *et al.*, 1993). The addition of more mass at the abluminal adventitial side of the tunica media may increase resistance under basal conditions and during maximal vasoconstriction, but does not modify R_{min}. A selective hypertrophic response at the luminal side of the vessel will, however, increase resistance under all conditions. In cardiology, it is well established that different conditions lead to eccentric and concentric cardiac hypertrophy, respectively, and that both processes have different functional and pathological consequences (Katz, 1990).

VII. Microvascular Rarefaction

It must be pointed out that peripheral resistance can also change due to rarefaction. In the discussion of hypertrophy in hypertension, the microcirculation and especially the smaller arterioles play a special role. Several reports described that at this level in established hypertension there is most likely no increase in wall mass of individual vessels but that the number of vessels is reduced (rarefaction) (e.g., Bohlen, 1989; LeNoble *et al.*, 1990a,b; Struijker Boudier, 1994). This combination suggests that the total mass of arterial smooth muscle may actually be reduced in hypertension.

VIII. Arterial Smooth Muscle Cell Size

An increase in arterial smooth muscle cell size can contribute to arterial wall hypertrophy. Arterial smooth muscle cell hypertrophy in hypertension has been suggested by observations that in some types of artery, media CSA was elevated without detectable increases in arterial smooth muscle cell number. Measurements of cell number, however, are more complicated than simply counting nuclear profiles. A stereological approach, referred to as the three-dimensional (3D) disector, determines cell volume by light microscopy on two adjacent sections in which the nuclear profiles and the cell membranes or the extracellular matrix material are stained (Sterio, 1984; Mulvany *et al.*, 1985; Gundersen *et al.*, 1988a,b). Accurate measurements of cell length and mean cell width can be obtained by scanning electron microscopy (Miller *et al.*, 1986, 1987). Both techniques have largely been restricted to small arteries and arterioles. For large arteries, cell volumes have been determined following enzymatic dispersion and protein staining or loading of the cells with calcium-sensitive dyes (Owens and Schwartz, 1982; Papageorgiou and Morgan, 1991). Using

isolated cells, a frequency distribution can be obtained which may be relevant in view of increasing suggestions of smooth muscle diversity and possible heterogeneous distribution of hypertrophic responses throughout the vascular wall. Reported values for arterial smooth muscle cell volume in normotensive rats and normotensive humans are in the order of 1200 to 1600 μm^3.

Little is known about the basic mechanisms that control smooth muscle cell size and there is no definitive molecular marker that distinguishes cellular hypertrophy from cell replication (Table IV). The

TABLE IV

"GROWTH" RESPONSES IN THE VASCULAR WALL AND THEIR DETECTION

Principle	Technique
Hyperplastic responses	
Dedifferentiation	Northern blot, *in situ* hybridization, immunohistochemistry
Protooncogene expression	Northern blot, *in situ* hybridization
Ornithine decarboxylase	Northern blot, biochemical assays
Proliferating cell nuclear antigen	Immunohistochemistry
DNA synthesis	[^{3}H]Thymidine autoradiography, BrdUrd histochemistry
DNA content	Bisbenzimide or ethidium bromide straining followed by fluorescence microscopy, cell sorting, or fluorimetry
Mitosis	Mitotic figures, "pulse chase"
↑ Cell number	DNA content (provided polyploidy can be dealt with), histology (number of cell layers; number of nuclear profiles/media cross section), stereology (3D-disector)
Cellular hypertrophy	
General	Elevated wall mass without signs of hyperplasia; DNA/mass
Proten synthesis	Incorporation of labeled amino acids; RNA/DNA
Cell size	Scanning electron microscopy; stereology (3D disector)
Wall hypertrophy	
Mass of defined segment	
Cross-sectional area	Planimetry after staining of elastic lamina
Wall thickness	Wall thickness measurement *in situ* can be converted to wall mass provided lumen diameter, segment length, and density are known
Expansion of extracellular matrix	
Collagen	Planimetry after staining with Sirius red
Elastin	Planimetry after staining with Lauson's solution
Collagen synthesis	Incorporation of [^{14}C]-hydroxyproline

incorporation of radioactive amino acids and changes in the RNA/DNA have been assessed but these do not exclude preparation of the cells for proliferation. Moreover, cells may synthesize DNA and replicate their chromosomes without completing cytokinesis, i.e., DNA can replicate without changing the number of cells or even the number of nuclei. This conclusion is based on the observation of larger polyploid cells in large arteries of some models of hypertension (Owens *et al.*, 1981; Schwartz, 1984; Black *et al.*, 1989; Papageorgiou and Morgan, 1991) and on observations in cell culture that indicate that certain mitogens fail to stimulate the proliferation of isolated smooth muscle cells through increased expression of autocrine mediators that inhibit progression through the cell cycle (Warner *et al.*, 1987; Libby *et al.*, 1988; Owens *et al.*, 1988a; Loppnow and Libby, 1990; Itoh *et al.*, 1993). Therefore, some of the "early" markers that are routinely used to demonstrate hyperplastic responses in tissues may partly be indicative of cellular hypertrophic changes. In summary, any conclusion concerning arterial smooth muscle cell hypertrophy seems to require the exclusion of hyperplasia or a direct measurement of cell volume.

Reported significant increases in arterial smooth muscle cell volume range between 17%, as observed in mesenteric resistance arteries of renal, one-kidney, one-clip hypertensive rats (Korsgaard and Mulvany, 1988), and 100%, as observed in the thoracic aorta of rats with intrarenal artery coarctation of the abdominal aorta (Papageorgiou and Morgan, 1991). Cellular hypertrophy does not reach statistical significance in subcutaneous resistance arteries of patients with essential hypertension (16%; Heagerty *et al.*, 1993; Korsgaard *et al.*, 1993), mesenteric or femoral resistance arteries of spontaneously hypertensive rats (Mulvany *et al.*, 1985; Bund *et al.*, 1991), or of mouse ren-2 transgenic rats (Thybo *et al.*, 1992).

IX. Arterial Smooth Muscle Cell Proliferation

Cell culture of arterial smooth muscle cells derived from SHR and from various strains of normotensive rats suggests differences between normotensive and hypertensive strains with respect to arterial smooth muscle cell growth control (Grunwald *et al.*, 1987; Scott-Burden *et al.*, 1991; Mikhail *et al.*, 1993; Saltis and Bobik, 1992). The ability of vascular smooth muscle to replicate *in vivo* and *in vitro* is well established. Unlike cardiac and skeletal muscle myocytes, they proliferate in response to serum or selected growth factors during cell culture. Also *in vivo*, cells that apparently originate from the tunica

media have been observed to proliferate extensively following luminal injury (e.g., Schwartz *et al.*, 1986; Bondjers *et al.*, 1991). This raises the possibility that arterial smooth muscle cell proliferation either secondary to some systemic effect or even as a primary change could contribute to arterial wall changes in hypertension and dictate the approaches to address this (Table IV).

The relevance of elevated replication of cultured smooth muscle cells from hypertensive animals for hypertension, however, may be limited. Cells have been derived primarily from the aorta, which may not be representative for other parts of the arterial tree (Stadler *et al.*, 1989; De Mey *et al.*, 1991c). The technique may actually select a subpopulation of cells and is accompanied by a high degree of dedifferentiation of the cells. Moreover, finding a difference between cells cultured from animals after the establishment of hypertension may only represent an acquired change due to the hypertension. This is a difficult issue to control since, as already discussed, elevated blood pressure has been reported as early as birth in some strains of SHR (Eccleston-Joyner and Gray, 1988).

Whatever the significance of the studies in culture may be, the relationship between isolated cells and cell replication *in vivo* is unclear not only from the strict morphological point of view but also with respect to influences of cell density, extracellular matrix components, neurogenic, autocrine, and paracrine mechanisms on cellular proliferation. Several approaches have been used to evaluate cellular proliferation in the arterial wall *in vivo* and to a lesser extent in isolated intact blood vessels in organ culture. The latter organoid cultures (Mauger *et al.*, 1975; Koo and Gotlieb, 1989; Angelini *et al.*, 1991; Boonen *et al.*, 1991; De Mey *et al.*, 1991c) were introduced in the hope that some of the advantages of *in vitro* research, such as experimental control of the chemical and physical environment, could be combined with the maintenance of tissue organization and cellular phenotype. Evaluation of changes in cell number in freshly isolated or cultured arterial specimens calls on the same techniques and faces the same type of problems (Table IV).

Just as we have noted that there are no unique markers of cell hypertrophy, rates of cell replication also cannot be based on measurements of components that are purported to be essential for cellular progression through the cell cycle. This includes ornithine decarboxylase (Thompson *et al.*, 1992), various protooncogenes, growth factors, and growth factor receptors. In contrast, proliferating cell nuclear antigen such as cyclin (DNA polymerase δ; Bravo *et al.*, 1987) may be a good candidate in this respect (Gordon *et al.*, 1990; Zeymer *et al.*, 1992). More widely used is the evaluation of DNA synthesis by analy-

sis of the nuclear incorporation of [^{3}H]thymidine or of the thymidine analog 5-bromo-2′-deoxyuridine (BrdUrd) by autoradiography and immunohistochemistry, respectively. Both techniques have been applied *in vitro* and *in vivo* (e.g., Yang *et al.*, 1989; Boonen *et al.*, 1991; De Mey *et al.*, 1991a,c). The relationship of this golden standard to cellular proliferation in the arterial wall is not perfect. The possibility that, at least, some of the observations relate to DNA repair (Toschi and Bravo, 1988) is hard to exclude. That DNA synthesis may lead to polyploid nuclei rather than to cellular division is not only a theoretical possibility but has been demonstrated in the aorta of several models of hypertension (Owens *et al.*, 1981; Black *et al.*, 1989; Owens, 1989). Assessment of DNA synthesis thus should be combined with an evaluation of nuclear DNA content. Fuelgen microdensitometry (Owens *et al.*, 1988b) and fluorescent-activated cell sorting (Boonen *et al.*, 1991; Papageorgiou and Morgan, 1991) can be used for this purpose. As already noted, based on DNA content, the hypertensive wall has more DNA, but this is misleading, as most of this DNA is in polyploid cells. Polyploidy, however, is confined to the aorta and large arteries. Measurements of cell replication in small vessels, therefore, should represent true hyperplasia, unless cells are lost due to apoptosis.

Given the uncertain relationship between possible early markers and actual cellular proliferation in the vascular wall, a firm definition of hyperplasia rests on an actual count of cell number. In some cases, the number of smooth muscle cell layers were determined (e.g., Eccleston-Joyner and Gray, 1988; Smeda *et al.*, 1988; Lee *et al.*, 1992a,b). More accurate are the stereological 3D disector techniques. Unfortunately, these are not widely applied in the field because they are judged too labor intensive. The approach determines both smooth muscle cell volume and the numerical density of the smooth muscle cells. Conclusions with respect to cell number, however, rest on knowledge of the actual volume of the tissue. The cross-sectional area of the muscular coat (tunica media) of blood vessels can be accurately measured but the actual length (and thus volume) of a particular segment is a more volatile parameter.

X. Hyperplasia in Hypertension

In severe (malignant) hypertension, extensive smooth muscle cell proliferation has been observed in the thoracic aorta (Owens and Reidy, 1985; Contard *et al.*, 1993) and can also be detected in resistance arteries (Johnson *et al.*, 1992; Fig. 2). It most likely represents a secondary response to wall injury inflicted by the marked and rapid rise in blood pressure. In mild hypertension, hyperplasia is not a gen-

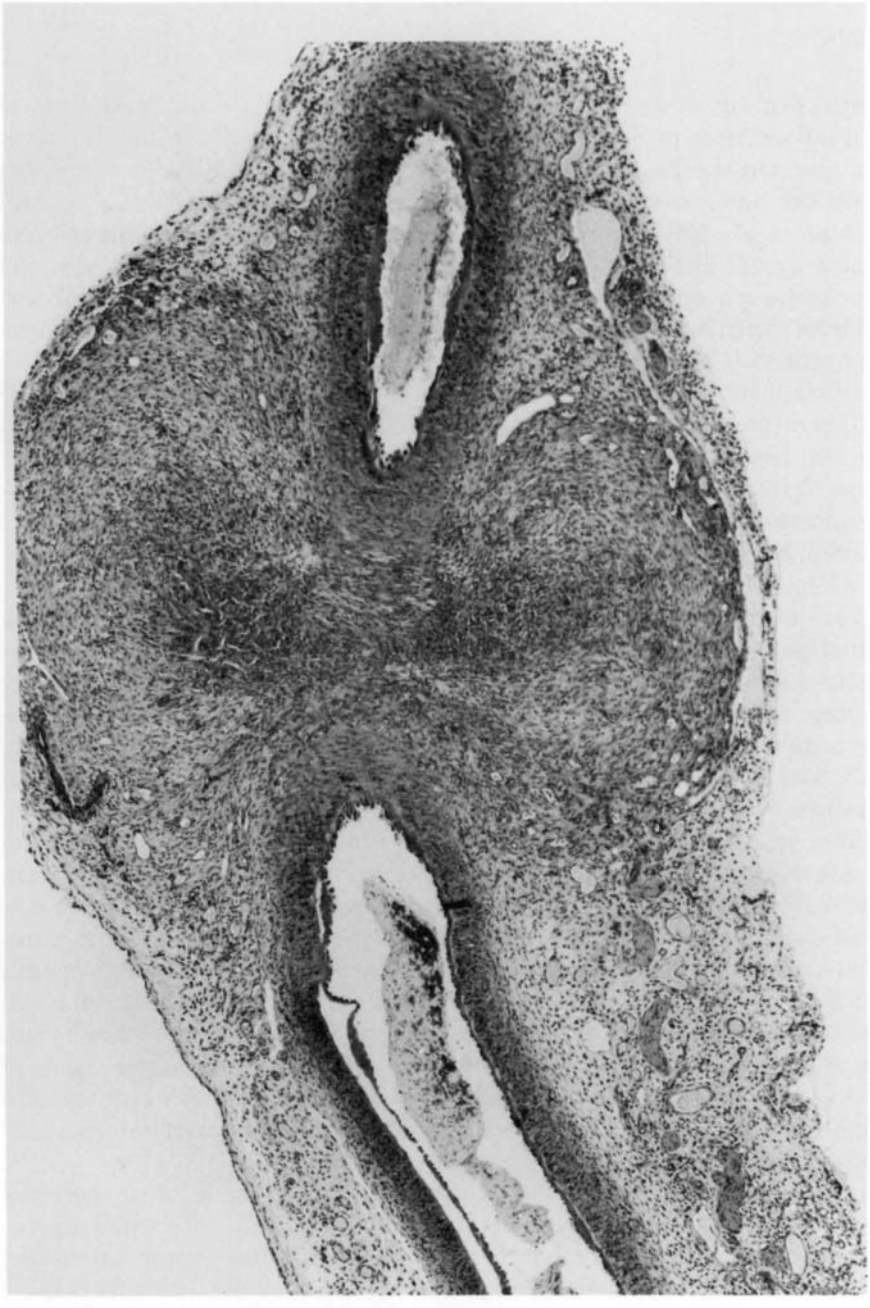

FIG. 2. Example of excessive smooth muscle cell proliferation in severe hypertension. The cross section (stained with hematoxylin) eosin was taken at a branching point between second and third order side branches of the mesenteric artery in a mouse ren-2 transgenic rat 10 weeks old having a systolic blood pressure of 236 mmHg. The cellular "infiltrate" was identified as smooth muscle on the basis of immunohistochemistry for SM α-actin (antibody SM1). Courtesy of G. Fazzi and H. Struijker Boudier (University of Limburg, Maastricht, The Netherlands).

eral finding. It has been observed in mesenteric, renal, and femoral small arteries of SHR (compared to WKY) with the 3D disector technique. On the basis of counts of nuclear profiles and of the number of smooth muscle cell layers in cross sections (Mulvany *et al.*, 1985; Bund *et al.*, 1991; Lee *et al.*, 1991), differences in cell number of up to 25% have been reported. In renal small arteries of SHR, hyperplasia is, however, not a consistent finding. Large elastic and muscular arteries of SHR display cellular hypertrophy/polyploidy rather than hyperplasia (Owens *et al.*, 1981; Owens and Schwartz, 1982; Lee *et al.*, 1983a,b; Owens, 1987). Furthermore, the same techniques failed to demonstrate significant hyperplasia in mesenteric small arteries of renal hypertensive rats (Korsgaard and Mulvany, 1988) or of mouse ren-2 transgenic rats (Thybo *et al.*, 1992) and in subcutaneous resistance arteries of essentially hypertensive patients (Heagerty *et al.*, 1993; Korsgaard *et al.*, 1993).

Mitogenic responses in the vasculature of hypertensive rats were also addressed by analysis of intraarterial DNA synthesis. In SHR, findings depended on the age of the animals. The pattern is as if elevated DNA synthesis (compared to WKY) is initiated at a very early stage in distal branches of the arterial tree and proceeds stream upwards during development of the animals and the hypertension (Fig. 3). At 1 week of age, DNA synthesis was elevated in mesenteric resistance arteries but not in proximal conduit arteries (Yang *et al.*, 1989). At 4 weeks of age, intraarterial DNA synthesis was significantly elevated in muscular conduit arteries but not in resistance arteries or in the aorta (Yang *et al.*, 1989). By 6 weeks of age, DNA synthesis was elevated in the thoracic aorta and in some of its major side branches but not in mesenteric or renal resistance arteries (De Mey *et al.*, 1991a). These signs of elevated mitogenicity, corresponding to a 100 to 200% increase in DNA synthesis, were obtained prior to more than a 10-mmHg pressure difference. Thereafter, significant elevation of DNA synthesis was restricted to the aorta where it was still statistically significant at 17 weeks of age but not at later points in time (Loeb and Bean, 1986).

Some data suggest that hyperplasia could precede, and therefore potentially cause, hypertension. In one-kidney, one-clip renal hypertensive rats, elevated intraarterial DNA synthesis may be a secondary response to high blood pressure (Rorive *et al.*, 1986). It is most pronounced during the blood pressure rise but is no longer significant when the hypertension is established. However, in two-kidney one-clip hypertension, elevated DNA synthesis in the aorta and in mesenteric small arteries precedes the development of hypertension and persists for some time in the established phase (Loeb and Bean, 1986;

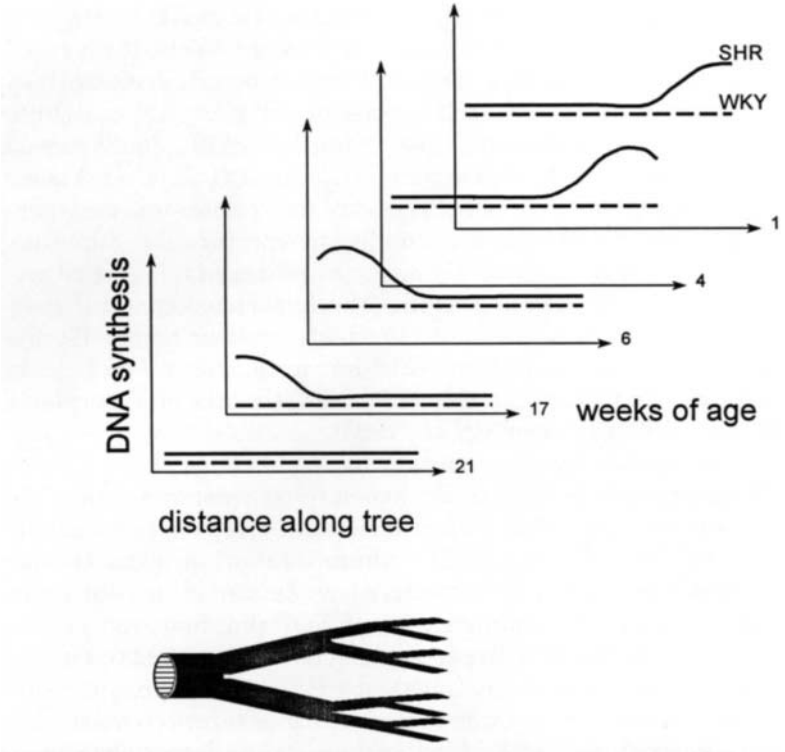

FIG. 3. Regional distribution and changes with time of intraarterial DNA synthesis (e.g., amount of thymidine incorporated/μg DNA × unit of time) in the arterial tree of normotensive (WKY) and spontaneously hypertensive rats (SHR) as suggested by previous findings (Loeb and Bean, 1986; Yang *et al.*, 1989; De Mey *et al.*, 1991a).

Lymn *et al.*, 1994). Interestingly, in two-kidney, one-clip hypertensive rats, increased incorporation of [^{3}H]thymidine was observed in mesenteric but not in subcutaneous resistance arteries (Lymn *et al.*, 1994). Furthermore, in this model the inhibitor of DNA synthesis, cytosine arabinoside, delays the development of hypertension (Loeb *et al.*, 1986). That in two-kidney, one-clip hypertension intraarterial DNA synthesis may be on the one hand an obligatory step and is on the other hand prevented by a broad variety of antihypertensive agents has been suggested to indicate that the DNA synthesis was triggered by a step involved in the excitation–contraction coupling in the smooth muscle (Loeb and Bean, 1986).

This relationship of elevated DNA synthesis to the development of hypertension may differ at different locations in the arterial tree and between models of hypertension. In SHR, the hyperplasia in resis-

tance arteries meets the new Koch's postulates (Table III) better than the late elevation of DNA synthesis seen in larger vessels. It may contribute to increased resistance, provided that it occurs preferentially at the luminal side of the vessel. It is present early, i.e., before significant elevation of blood pressure (Lee, 1985; Lee *et al.*, 1988; Smeda *et al.*, 1988; Yang *et al.*, 1989; De Mey *et al.*, 1991a). It is not known whether it cosegregates with blood pressure in F2 cross-breeding generations, but correction of the abnormality by chronic ACE inhibition treatment (Christensen *et al.*, 1988, 1989) or neonatal sympathectomy (Lee *et al.*, 1987, 1991) prevents the hypertension. Antihypertensive therapy does, however, not invariably prevent or reverse hyperplasia. β-blockers, diuretics, calcium antagonists, and most vasodilators do, for instance, not modify the estimates of hyperplasia (e.g., Lee *et al.*, 1992b; Tsoporis *et al.*, 1993).

The hypothesis that resistance artery hyperplasia may be the direct cause of hypertension in SHR is weakened by at least two sets of observations. In one study, induction of resistance artery hyperplasia by neonatal nerve growth factor (NGF) administration in normotensive rats was not accompanied by hypertension (Zettler *et al.*, 1991). In another study, neonatal administration of NGF did, however, not result in either hyperplasia or hypertension (Lee *et al.*, 1992a). On the other hand, during chronic treatment of SHR with minoxidil, resistance artery hyperplasia is exaggerated but pressure is persistently reduced (Tsoporis *et al.*, 1993). Furthermore, while hyperplasia may be absent in the resistance arteries of the kidneys of SHR (Skov *et al.*, 1992), transplantation of kidneys of SHR in normotensive rats leads to hypertension and transplantation of WKY kidneys in SHR lowers blood pressure (Guidi and Hollenberg, 1989).

In summary, there is only suggestive evidence that the elevation of vessel wall smooth muscle replication can precede, and therefore potentially cause, hypertension.

XI. Remodeling

As noted earlier, an absolute increase in arterial wall mass, be it as a result of cellular hypertrophy or hyperplasia, is not a uniform finding in hypertension and must be accompanied by a reduction of the luminal diameter to explain the elevation of R_{min}. Subcutaneous resistance arteries (Heagerty *et al.*, 1993; Korsgaard *et al.*, 1993) and mesenteric resistance arteries (Short and Thompson, 1959) of essentially hypertensive patients have been found to be narrower in the absence of a statistically significant increase of media CSA, smooth muscle cell volume, or number. This structural alteration is referred

to as "remodeling." The term remodeling was initially introduced by Baumbach and Heistad (1989) to explain the reduction of the external diameter of cerebral small arteries in SHRSP despite a significant increase in wall volume. It has been suggested to represent a rearrangement of a normal number of normal-sized cells in the wall, resulting in a narrower lumen. As such, remodeling does not require growth and may proceed independently but concomitantly with changes in cell size or cell number. In a review article, Heagerty *et al.* (1993) suggested that the remodeling of small arteries is an adaptive response to the increased blood pressure.

At present there is no plausible mechanism to explain the suggested rearrangement of cells in response to pressure elevation and there is little experimental evidence to support the proposed sequence of events (formation of a normal organization of a normal wall mass that is subsequently followed by rearrangement). In SHR, SHRSP, and mouse ren-2 transgenic rats which have been reported to display small artery remodeling, there is, for instance, no evidence that wall organization and cellular arrangement were normal at an earlier developmental stage. The problem has been approached by protecting the femoral vascular bed of SHR from pressure elevation. Bund *et al.* (1991) applied in young rats a loose ligation on an iliac artery that became pressure limiting at later developmental stages. In small arteries from the protected bed, media thickness, media CSA, and the w/l ratio remained comparable to those in vessels from normotensive WKY. Lumen diameter was not modified but did not differ either between vessels from the unprotected femoral vascular bed of SHR and WKY. We applied unilateral pressure-limiting stenoses on iliac arteries of adult SHR and WKY and observed in both strains a marked reduction of media CSA largely due to a reduction of the size of the arterial smooth muscle cells (De Mey *et al.*, 1993). In our experiments, marked pressure reduction in the adult resulted in a reduction of lumen diameter rather than in an increase.

Collectively, these findings, along with the cellular hypertrophic responses found in experimental (renal) hypertension and following chronic infusion of angiotensin II, suggest that pressure alterations in juvenile and adult animals modify arterial smooth muscle volume more readily than the arrangement or alignment of the cells in the wall. Partially constricting arterial ligations may, however, modify arterial structure through other mechanisms than reduction of mean pressure. Ligated vessels have a more dramatic reduction in pulse pressure than mean pressure. Moreover, ligation is accompanied by a significant reduction of the density of periarterial sympathetic nerves and of regional blood flow and shear stress. Especially the latter may

be a more powerful determinant of arterial diameter than pressure (Langille and O'Donnel, 1986; Langille *et al.*, 1990).

The absence of experimental support for rearrangement of arterial smooth muscle cells raises the interesting possibility that structural narrowing of vessels with a normal wall mass represents an inappropriate development of the arterial lumen rather than a secondary encroachment. This hypothesis is strengthened by the elevated R_{min}, corresponding to a 7% smaller lumen, in the hindquarter vascular bed of 4-week-old prehypertensive SHR (Adams *et al.*, 1989; Table II). Pressure elevation may then serve to maintain adequate organ perfusion. Although direct support for remodeling seems to be lacking, "reverse" remodeling is substantiated by findings following antihypertensive therapy. This results in an increase of the lumen diameter of human subcutaneous resistance arteries without alteration of media CSA (Heagerty *et al.*, 1988).

XII. A Biased View

From the preceding discussion, it may have become clear that arterial growth responses in hypertension (i) are rather limited, (ii) consist of hyperplasia when occurring in the neonate and of cellular hypertrophy and polyploidy in more mature animals, and (iii) must be concentric or accompanied by remodeling to result in all hemodynamic characteristics of hypertension. Remodeling seems, at least in SHR and SHRSP, to occur primarily in small vessels that undergo a hyperplastic response at an early developmental stage. Given the just-mentioned reservations concerning the techniques that are presently used, e.g., none of the current techniques can reproducibly detect a 10% difference in cell number, it is not surprising that interests shifted to remodeling at the expense of increased growth, despite the present absence of a candidate mechanism. These matters have been addressed in a comprehensive modeling study by Korner and Angus (1992). They calculated that a small increase in cell number at the luminal surface of small resistance-sized arteries suffices to result in the elevation of R_{min} that precedes the development of hypertension in young SHR (Table II). In their view, upstream structural changes could then develop as an adaptive response to the elevated pressure aimed at maintaining adequate tissue perfusion (e.g., Fig. 3). This suggestion is attractive because it comprises both primary and secondary arterial structural changes, because it links growth to luminal narrowing, and because it takes into account the regional and temporal heterogeneity noted with respect to the distribution of cellular hypertrophy and hyperplasia. It is furthermore attractive because it

contains hidden suggestions regarding the mechanism that may be involved (Fig. 4). The endothelium, the renin–angiotensin system, and the sympathetic nervous system are candidate mechanisms by which centripetal arterial growth may be achieved.

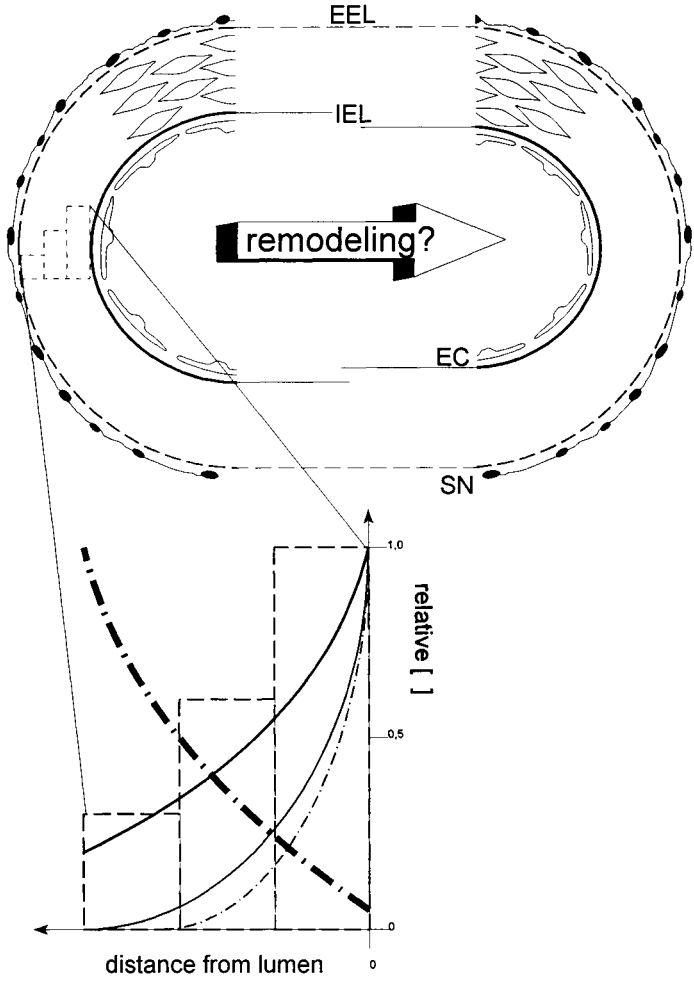

FIG. 4. Putative mechanisms of concentric centripetal smooth muscle cell proliferation in arteries and subsequent encroachment of the arterial wall on the arterial lumen. The model is based on concentration gradients within the wall for angiotensin II (-), plasma-derived epinephrine (-), endothelium-derived mitogens (-••-), sympathetic nerve-derived growth inhibitors (-•••), and, for example, adrenoceptors (□), but is not limitative in this respect. EEL, external elastic lamina; IEL, internal elastic lamina; EC, endothelial cells; SN, sympathetic nerves. Based on suggestions by Korner and Angus (1992).

XIII. Role of the Endothelium

The endothelium has at least the capacity to release a broad variety of mitogens and growth inhibitors (for review see, e.g., Schwartz and Liaw, 1993). It has been noted by us and others to control the mitogenic responses of the isolated arterial wall to serum and recombinant growth factors (Koo and Gotlieb, 1989; Angelini *et al.*, 1991; De Mey *et al.*, 1991b,c; Schiffers *et al.*, 1994a,b). In view of its capacity to sense shear stress (Davies and Tripathi, 1993), it has also been implicated in the control of the structural diameter of arteries of growing animals (Langille and O'Donnel, 1986; Langille *et al.*, 1989, 1990). Any dysbalance between endothelial production of growth promoters and inhibitors and between endothelial modulation of arterial wall mass and diameter is an attractive candidate for centripetal smooth muscle cell proliferation and hemodynamically relevant arterial narrowing (or inappropriate developmental widening).

XIV. Role of the Renin–Angiotensin System

A large body of evidence supports a role for angiotensin II in structural arterial changes in hypertension. This is not restricted to two-kidney, one-clip renal hypertension which is characterized by elevated plasma renin activity but applies also to models such as SHR in which plasma renin is normal or even reduced. This can be due to a specific role for the intravascular renin–angiotensin system and to exaggerated responses to normal levels of angiotensin II.

Angiotensin II is available at sites of smooth muscle growth, especially smooth muscle cells in the inner part of the wall. Angiotensin I is converted to angiotensin II by an angiotensin-converting enzyme which is far more abundant in the endothelium than in other vascular cell types (Campbell, 1985; Kifor and Dzau, 1987; Dzau, 1988; Tang *et al.*, 1990). It is likely that there is a concentration gradient of angiotensin II in the arterial wall from lumen to adventitia. The trophic consequences of this may be reinforced by stimulatory effects of angiotensin II on endothelial production and release of various potential paracrine growth mediators (Schelling *et al.*, 1991, Scott-Burden *et al.*, 1992; Hahn *et al.*, 1993a,b; Schwartz and Liaw, 1993).

Angiotensin II has been shown to cause hypertrophy and proliferation of isolated aortic smooth muscle cells of WKY and SHR, respectively (Bunkenburg *et al.*, 1992; Sachinidis *et al.*, 1992), and proliferation of passaged renal arteriolar smooth muscle and mesangial cells (Anderson *et al.*, 1993). The peptide also induces wall hypertrophy in isolated arterial segments (Schiffers *et al.*, 1993). Chronic infusion of

low, initially subpressor doses of angiotensin II cause a slow rise in blood pressure that is associated with an increase of the w/l ratio in small arteries, resulting from an increase in cell size [cellular hypertrophy (Brown *et al.*, 1981; Lever, 1986; Griffin *et al.*, 1991)]. Small artery hypertrophy was also seen in animals treated with both angiotensin II and hydralazine in order to prevent the rise in blood pressure (Griffin *et al.*, 1991). The wall hypertrophy seen in small arteries of rats subjected to several days of angiotensin II was not observed in larger arteries, such as the aorta, and was not found after comparable pressure elevation by intravenous infusion of the α_1-adrenergic agonist phenylephrine (Boonen *et al.*, 1993). Furthermore, chronic treatment of SHR with inhibitors of angiotensin-converting enzyme (Freslon and Guidicelli, 1985; Christensen *et al.*, 1988, 1989; King *et al.*, 1992) or with antagonists of AT-1 receptors (Oddie *et al.*, 1993; Soltis, 1993) prevents and reverses arterial structural changes and hypertension. This prevention and reversal of arterial structural changes include elevated intraarterial DNA synthesis (Loeb and Bean, 1986), large artery polyploidy (Black *et al.*, 1989), and the increase of w/l in small arteries. Effects on small artery hyperplasia were more variable. Structural effects of ACE inhibitors are more marked than those of other types of antihypertensive agents. Furthermore, the antihypertensive effect of ACE inhibitors in SHR persists for a reasonably long period of time when the treatment is withdrawn (Thybo *et al.*, 1994; King *et al.*, 1992). These observations have led to the suggestion that angiotensin II induces pressure-independent trophic actions that subsequently help to raise pressure and that inhibition of the renin–angiotensin system chronically reduces elevated blood pressure by prevention and reversal of arterial structural changes. It has been highlighted, however, that the structural effects of ACE inhibitors may be a secondary consequence of the pressure reduction. The lowering of pressure is indeed larger than with other antihypertensive regimens during which pressure-related antihypertrophic effects may be offset by direct mitogenic actions of some of the agents or by baroreflex-mediated activation of the sympathetic nerves and of the renin–angiotensin system.

XV. Role of the Sympathetic Nervous System

Experimental evidence also suggests a role for catecholamines in the development of both structural changes and hypertension in genetic hypertension. Because sympathetic nerves are located at the media adventitia border, they may seem unlikely candidates to promote centripetal vascular growth. First, if catecholamines, or another

sympathetic (co)transmitter, act in conjunction with angiotensin II (e.g., van Kleef *et al.*, 1992), the just-mentioned mechanism may apply. Second, the antitrophic actions of sympathectomy are more marked when combined with adrenal medullectomy or chronic α_1 blockade (Lee *et al.*, 1991; Korner *et al.*, 1993b). This suggests a role for circulating catecholamines. For these, a concentration gradient is likely to exist in the vascular wall because of their supply from the blood stream and their rapid uptake by the sympathetic nerve endings (Fig. 4). Third, a series of experiments performed by Osswald and colleagues suggests that periarterial nerves in the adult actually exert an antitrophic influence on vascular smooth muscle and that this is due to adenosine derived from neurogenically released ATP (Branco *et al.*, 1984; Albino-Teixeria *et al.*, 1990). In view of this there may be a gradient for an antitrophic agent from adventitia to lumen (Fig. 4). Fourth, the arterial media contains several types of postjunctional adrenoceptors. These may be unevenly distributed throughout the wall. In vessels in which postjunctional α_2 and β-adrenoceptors are present and coupled to contractility, the former are situated within the proximity of sympathetic nerves (e.g., rat and human subcutaneous small arteries) (Nielsen *et al.*, 1990) and the latter beyond the reach of released norepinephrine (e.g., rat mesenteric small arteries) (Eerdmans *et al.*, 1991). Thus, the distinct location of nerves in the vascular wall may contribute to an uneven distribution of adrenoceptor (sub)types. When a noninnervated (sub)type would be primarily involved in trophic effects this may participate in concentric centripetal arterial growth. This remains to be firmly demonstrated.

In SHR, the density of periarterial sympathetic nerves is higher than in normotensive rats (Head, 1989, 1991). Some findings also indicate that the sympathetic nervous system develops more rapidly in SHR than in WKY (Gray, 1984; Gray and De Mey, 1985). These differences seem to be due to stronger expression and synthesis of NGF in arterial smooth muscle of young SHR. The hyperinnervation of SHR blood vessels meets the new Koch criteria of pathogenesis rather well (Table III). Catecholamines have been shown to exert trophic actions in isolated arterial smooth muscle cells. Sympathetic nerves have been shown to stimulate arterial wall growth in young rabbits (Bevan, 1975; Bevan and Tsuru, 1979, 1981). Sympathectomy, involving anti-NGF antibodies and guanethidine, partly prevents the development of both resistance artery structural changes and of hypertension in SHR (Lee *et al.*, 1987, 1991; Mangiarua and Lee, 1992; Korner *et al.*, 1993a,b). Early hyperplastic arterial changes are seen almost exclusively in densely innervated small arteries. Late hypertrophic changes, which may be a secondary response to elevated pressure

anyway, develop in vessels that are poorly innervated or not innervated at all, such as the rat aorta.

Some findings, however, cast doubt on the role of the sympathetic nerves. In established hypertension, chronic administration of the α_1 antagonist prazosin (Jonsson *et al.*, 1992) has only minimal effect on blood pressure. It significantly reduces aortic wall mass but does not modify resistance artery structure. Unlike angiotensin II, intravenous infusion of the α_1 agonist phenylephrine fails to increase the wall mass in small resistance arteries despite inducing a 15% increase in aortic wall mass (Boonen *et al.*, 1993). These observations suggest that potential structural effects of sympathetic nerves on the vascular wall may not be mediated by α_1-adrenoceptors on the smooth muscle cells. Furthermore, an elevated number of arterial smooth muscle cell layers has been reported for the carotid artery of newborn SHR (Eccleston-Joyner and Gray, 1988), i.e., prior to significant innervation of the arterial system and in a vessel that in the adult is devoid of any sympathetic nervous supply. In addition, neonatal treatment of normotensive rats with NGF does not lead to hypertension (Zettler *et al.*, 1991; Lee *et al.*, 1992a). Collectively, findings with respect to the sympathetic nervous system suggest that it is most likely not the sole determinant of arterial structural changes. A role in conjunction with the renin–angiotensin system is attractive in view of the extensive interactions between both systems in the central nervous system, at the level of the sympathetic nerve endings, and at the level of the smooth muscle cells or of the endothelial cells.

XVI. Neointimal Proliferation

Arterial smooth muscle cell proliferation in hypertension is, when present, far less marked than neointimal cell proliferation seen following balloon catheter injury or during ductus arteriosus closure. Both models allow, however, for evaluation of some of the suggestions that were formulated earlier. The structural response to mechanical injury can be reduced by various types of ACE inhibitors, by AT-1 receptor antagonists, and by α_1-adrenoceptor antagonists (Powell *et al.*, 1989; Jackson and Schwartz, 1992; Timmermans *et al.*, 1993). This also indicates that in this model the renin–angiotensin system and adrenergic mechanisms interact. This is further suggested by the observation that the α_1 antagonists prazosin and doxazosin can reduce the mitogenic effect in intact arteries observed during intravenous infusion of angiotensin II (van Kleef *et al.*, 1992).

In the ductus arteriosus, neointimal cell proliferation during the perinatal period ultimately leads to closure of the vessel (de Reeder

et al., 1989; Slomp *et al.*, 1992). The notion elaborated earlier that concentric centripetal cell proliferation can reduce lumen diameter may be strengthened by this observation. However, extensive neointimal cell proliferation following luminal mechanical injury of the vascular wall does not invariably lead to encroachment of the wall upon the lumen (Glagov *et al.*, 1992; Post *et al.*, 1994). This is especially the case after development of a pseudoendothelium on top of the neointima or after coverage of the neointima by regenerating endothelium. It has been suggested that this failure may be due to remodeling of the underlying media (widening in this case) in an attempt to maintain adequate perfusion of the supplied organ.

XVII. Other Cardiovascular Diseases

Small artery remodeling due to pressure-independent centripetal trophic actions of the endothelium, angiotensin II, and catecholamines may not be restricted to hypertension. In heart failure, for instance, circulating levels of several potential mitogens, including endothelin, angiotensins, and catecholamines, are elevated (Riegger *et al.*, 1988; Margulies *et al.*, 1990; Svanegaard *et al.*, 1993; Noll *et al.*, 1994). In this condition, endothelial inhibitory influences, as judged from endothelium-dependent relaxations, are reduced (Drexler and Lu, 1992; Kiuchi *et al.*, 1993; Noll *et al.*, 1994; Teerlink *et al.*, 1994). Furthermore, peripheral vascular resistance is elevated in heart failure (Drexler and Lu, 1992; Parker, 1992). Activation of neurohumoral mechanisms may initially result in vasoconstriction that maintains adequate perfusion of vital organs. Over time this may be fixed in an alteration of arterial structure.

XVIII. Conclusion

Structural changes in the vessel wall that, in theory, could be causal in hypertension include rarefaction of microvessels, remodeling of vessel walls in the resistance arteries to reduce the lumen size without altering wall mass, or absolute changes in wall mass itself. All of these changes have been observed in different models of hypertension, but evidence that such changes can cause hypertension remains inconclusive. Such structural changes could result in the necessary hemodynamic changes provided that they occur at the right time and at the right place, i.e., before significant pressure elevation and at the luminal side of resistance-sized arteries. Moreover, with the possible exception of smooth muscle hyperplasia, the molecular mediators and even the processes involved in structural changes in resis-

tance vessels remain poorly defined. The endothelium, the renin–angiotensin system, and the sympathoadrenal axis, however, are good candidates to be involved in a process that could be central to hypertension.

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Epilogue

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This book represents a snapshot of a field that is likely to undergo rapid change in the next few years. Those changes will be driven by the smooth muscle cell itself; that is, by its unique biology. Others will be driven by the burgeoning accumulation of new tools that we collectively term molecular biology.

Perhaps most interesting to consider are those changes that will be driven by the smooth muscle cell itself. An area entirely neglected by this book is the mechanism of smooth muscle contractility. Our attention, however, to smooth muscle developmental biology will inevitably lead to new insights integrating the kinds of proteins being made by the smooth muscle cell and the control of the synthesis of those proteins with mechanisms of smooth muscle contraction. Moreover, as pointed out in a review by the Somlyos, our understanding of smooth muscle contractility will depend not only on understanding the contractile apparatus, but on the signaling mechanisms as well.

Another area of smooth muscle biology likely to show growth in the next few years is our understanding of smooth muscle diversity. Chapters in this book have stressed smooth muscle diversity within individual vessels. Thus, we have learned about differences between intima and media or even differences between smooth muscle cells located in the media itself. But smooth muscle appears in many different places in the body. It appears in different vessels and, of course, in different organ systems. Indeed, a striking issue growing out of the chapter by Gittenberger-de Groot and colleagues is the fact that smooth muscle cells invest endothelial cells wherever the endothelial cells form structures. Much the same thing, however, could be said about smooth muscle investment of endodermal epithelium. At virtually any site in the body where the endoderm forms tubes, those tubes become surrounded by smooth muscle. This subject has been studied to some extent by Cunha. He found that there is a mutual cross-talk between smooth muscle cells in the connective tissue of an organ and the differentiated state of the overlying epithelium. Indeed, by putting epithe-

lial cells on different smooth muscle-derived substrates, one can cause the epithelial cells to differentiate into different tissue types!

Aside from the fascinating issues of how smooth muscle cells are recruited, very little is known about the differences between smooth muscle cells associated with endoderm and smooth muscle cells associated with endothelium. There have been many reports of physiologic differences between these cells, but it is likely that there will be differences at the level of gene expression as well. Current molecular biologic methods allow us to look basically at one gene at a time, although techniques like differential hybridization, subtractive hybridization, or differential display promise that we can look at larger numbers of genes in the future. The ability to compare patterns of gene expression and indeed to identify new genes will change dramatically, however, as the ability to rapidly sequence domains of expressed proteins becomes more generally available.

Finally, and most difficult to consider, is the possibility that there will be significant advances in our understanding of the pathology of smooth muscle cells. The problems with this area have been well illustrated by the chapters in this book devoted to smooth muscle pathology and by the overview by Ross. The truth is that very little is known about smooth muscle proliferation in hypertension, atherosclerosis, or even restenosis. A great deal of clinical effort is being devoted now to inhibiting proliferation using sophisticated tools such as transfection with tumor suppressor genes or expression of cytocidal forms of thymidine kinase. Such approaches may tell us something about the importance of proliferation, if only because a successful clinical trial would imply that proliferation had, in fact, been important.

Perhaps most promising, if only because so little is currently known, however, is the possibility that new genetic approaches will provide insights into smooth muscle biology. The most likely direction for advances in genetics relevant to smooth muscle will come from the rapidly moving front of hypertension genetics. We know that the heritability of hypertension is controlled by a fairly small number of genes. It is likely that many of these genes will be identified as soon as the map of the rat genome is published. This map, combined with studies of inbred strains derived from spontaneously and other genetically hypertensive rats, will lead to the identification of the genes responsible for hypertension, and one might imagine that at least some of these will turn out to be genes whose function is located in the smooth muscle cell itself.

This discussion of smooth muscle genes brings us full circle with the introduction. At the beginning we suggested that the most interesting advances in smooth muscle biology might well come from the interface between smooth muscle-specific functions and the advancing tools of molecular biology. The next few years should be very interesting.

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