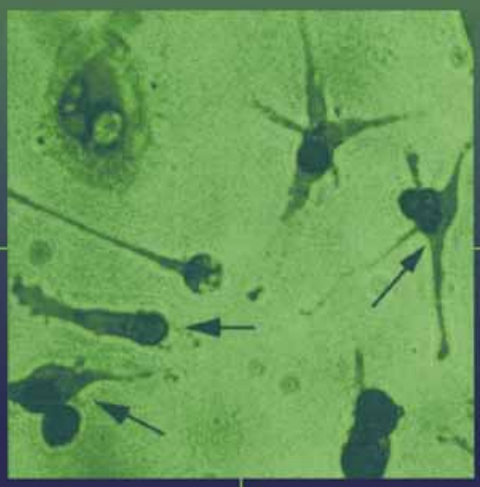

H. Mori • H. Matsuda (Eds.)

Cardiovascular Regeneration Therapies Using Tissue Engineering Approaches

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ЗНАНИЕ

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With 137 Figures

Springer

Hidezo Mori, M.D., Ph.D.
Director, Department of Cardiac Physiology
National Cardiovascular Center Research Institute
5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

Hikaru Matsuda, M.D., Ph.D.
Professor, Division of Cardiovascular Surgery, Department of Surgery
Osaka University Graduate School of Medicine
2-2-E1 Yamadaoka, Suita, Osaka 565-0871, Japan

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Preface

The cardiovascular system transports oxygen and nutrients to all parts of the body; therefore, any impediment to this system through, for example, a circulatory disorder, represents a serious threat to organs, tissues, and cells. Obstructive diseases of vessels with a diameter of more than 1 mm can be treated by conventional surgical and interventional approaches; however, blockages in small vessels with a diameter of less than 1 mm cannot be treated by conventional methods. As a consequence, therapeutic angiogenesis and vasculogenesis for the treatment of ischemic diseases have been widely studied in the last decade. These methods may contribute to the repair of intractable cardiovascular diseases with a main vascular involvement in the body's smallest vessels.

In this book, Hikaru Matsuda and I have tried to summarize recent Japanese developments in the field of cardiovascular regeneration therapies using tissue engineering. The Ministry of Health, Labor, and Welfare of Japan has been encouraging the National Cardiovascular Center Research Institute to promote cardiovascular regeneration therapies using such approaches. Therefore, it is with the financial aid and support of research grants, such as that for Cardiovascular Disease (13C-1 and 16C-6), Health and Labor Sciences Research Grants (RHGTEFB-genome-005, RHGTEFB-saisei-003, and CRCD-junkanki-009) and a grant from NEDO of Japan, that significant progress has been possible.

With an eye on the fundamental tools of angiogenic cytokines, cardiovascular stem cells, and tissue engineering tools, we have arranged the contents of this book as follows. In Chapter 1, bone marrow-derived vascular precursor cells and the ways in which they are potentiated are highlighted. Takayuki Asahara, who found endothelial progenitor cells in circulating blood, and several other leading medical scientists, describe the experimental benefits of bone-marrow cells for treating various ischemic diseases. In Chapter 2, Teruo Okano and Tatsuya Shimizu describe myocardial sheets, while their colleagues look at their applications. Also, Jun Yamashita discusses the possible applications of embryonic stem cells. In Chapter 3, hybrid vessels and heart valves are analyzed by Akio Kishida and colleagues using tissue engineering technologies. In Chapter 4, Ryozo Nagai, Masataka Sata, and colleagues consider new aspects of angiogenic growth factors. Finally, Chapter 5 features the clinical results of therapeutic angiogenesis and vasculogenesis, as described by Tsutomu Imaizumi and colleagues.

It will be a great honor if this book can contribute to the development of therapeutic angiogenesis and vasculogenesis for the treatment of ischemic diseases, and promotes young doctors' understanding of current academic work in this field.

November 15, 2004

Hidezo Mori, M.D., Ph.D.
Director, Department of Cardiac Physiology
National Cardiovascular Center Research Institute

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Chapter 4: NEW ASPECTS OF ANGIOGENESIS

Chapter 5: CLINICAL RESULTS OF THERAPEUTIC ANGIOGENESIS AND VASCULOGENESIS

Contributors

CHAPTER 1

VASCULAR PRECURSOR CELLS AND THEIR POTENTIATION

EPC and Their Potentiation by Adenovirus Gene Delivery

Hideki Iwaguro[1] and Takayuki Asahara[1,2]

[1]Department of Regenerative Medicine, Division of Basic Clinical science, Tokai University School of Medicine, Kanagawa, Japan.
[2]Department of Regenerative Medicine, Institute of Biomedical Research and Innovation/RIKEN Kobe Center for Developmental Biology, Kobe, Japan.

Summary. The isolation of endothelial progenitor cells (EPCs) derived from bone marrow (BM) was an outstanding event in the recognition of 'de novo vessel formation' in adults occurring as physiological and pathological responses. The finding that EPCs migrate to sites of neovascularization and differentiate into endothelial cells (ECs) *in situ* is consistent with "vasculogenesis", a critical paradigm that is well described for embryonic neovascularization, but proposed recently in adults in which a reservoir of stem or progenitor cells contribute to vascular organogenesis. EPCs have also been considered as therapeutic agents to supply the potent origin of neovascularization under pathological conditions. This review highlights an update of EPC biology as well as their potential use for therapeutic regeneration.

Key words. Endothelial progenitor cells, Angiogenesis, Vasculogenesis, ischemia, Gene transfer, Vascular endothelial Growth factor, Neovascularization

Introduction

Tissue regeneration by somatic stem/progenitor cells has been recognized as a maintenance or recovery system of many organs in the adult. The isolation and investigation of these somatic stem/progenitor cells have provided how insight as to these cells contribute to postnatal organogenesis. On the basis of the regenerative potency, these stem/progenitor cells are expected to develop as a key strategy of therapeutic applications for the damaged organs.

Recently, endothelial progenitor cells have been isolated from adult peripheral blood (Asahara T, et al. 1997). EPCs are considered to share common stem/progenitor cells with hematopoietic stem cells and shown to derive from bone marrow (BM) and to incorporate into foci of physiological or pathological neovascularization (Asahara T et al. 1999, Gunsilius E, et al. 2000, Crosby J.R, et al. 2000). The finding that EPCs migrate to sites of neovascularization and differentiate into endothelial cells *in situ* is consistent with "vasculogenesis", a critical paradigm that is well described for embryonic neovascularization. Recent findings proposed in adults suggest that a reservoir of stem/progenitor cells contribute to post-natal vascular organogenesis. The discovery of EPCs has therefore drastically changed our understanding of adult blood vessel formation (Fig.1).

EPC transplantation constitutes a novel therapeutic strategy that provide a robust source of viable endothelial cells (ECs) to supplement the contribution of ECs resident in the adult vasculature that migrate, proliferate, and remodel in response to angiogenic cues, according to the classic paradigm of angiogenesis developed by Folkman and colleagues (Folkman J, 1993). Just as classical angiogenesis may be impaired by certain pathologic phenotypes, EPC function (i.e., mobilization from the bone marrow and incorporation into neovascular foci) may be impaired by aging, diabetes, hypercholesterolemia, and hyperhomocysteinemia. Gene transfer to EPCs during ex vivo expansion represents a potential approach to enhance EPC function. We present research protocols for transferring vascular endothelial growth factor (VEGF) gene to EPCs to achieve angiogenic phenotypic modulation of EPC function.

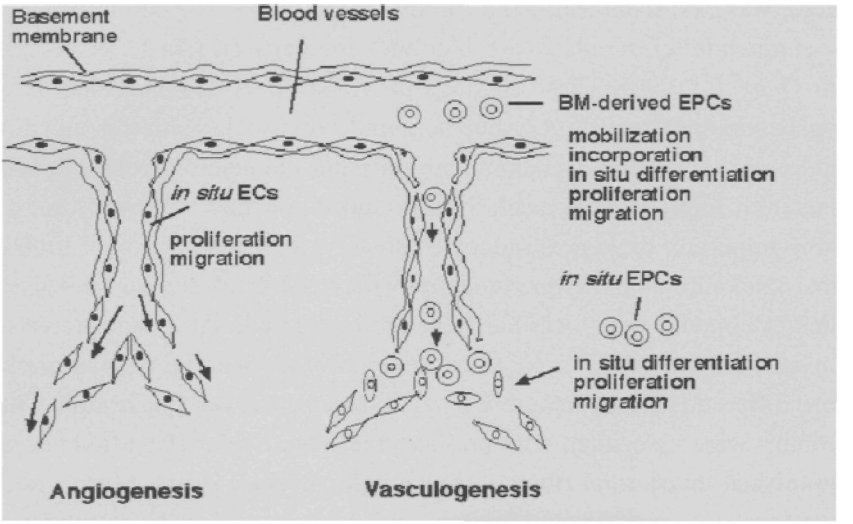

Fig. 1. Post-natal neovascularization. Post-natal neovascularization in the physiological or pathological events is consistent with neovessel formation contributed by angiogenesis and vasculogenesis at the various rates between their two mechanisms. Angiogenesis and vasculogenesis are due to the activations of *in situ* ECs and BM-derived or *in situ* EPCs, respectively.

The potential of EPC transplantation

The regenerative potential of stem/ progenitor cells is currently under intense investigation. *In vitro*, stem/ progenitor cells possess the capability of self-renewal and differentiation into organ-specific cell types. When placed *in vivo*, these cells are then provided with the proper milieu that allows them to reconstitute organ systems. The novel strategy of EPC transplantation (cell therapy) may therefore supplement the classic paradigm of angiogenesis developed by Folkman and colleagues. Our studies indicated that cell therapy with culture-expanded EPCs can successfully promote neovascularization of ischemic tissues, even when administered as "sole therapy," i.e., in the absence of angiogenic growth factors. Such a "supply-side" version of therapeutic neovascularization, in which the substrate (EPCs/ECs) rather than ligand (growth factor) comprises the therapeutic

agent, was first demonstrated by intravenously transplanting human EPCs to immunodeficient mice with hindlimb ischemia (Kalka C, et al. 2000, Shi Q, et al. 1998). These findings provided novel evidence that exogenously administered EPCs rescue impaired neovascularization in an animal model of critical limb ischemia. Not only did the heterogonous cell transplantation improve neovascularization and blood flow recovery, but also led to important biological outcomes notably, a 50% reduction of limb necrosis and auto-amputation compared with controls. A similar strategy applied to a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs localized to areas of myocardial neovascularization, differentiate into mature ECs and enhance neovascularization. These findings were associated with preserved left ventricular (LV) function and diminished myocardial fibrosis (Kawamoto A, et al. 2001). Murohara and associates reported similar findings in which human cord blood-derived EPCs also augmented neovascularization in hindlimb ischemic model of nude rats, followed by in situ transplantation (Murohara T, et al. 2000).

Other researchers have more recently explored the therapeutic potential of freshly isolated human CD34+ MNCs (EPC-enriched fraction). Shatteman and associates conducted local injection of freshly isolated human CD34+ MNCs into diabetic nude mice with hindlimb ischemia, and showed an increase in the restoration of limb flow (Schatteman GC, et al. 2000). Similarly Kocher and associates attempted intravenous infusion of freshly isolated human CD34+ MNCs into nude rats with myocardial ischemia, and found preservation of LV function associated with inhibition of cardiomyocyte apoptosis (Kocher AA, et al. 2001). Thus 2 approaches of EPC preparation (i.e., both cultured and freshly-isolated human EPCs) may provide therapeutic benefit in vascular diseases, but as described below, will likely require further optimization of techniques to acquire the ideal quality and quantity of EPCs for EPC therapy.

Future strategy of EPC cell therapy

Ex vivo expansion of EPCs cultured from PB-MNCs of healthy human volunteers typically yields $5.0x10^6$ cells per 100ml of blood on day 7. Our animal studies suggest that heterologous transplantation requires systemic

injection of $0.5\sim2.0x10^4$ human EPCs /g body weight of the recipient animal to achieve satisfactory reperfusion of an ischemic hindlimb. Rough extrapolation of these data to human suggests that a blood volume of as much as 12 liters may be necessary to obtain adequate numbers of EPCs to treat critical limb ischemia in patients (Fig. 2).

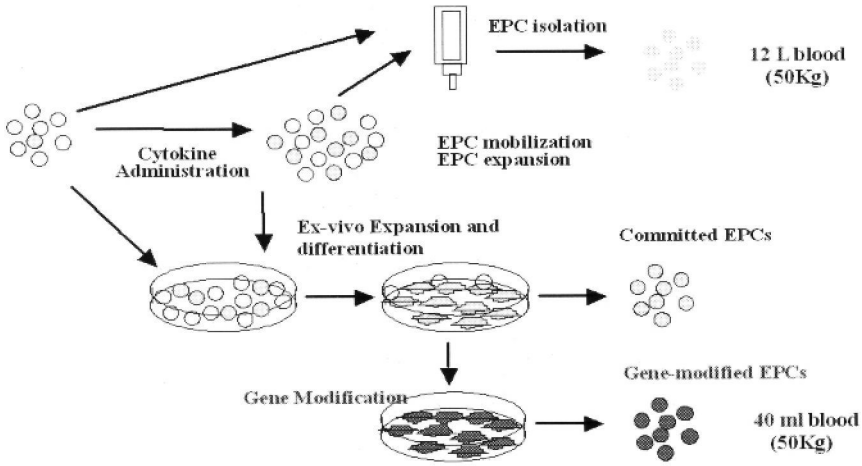

Fig. 2. EPC therapy using autologous EPCs derived from BM for vascular regeneration. Transplantation of BM- or mobilized PB-MNCs are considered 'crude EPC therapy'under, as EPCs are not selected. BM-MNCs have been already under clinical application. Following the manipulation to acquire the optimized quality and / or quantity, e.g., sorting by surface markers, ex vivo culture-expansion and / or gene transfection, EPC therapy is expected to be the useful strategy for vascular regeneration.

Therefore, the fundamental scarcity of EPCs in the circulation, combined with their possible functional impairment associated with a variety of phenotypes in clinical patients, such as aging, diabetes, hypercholesterolemia, and homocyst(e)inemia (vide infra), constitute major limitations of primary EPC transplantation. Considering autologous EPC therapy, certain technical improvements that may help to overcome the primary scarcity of a viable and functional EPC population should include; 1) local

delivery of EPCs 2) adjunctive strategies (e.g. growth factor supplements) to promote BM-derived EPC mobilization (Takahashi T, et al. 1999, Asahara T, et al. 1999), 3) enrichment procedures, i.e., leukapheresis or BM aspiration, or 4) enhancement of EPC function by gene transduction (gene modified EPC therapy, vide infra) 5) culture-expansion of EPCs from self-renewable primitive stem cells in BM or other tissues. Alternatively, unless the quality and quantity of autologous EPCs to satisfy the effectiveness of EPC therapy may be acquired by the technical improvements described above, allogenic EPCs derived from umbilical cord blood or culture-expanded from human embryonic stem cells (Levenberg S, et al. 2002), may be available as the sources supplying EPCs.

Gene modified EPC therapy

A strategy that may alleviate potential EPC dysfunction in ischemic disorders is considered reasonable, given the findings that EPC function and mobilization may be impaired in certain disease states (Kalka C, et al. 2000). Genetic modification of EPCs to overexpress angiogenic growth factors, to enhance signaling activity of the angiogenic response, and to rejuvenate the bioactivity and/or extend the life span of EPCs, can constitute such potential strategies.

EPC gene transfer and in vitro & in vivo study

The methods described below outline (1) EPC gene transfer, (2) Proliferative activity assay, and (3) In vitro incorporation of Tf-EPCs into HUVEC monolayer.

EPC gene transfer

After 7 days in culture (see note 1), cells were transduced with an adenovirus encoding the murine VEGF 164 gene (Ad/VEGF) or lacZ gene (Ad/ßgal). To establish optimal conditions for EPC, adenovirus gene transfer serum concentration, virus incubation time and virus concentration

were evaluated using X-gal staining to detect ß-galactosidase expression (Fig. 3).

Human EPCs were transfected with 1,000 MOI of Ad/VEGF or Ad/ßgal for 3 hours incubation in 1% serum media. After an adenovirus transduction, cells were washed with PBS and incubated in EBM-2 media for 24 hours prior to transplantation experiments.

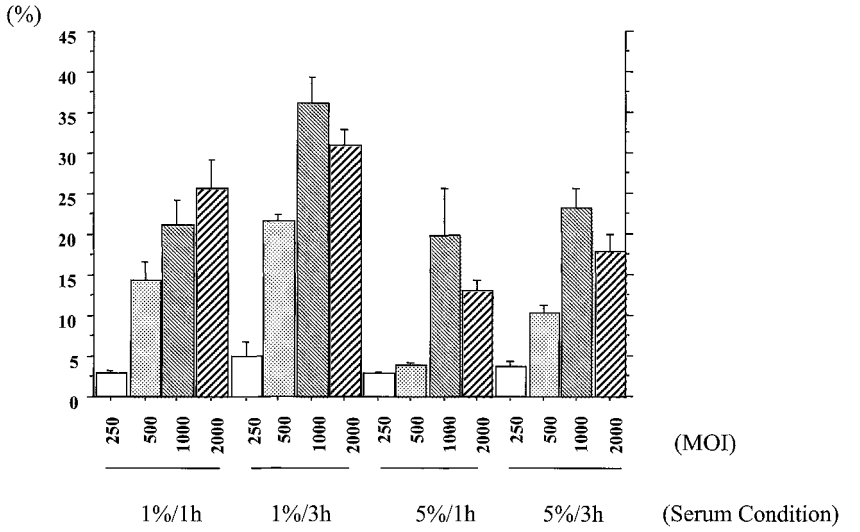

Fig. 3. Profile of transfection efficiencies for Ad/ß-gal in *ex-vivo* expanded human EPCs. Four different multiplicities of infection (MOI, 250, 500, 1000, and 2000) were tested in 2 different media conditions (1% or 5% serum EBM-2), for 1 or 3 hours (h) incubation. Error bars represent SEM of triplicate experiments. Following these preliminary experiments, human EPCs were transduced with 1,000 MOI Ad/VEGF or Ad/ßgal for 3 hrs in 1% serum media (see note 2). *$p<0.01$, non-Tf *vs* Tf/ ß-EPCs.

Proliferative activity assay

At 24 hours post-gene transfer, reseed EPCs transduced with Ad/VEGF (Tf/V-EPCs),Ad/ß-gal(Tf/ß-EPCs),or non-transduced EPCs (non-Tf/EPCs) on 96-well plates coated with human fibronectin for the proliferative activ-

ity using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (see note 2).

After 48 hours in culture, add MTS/PMS (phenazine methosulfate) solution to each well and incubate for 3 hours. Light absorbance at 490 nm was detected by ELISA plate reader.

In our previous experiments, MTS assay was employed to determine proliferative activity of transduced EPCs. By using 5% serum conditioned media, we found that proliferative activity of Ad/VEGF-transduced EPCs exceeded proliferative activity of Ad/ß-gal (0.48±0.03 vs. 0.37±0.01 corrected absorbance at 490 nm, $p<0.01$) and non-transduced EPCs (non-Tf=0.32±0.02, $p<0.05$) in vitro (Fig. 4).

*p<0.01 vs non-Tf and Tf/ß-EPC

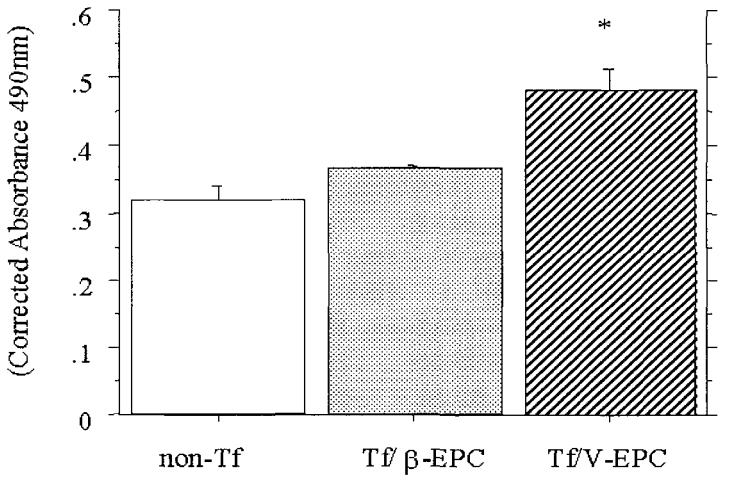

Fig. 4. Proliferative activity assay. The proliferative activity of EPCs transduced in 5% serum was measured by MTS assay after 48 hrs in culture. The increase in mitogenic response of EPCs transduced with Ad/VEGF (Tf/V-EPCs) was statistically significant in comparison with EPCs transduced with Ad/ßgal (Tf/ß-EPCs) and non-transduced EPCs (non-Tf). *p<0.01, Tf/ßgal *vs* non-Tf.

In vitro incorporation of Tf-EPCs into HUVEC monolayer

At 24 hours post-gene-transfer, Tf/V-EPCs and Tf/ß-EPCs were stained with fluorescent carbocyanine DiI. DiI-labeled EPCs (see note 3) were incubated in a monolayer of human umbilical vein endothelial cells (HUVECs) in 4-well culture slides for 12 hours with or without pretreatment with tumor necrosis factor (TNF)-α (1ng/ml) .

Three hours after incubation, remove non-adherent cells by washing with PBS, apply new media, and maintain the culture for an additional 24 hours. And then, count the total number of adhesive EPCs in each well in a blinded manner under a 200X magnification field of a fluorescent microscope.

At 24 hours post-transduction, label EPCs with the fluorescent marker, DiI, for cell tracking. Incubate DiI- labeled, VEGF-transduced EPCs in a HUVEC monolayer for 12 hours with or without pre-treatment with TNF-α (1ng/ml) (Fig. 5a).

In our previous experiments, in the quiescent HUVEC monolayer, adhesion of DiI-labeled EPCs were not significantly different between Tf/V-EPCs and Tf/ß-EPC transplanted animals (2.7±0.2 vs. 2.2±0.3, p=ns) (Fig. 5b). In activated HUVECs, however, adhesion of DiI-labeled Tf/V-EPCs exceeded that of Tf/ß-EPCs (4.3±0.4 vs. 2.9±0.3, $p<0.01$). Alternatively, the same cells were incubated in new media, and maintained for 24 hours in HUVEC monolayer to confirm incorporation *in vitro* and *in vivo*. In the quiescent HUVEC monolayer, incorporation of DiI-labeled Tf/V-EPCs exceeded that of Tf/ß-EPCs (7.0±0.5 vs. 3.5±0.5, $p<0.01$) (Fig. 5b). In activated HUVECs, incorporation of DiI-labeled Tf/V-EPCs also exceeded that of Tf/ß-EPCs (13.8±0.8 vs. 5.3±0.6, $p<0.001$).

In vivo cell transplantation to ischemic animal model

We have recently shown for the first time that gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia (Iwaguro H, et al. 2002). Transplantation of heterologous EPCs transduced with adenovirus encoding human VEGF165 not only improved neovascularization and blood flow recovery, but also had meaningful biological consequences, i.e., limb necrosis and auto-amputation were reduced by 63.7% in

comparison with controls. Notably, the dose of EPCs needed to achieve limb salvage in these *in vivo* experiments, was 30 times less than that required in the previous experiments involving unmodified EPCs. Thus, combining EPC cell therapy with gene (i.e., VEGF) therapy may be one option to address the limited number and function of EPCs that can be isolated from peripheral blood in patients.

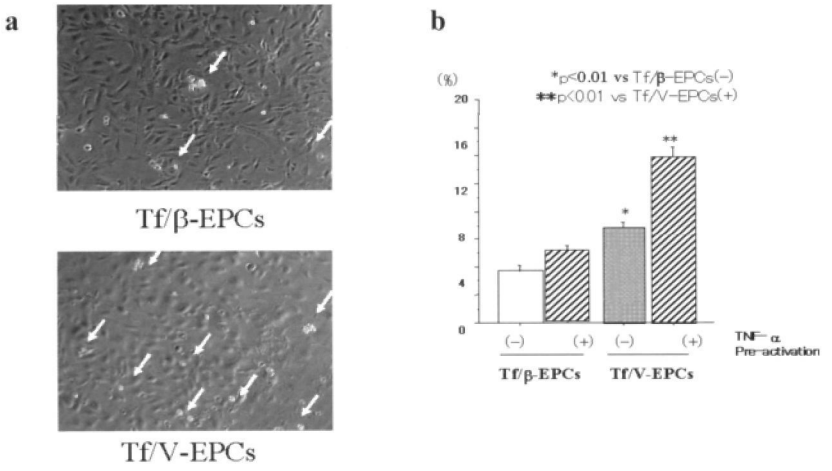

Fig. 5. *In vitro* incorporation of Tf-EPCs in HUVEC monolayer. **a** Representative macroscopic photographs of Tf/V-EPCs and Tf/ß-EPCs in HUVEC monolayer at 24 hrs after transduction with Ad/VEGF or Ad/ßgal, respectively. Left panel is the Tf/ß-EPCs and right panel is the Tf/V-EPCs in HUVEC monolayer, both pretreated with TNF-α stimulation. White bars indicate 50μm length. **b** Quantitative analysis of EPC adhesion observed 3 hrs and incorporation observed 24 hrs after transduction with (+) and without (-) pre-treatment of TNF-α. *$p<0.01$, Tf/ß-EPCs *vs* Tf/V-EPCs.

Conclusion

As the concept of gene-modified EPCs in adults and postnatal vasculogenesis are further established, clinical applications of EPCs to regenerative medicine are likely to follow. To acquire the more optimized quality and quantity of EPCs, several issues remain to be addressed, such as the development of more efficient method of EPC purification and expansion, the methods of administration and senescence in EPCs.

This strategy will provide the safe and potent methodology of gene therapy for vascular regeneration.

Notes

1. Cell culture number of initial plate (approximately):
   a 10 cm dish : 3.0-5.0 x 10^7 cells
   a 35 mm dish : 0.8-1.0 x 10^7 cells
   a 4-well dish : 2.0-8.0 x 10^6 cells
2. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay: Non-radioactive cell proliferation is a method for determining the number of viable cells in proliferation or chemosensitivity assays. The following procedure is recommended for the preparation of reagents sufficient for one 96 well plate containing cells cultured in a 100 μl volume. After add 20 μl of combined MTS/PMS solution (basic 100 μl of PMS solution to the 2.0 ml of MTS solution) into each well of the 96 well assay plate containing the sample, incubate the plate for 1-4 hours at 37°C in a humidified 5% CO2 atmosphere.
3. DiI-acetylated-LDL incorporation: Purified low density lipoprotein was acetylated and then labeled with the fluorescent probe, DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate) for labeling endothelial cells and macrophages. Aseptically dilute the DiI-Ac-LDL to 10 μg/ml in standard media, and add it to live cells and incubate for 2~4 hours at 37°C. Remove media containing DiI-Ac-LDL, wash twice by DPBS, and count cells for each experiment.

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Potentiation of Regenerative Therapy by Non-Viral Vector, Gelatin Hydrogel

Noritoshi Nagaya[1], Naoto Fukuyama[2], Yasuhiko Tabata[3], Hidezo Mori[4]

[1]Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, Osaka, Japan, [2]Department of Physiology, Tokai University School of Medicine, Kanagawa, Japan, [3]Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, and [4]Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka, Japan

Summary. Both gene therapy and cell transplantation are promising approaches for therapeutic angiogenesis. However, gene therapy must overcome biohazard of viral vectors, transfection efficiency, and premature tissue-targeting. Conventional cell therapy is insufficient in some cases because of small cell numbers, poor survival, impaired differentiation, etc. Endothelial progenitor cells (EPCs) play an important role in modulating angiogenesis and vasculogenesis. Here, we present a new concept for hybrid cell-gene therapy using a nonviral vector, gelatin. Genetically-modified EPCs may serve, not only as a tissue-engineering tool to reconstruct the vasculature, but also as a vehicle for gene delivery to injured endothelium. Thus, hybrid cell-gene therapy may be a new therapeutic strategy for the treatment of intractable cardiovascular diseases.

Key words. Cell therapy, Transplantation, Angiogenesis, Adrenomedullin, Gene therapy

Therapeutic angiogenesis is a promising strategy for the treatment of intractable cardiovascular diseases such as ischemic heart disease, peripheral vascular disease, and pulmonary arterial hypertension. Although gene therapy has been shown to be an effective approach for angiogenesis, it is still unsatisfactory because of biohazard of viral vectors, transfection efficiency, and premature tissue-targeting (St George JA 2003). Therefore, highly efficient and safe gene transfer is desirable. Recently, we developed a novel nonviral vector, gelatin which allows highly efficient and long-lasting gene transfer. Because positively charged biodegradable gelatin hold negatively charged plasmid DNA in its positively charged lattice structure, (Fukunaka Y 2002, Nagaya N 2003) DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer (Tokunaga N 2004).

Recently, transplantation of stem cells or progenitor cells has been shown to regenerate a variety of tissues. Endothelial progenitor cells (EPCs) have been discovered in adult peripheral blood (Asahara T, et al. 1997). EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, then migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ(Kawamoto A, et al. 2001). Transplantation of EPC induces therapeutic angiogenesis in ischemic heart or limb (Kawamoto A 2001. Murohara T, et al. 2000). However, some patients are refractory to conventional cell therapy because of insufficient cell number, poor survival, impaired differentiation, etc. Thus, a novel therapeutic strategy to enhance the angiogenic property of EPCs is desirable. Here, we present a new concept for hybrid cell-gene therapy using a nonviral vector, gelatin. Gene-modified EPCs may serve not only as a tissue-engineering tool to reconstruct the vasculature, but also as a vehicle for gene delivery to injured endothelium.

This chapter focuses on gelatin-mediated in vivo and in vitro gene transfer, and the rationale and preliminary results of combining cell (EPCs) and gene therapy for regenerative medicine.

Nonviral vector, gelatin

Tabata Y, et al. discovered biodegradable gelatin which controls the release of growth factors such as basic fibroblast growth factor. Positively charged biodegradable gelatin ionically link with negatively charged protein (Tabata Y 1999, Tabata Y 1987). Thus, the gelatin has been widely used as a carrier of proteins because of its capacity to delay protein degradation. Plasmid DNA is known to be negatively charged. Thus, we used the gelatin as a vector for gene therapy. Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of approximately 30μm, water content of 95% and an isoelectric point (pI) of 9 after swelling in water (Tabata Y 1999, Tabata Y 1987). After a 2-hour incubation period, positively charged gelatin held negatively charged plasmid DNA in its positively charged lattice structure (Fig.1**a** and **b**). DNA particles are released from the gelatin through its degradation.

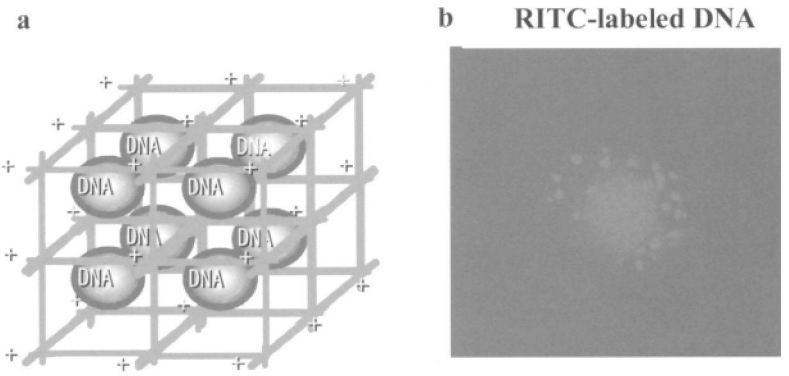

Fig. 1a, b. a Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. **b** A number of RITC-labeled AM DNA particles were incorporated into gelatin.

Angiogenic potential of adrenomedullin

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (Kitamura K 1993). AM and its receptor are expressed mainly in vascular endothelial cells and vascular smooth muscle cells. AM not only induces vasorelaxation but also regulates growth and death of these vascular cells (Nagaya N, et al. 2000. Nishimasu H, et al. 2001). A recent study has shown that vascular abnormalities are present in homozygous AM knockout mice, suggesting that AM is indispensable for vascular morphogenesis (Shindo T, et al. 2001). Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, is a multistep process that involves migration and proliferation of endothelial cells, remodeling of the extracellular matrix and functional maturation of the newly assembled vessels. Recently, AM induced tyrosine phosphorylation of Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase1/2 (ERK1/2) in human umbilical vein endothelial cells (Kim W, et al. 2003). Both signals play important roles in regulation of multiple critical steps in angiogenesis and vasculogenesis; endothelial cell survival, proliferation, migration, and capillary-like structure formation. These findings raise the possibility that AM gene transfer plays a role in modulating vasculogenesis and angiogenesis in ischemic tissues.

In vivo transfection

We examined the usefulness of gelatin as a nonviral vector for in vivo gene transfer (Tokunaga N, et al. 2004). AM plasmid DNA was used for therapeutic angiogenesis. We demonstrated that AM DNA was incorporated into positively charged gelatin. Interestingly, AM immunoreactivity surrounding AM DNA-gelatin complexes in the skeletal muscles was intense (Fig.2**a**). AM production of AM-gelatin group was enhanced compared with that of naked AM DNA group (Fig.2**b**). Furthermore, gelatin allowed long-lasting AM expression after gene transfer. These results suggest that biodegradable gelatin may serve as a nonviral vector for gene transfer. In fact, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hindlimb ischemia than naked AM DNA

(Fig.3), as evidenced by significant increases in histological capillary density, calf blood pressure ratio, and laser Doppler flow. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. AM DNA-gelatin complexes were distributed mainly in connective tissues. We have recently demonstrated that gelatin-DNA complexes are readily phagocytosed by macrophages, monocytes, endothelial progenitor cells etc, resulting in gene expression within these phagocytes (Tabata Y and Ikeda Y 1987). These findings raise the possibility that AM secreted from these cells acts on muscles in a paracrine fashion. Unlike AM production in the Naked AM group, AM overexpression in the AM-gelatin group lasted for longer than two weeks. Thus, it is interesting to speculate that delaying gene degradation by gelatin may be responsible for the highly efficient gene transfer. These results suggest that the use of gelatin, which is considered to be less biohazardous than viral vectors, enhances the angiogenic potential of AM DNA. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular diseases.

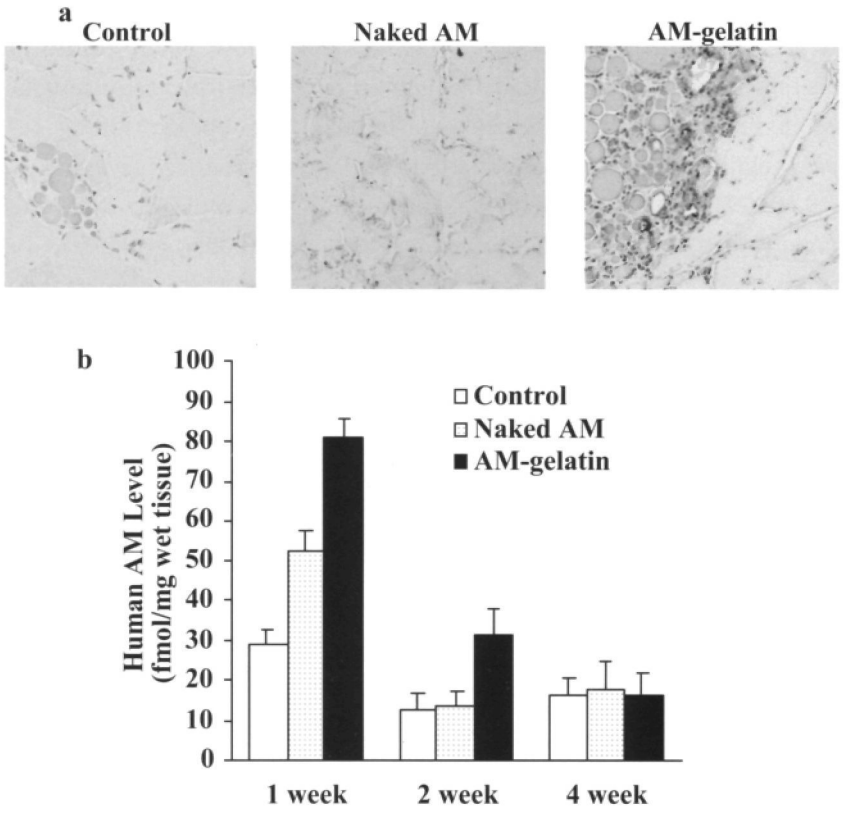

Fig. 2a, b. a Immunohistochemistry for AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification x200. **b** Time course of AM production in ischemic muscles after gene transfer. Data are mean ± SEM. *P < 0.01 vs Control group; †P < 0.01 vs Naked AM group.

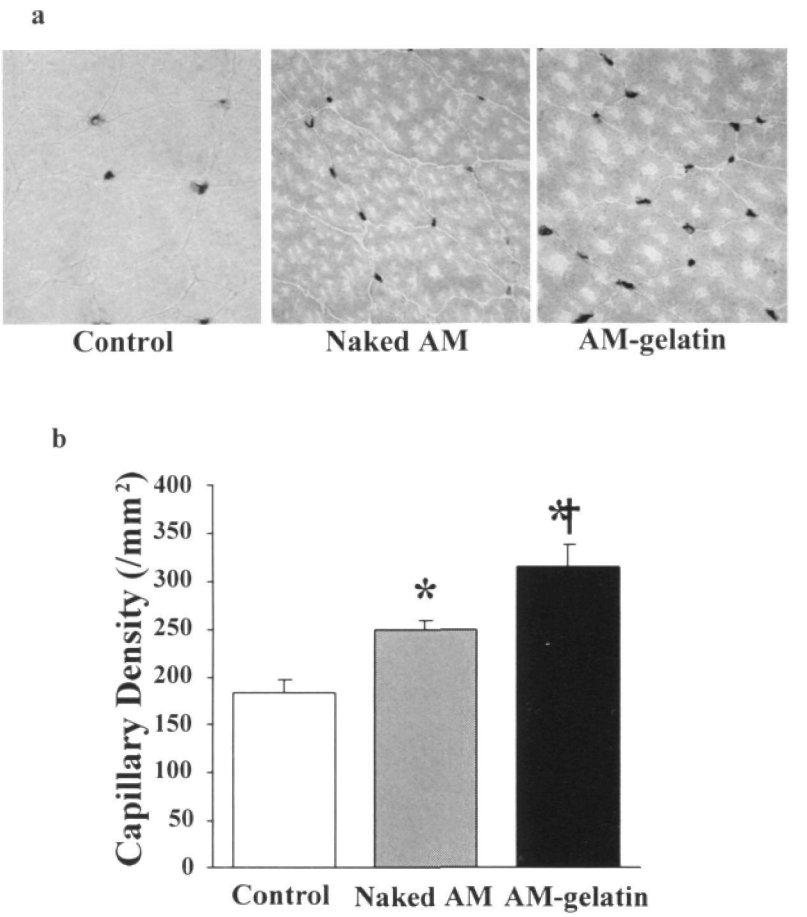

Fig. 3a, b. a Representative examples of alkaline phosphatase staining in ischemic hindlimb muscles. Magnification x200. **b** Quantitative analysis of capillary density in ischemic hindlimb muscles. Data are mean ± SEM. *P < 0.05 vs Control group; †P < 0.05 vs Naked AM group.

In vitro transfection

We examined the usefulness of gelatin as a nonviral vector for in vitro gene transfer (Nagaya N, et al. 2003). Here we demonstrate that EPCs have a phagocytosing action which allows nonviral gene transfer into EPCs. To produce ionically linked plasmid DNA-gelatin complexes, we prepared positively charged biodegradable gelatin. Positively charged gelatin was readily complexed with negatively charged plasmid DNA after 24-hr incubation. Then, EPCs were cultured with green fluorescent protein (GFP) plasmid DNA-gelatin complexes. Interestingly, fluorescence microscopy revealed that GFP was expressed in EPCs after a 72-hr incubation period. Quantitative analyses confirmed a high incidence (approximately 70%) of GFP expression in EPCs. Transmission electron microscopy demonstrated that EPCs were phagocytosing DNA-gelatin complexes after 12-hr incubation (Fig.4**a**). These results suggest that EPCs phagocytose plasmid DNA-gelatin complexes in co-culture, which allows nonviral, highly efficient gene transfer into EPCs. A number of DNA particles labeled by rhodamine B isothiocyanate (RITC) were incorporated into gelatin. RITC-labeled DNA particles were gradually released from gelatin within EPCs through gelatin degradation (Fig.4**b**). After 72-hr incubation, RITC-labeled DNA particles released from gelatin were distributed in the cytoplasm, but not the nucleus, of EPCs (Fig.4**c**). These results suggest the ability of EPCs to take up DNA-gelatin complexes and dissolve the gelatin, freeing the DNA into EPCs. Unlike EPCs, neither human mature pulmonary artery endothelial cells (HPAECs) nor human umbilical vein endothelial cells (HUVECs) phagocytosed DNA-gelatin complexes.

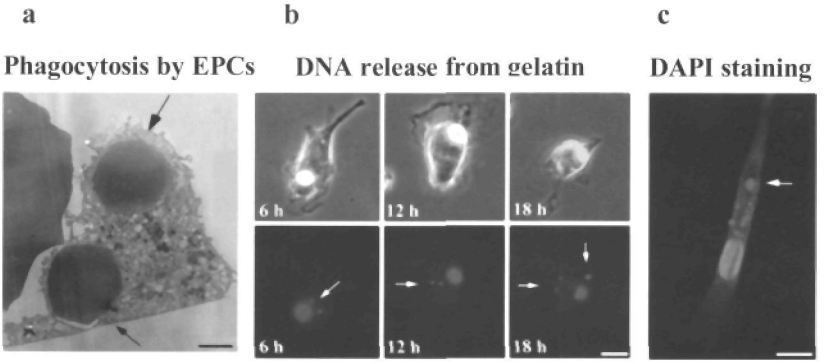

Fig. 4a, b, c. Ex vivo gene transfer into EPCs based on phagocytosing action. **a** Transmission electron microscopy revealed that EPCs had phagocytosed GFP DNA-gelatin complexes (arrows). **b** Time-course of changes in DNA-gelatin complexes within EPCs. RITC-labeled DNA particles (red, arrows) were released from gelatin through its degradation. **c** After 72-hr incubation, RITC-labeled DNA particles released from phagocytosed gelatin (arrow) were distributed in the cytoplasm, but not the nucleus, of EPCs. The nuclei of EPCs were identified by DAPI staining. Scale bars: 10μm (**a**); 5μm (**b** and **c**)

When EPCs were cultured with AM plasmid DNA-gelatin complexes, intense immunostaining for AM was observed in EPCs impregnated with AM DNA-gelatin (Fig.5**a**). After 72-hr incubation with AM DNA-gelatin complexes, EPCs markedly secreted AM into the culture medium (10-fold increase compared to EPCs alone, Fig.5**b**). AM overproduction lasted for more than 16 days after gene transfer. Finally, we examined the effects of AM gene transfer on EPC proliferation in vitro using MTS assay. Proliferative activity of AM DNA-transduced EPCs exceeded that of nontransduced EPCs. In addition, AM gene transfer inhibited apoptosis of EPCs in vivo and in vitro. This can be explained by recent findings that AM inhibits cell apoptosis through the PI3K/Akt pathway (Okumura H, et al. 2004). Thus, transfection of AM gene may strengthen therapeutic potential of EPCs.

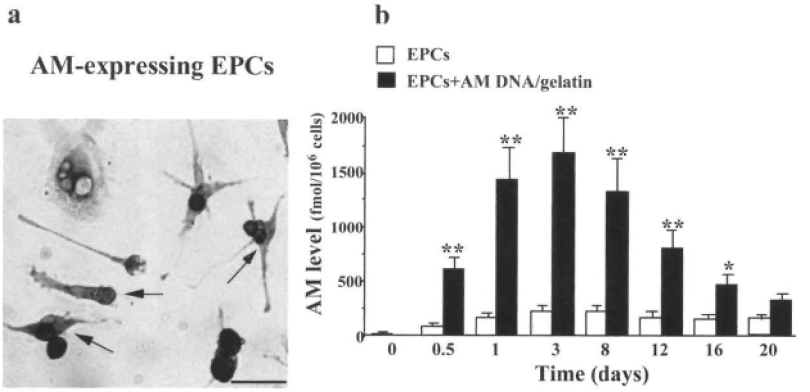

Fig.5a, b. a Immunohistochemistry for AM in EPCs after gene transfer. **b** Time course of AM secretion from EPCs during coculture with AM DNA-gelatin complexes. Each bar represents the mean ± SEM. *P < 0.05; **P < 0.001 versus non-transduced EPCs.

Hybrid cell-gene therapy

Here, we present a new concept for cell-based gene delivery into the vasculature, consisting of three processes (Nagaya N, et al. 2003). First, positively charged gelatin is readily complexed with negatively charged plasmid DNA. Second, EPCs phagocytose ionically linked plasmid DNA-gelatin complexes in co-culture, which allows nonviral gene transfer into EPCs with high efficiency. Third, transplanted gene-modified EPCs are incorporated into injured vascular beds. This novel gene delivery system has great advantages over conventional gene therapy: nonviral, noninvasive, and highly efficient gene targeting into the vasculature. These benefits may be achieved mainly by the capability of EPCs to phagocytose DNA-gelatin complexes and to migrate to sites of injured endothelium.

Primary pulmonary hypertension (PPH) is a rare, but life-threatening disease characterized by the progression of pulmonary hypertension, ultimately producing right ventricular failure and death. Median survival in

patients with PPH is considered to be 2.8 years from the time of diagnosis. Thus, novel and effective therapy is desirable for the treatment of pulmonary hypertension. Dysfunction of the endothelium may play a role in the pathogenesis of pulmonary hypertension such as PPH. Thus, pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension.

We present cell-based gene delivery into the pulmonary vasculature. EPCs are mobilized from within the bone marrow into the peripheral blood in response to tissue ischemia or injury, then migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ. These findings raise the possibility that transplanted EPCs may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. AM is a potent vasodilator peptide which also inhibits cell apoptosis and enhances endothelial cell differentiation. Thus, we investigated the effects of AM gene-modified EPCs on pulmonary hypertension in rats.

GFP-expressing EPCs (1×10^6/rat) were intravenously administered into rats with monocrotaline-induced pulmonary hypertension. Three days after transplantation, GFP-expressing EPCs were incorporated into the walls of pulmonary arterioles in monocrotaline rats and composed pulmonary vasculature. Transplanted GFP-expressing EPCs were distributed on the walls of pulmonary arterioles and capillaries. AM gene-transduced EPCs were similarly incorporated into the pulmonary vasculature. Immunohistochemical analyses of rat and human CD31 demonstrated that the transplanted EPCs were of endothelial lineage and constituted vessel structure similar to rat endothelial cells. However, transplanted EPCs were rarely distributed to other tissues such as cardiac ventricles, kidneys, aorta, and brain (data not shown).

Next, we examined the effects of AM-expressing EPCs on pulmonary hemodynamics. Monocrotaline rats received intravenous injection of 1×10^6 EPCs, 1×10^6 AM-expressing EPCs, or culture medium. Pulmonary hypertension developed three weeks after monocrotaline injection. Mean pulmonary arterial pressure was not strikingly decreased in the EPC group

(-14%), but was significantly lower in the AM-EPC group (-29%) compared to that in the CONTROL group. Pulmonary vascular resistance was significantly lower in both the EPC group (-16%) and AM-EPC group (-39%) than that in the Control group. Importantly, the AM-EPC group showed significantly greater improvement in pulmonary vascular resistance than the EPC group. Finally, we examined the effects of hybrid cell-gene therapy on survival in MCT-injected rats. AM-expressing EPCs were used immediately after 72-hr incubation with AM DNA-gelatin complexes. Kaplan-Meier survival curves demonstrated that MCT rats transplanted with AM-expressing EPCs had a significantly higher survival rate than those given culture medium or EPCs alone.

These findings suggest that gene-modified EPCs using gelatin may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. This hybrid cell-gene therapy may apply for intractable cardiovascular diseases including ischemic heart disease. Thus, genetic manipulation of stem cells opens new avenues for regenerative medicine.

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Regeneration of Myocardium Using Bone Marrow Cells

Shinji Tomita[1], Takeshi Nakatani[2]

[1]Cardiothoracic Surgical Unit, Auckland City Hospital, Auckland, New Zealand [2] Department of Organ Transplantation, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka, 565-8565, Japan

Summary. Bone marrow cells are advantageous for exogenous cell transplantation to treat end-stage heart failure regard to autologous source, no ethical issue, capacity to regenerate myocardium, induction of angiogenesis. Based on basic research showing regenerating myocardium using bone marrow, clinical trials in several places were conducted like gold rush in recent years. Endogenous-stem cell therapy may be also a promising strategy. Self-renewal of myocardium may be partly derived from bone marrow and the myocardium itself, which was thought to be a terminally differentiated organ. The past new technologies have been developed and their use expanded despite a lack of concrete evidence regarding their effectiveness. However, we still have a lot of unanswered questions including optimal cell population, cell density, and exact mechanism responsible for the improvement of cardiac dysfunction, fate of fusioned cells, cardiac environmental factors, regulation of proliferation and differentiation of transplanted cells, efficient cell tracking method in human. People involved in this field must be careful as they proceed, as inappropriately designed research might ruin the future of the field of regenerative medicine. Cell-based therapy will continue to expand at a rapid rate over the next decade. Whether the benefits of cell-based therapy are evident in the future remains to be seen.

Key Word. Heart, Bone marrow cells, Cell-based therapy, Exogenous-stem cell, Endogenous-stem cell

Introduction

Once the heart is damaged, some cardiomyocytes will necrose and residual cardiomyocytes may compensate heart function with hypertrophy. However, heart failure may eventually develop in the end stage. Although heart transplantation is an effective treatment for end-stage heart failure, the shortage of donors is a major limiting factor. (Hosenpud JD, et al. 1998). While heart transplantation currently remains the best choice, other alternatives, such as mechanical support and drug therapy, are being investigated. In our hope exogenously transplanted cells may compensate loss of cardiomyocytes to improve the damaged heart function. Since then a variety of cell-types have been investigated enthusiastically (Li RK, et al. 1999, Li RK, et al. 1996, Taylor DA, et al. 1998).

Exogenous cell transplantation

Bone marrow cells have many advantages compared to other cell sources. The technique of bone marrow aspiration is an established procedure in hematology. There are no immunological or ethical issues because of the autologous source. Bone marrow comprises two major systems; one haematopoietic and the other mesenchymal. Bone, cartilage, and fat derive from "mesenchymal stem cells". However, the lack of a universal way to identify "mesenchymal stem cells" represents a major obstacle.

In 1999, Makino S, et al. (Makino S, et al. 1999) and our group (Tomita S, et al. 1999) reported that cardiomyocytes could be generated from 5-azacytidine-treated bone marrow cells. Bittner et al reported that intravenously transplanted bone marrow cells could also differentiate into cardiomyocytes (Bittner RE, et al. 1999).

Many groups have tried to purify adult stem cells of several phenotypes, including cardiomyocytes, endothelial cells, and smooth muscle cells. Orlic et al. injected Lin-C-kit+cells to regenerate the infarcted myocardium (Orlic D, et al. 2001). Jackson et al. used side population (SP) cells (Jackson KA, et al. 2001). More recently, Beltrami AP, et al. reported Lin-C-kit+cells with the properties of cardiac stem cells. When injected into an ischemic heart, these cells reconstitute the myocardium including new ves-

sels and myocytes (Beltrami AP, et al. 2003). In contrast, Murry CE, et al. (Murry CE, et al. 2004) and Balsam LB, et al. (Balsam LB, et al. 2004) demonstrated that haematopoietic stem cells (Lin-c-kit+, Lin-c-kit+Sca+) did not transdifferentiate into cardiomyocytes in myocardial infarcts using cardiomyocyte-restricted and ubiquitously expressed reporter transgenes. Researchers are attempting to manipulate stem cells in vitro and in vivo. However, currently nobody has established the best way to expand human cardiomyocytes or cardiomyoblasts to a number that would be sufficient for clinical application.

Historically allograft cells were used for research in early the 1990's because of easiness; however, those cells were eliminated due to immunorejection despite cyclosporine therapy (Li RK, et al. 1997). Therefore, many researchers recently have utilized autologous or syngenic animal model to test applicability to clinical setting. However, if we can utilize allograft or xenogeneic cells without immunorejection, we will be able to expand cell-based therapy much more efficiently and industrially. There are several reports showing the possibility of immunotolerance or no immunorejection using bone marrow cells. Liechty KW, et al. transplanted human mesenchymal stem cells into sheep. They observed human cells inside of sheep without immunorejection (Liechty KW, et al. 2000). Chiu's group highlighted the possibility of xenonenic bone marrow stromal cells without rejection (Saito T, et al. 2002). Embryonal stem cell (ES cell) research may be an alternative in the future. In contrast to adult stem cells it is easier to expand ES cells in vitro. They provide a good tool to investigate the mechanism of cardiac differentiation at the genetic level. However ethical and immunological issues provide major hurdles that need to be overcome before pursuing clinical applications.

Based on animal data Menasche's group in France conducted the first clinical trial using skeletal myoblast. They showed that possible efficacy but faced on a critical issue: arrhythmia (Menasche P, et al. 2003). Four out of 10 patients had ventricular tachycardia and required an Automatic Intracardiac Defibrillator (AICD). They speculated that formation of gap junctions between host and donor cells might cause arrhythmia. The AICDs were not activated due to arrhythmia after implantation. In further trials, some institutions may implant AICDs prophylactically before cell

transplantation. The interpretation of this reported adverse effect of myoblast transplantation is hampered by the differing methods of cell culture used in different institutions. Standardization of the methods employed for cell culture by Good Manufactural Practice (GMP) is essential for the analysis and reproducibility of outcomes. However it is difficult to generate guidelines and regulations due to the rapid development occurring in this field. So far, there are a lot of human and animal studies using bone marrow cells, which did not detect any harmful effect regarding arrhythmia (Fujii H, et al. 2004). This issue should be investigated more deeply in further studies.

A number of studies from Japan have contributed to the regenerative medicine field. Noishiki Y, et al. reported that bone marrow cells seeded PTFE grafts and produced rapid endothelialisation inside the graft (Noishiki Y, et al. 1996). Asahara T, et al. in Boston proposed endothelial progenitor cells (EPC) originating from bone marrow contribute to angiogenesis and vasculogenesis in ischaemic myocardium (Asahara T, et al. 1999, Asahara T, et al. 1997). Murohara's group promoted bone marrow mononuclear cells (BMMNC) instead of EPC (Shintani S, et al. 2001) for their clinical research into ischaemic disease (Tateishi-Yuyama E, et al. 2002). BMMNC do not require special techniques for culture in contrast to myoblasts and EPC. Autologous donation avoids problems related to ethics and immune rejection. There is some evidence that transplanted BMMNC contribute to revascularization. Many people believe that cytokines released by bone marrow cells play a major role in angiogenesis (Tateishi-Yuyama E, et al. 2002). These benefits have conferred a significant advantage to bone marrow cells over other cell types and resulted in rapid expansion of this method into clinical application from Japan (Hamano K, et al. 2002) to other countries (Tse HF, et al. 2003). Their main concept is to rescue the ischemic heart through the mechanism of vasculogenesis and angiogenesis by bone marrow cells.

Based on easiness to utilize bone marrow cells and the hypothesis that bone marrow cells can regenerate the heart, many clinical trials have been conducted in recent years, too (Assmus B, et al. 2002, Strauer BE, et al. 2001). They are trying to merge the two concepts: one is for angiogenesis and the other is for myogenesis. Because we are not sure what exact

mechanism to improve regarding the impaired function of the heart, even in animal studies, we do not know what the major contributor is in human.

Endogenous-stem cell therapy

For many years people believed that adult cardiomyocytes cannot proliferate and regenerate themselves. In 2001 Anversa's group reported that even adult cardiomyocytes are capable of proliferation and self regeneration in the diseased heart and opened the door to endogenous-stem-cell therapy (Beltrami AP, et al. 2001). Other groups have also reported regeneration of cardiomyocytes in varying amounts (Laflamme MA, et al. 2002). These studies have indicated the existence of endogenous-stem cells which can contribute to the self-renewal of myocardium. In contrast to exogenous cell therapy methods, endogenous therapy does not require cell culture or surgical techniques.

In 2001, Orlic D, et al. reported that granulocyte colony-stimulating factor (GCSF) and stem-cell-factor (SCF) improved heart function and the survival rate after myocardial infarction (Orlic D, et al. 2001). We confirmed that bone marrow was a source of regenerated cardiomyocytes using a GFP chimera mouse infarction (Fukuhara S, et al. 2002) and doxorubicin model (Tomita S, et al. 2004) and that GCSF promoted bone marrow cells to migrate. However, it does not seem that migrated bone marrow cells support pump function directly because of the very small number of bone marrow- derived cardiomyocytes found in the heart (Fukuhara S, et al. in press). GCSF has a variety of biological roles, including effects on healing through the enhancement of proliferation. We hypothesized that GCSF directly affected diseased myocardium and enhanced proliferation of Troponin I positive cells through GCSF-receptor (Hamamoto M, et al. in press). More recently, Beltrami AP, et al. reported cardiac stem cells exist in the heart (Beltrami AP, et al. 2003).

Sata M, et al. reported that hematopoietic stem cells may contribute to atherosclerosis (Sata M, et al. 2002). Recently a Korean group conducted GCSF treatment for patients who had acute myocardial infarction followed by intracoronary stenting. They stopped enrollment because of unexpectedly high rate of in-stent restenosis in those patients who received GCSF

(Kang HJ, et al. 2004). They raised the important point that endogenous-stem cells could migrate into both injured myocardium and atherosclerotic lesions. If we can elucidate the physiological mechanism of endogenous-stem cell migration, we may be better able to control this process in the future treatment of myocardial injury.

Cardiac environmental Factors

Several reports have emphasized the importance of cardiac environmental factors in the cardiac differentiation of stem cells (Liechty KW, et al. 2000, Wang JS, et al. 2000). There are many possible factors, including myocardial injury, paracrine factors from the host, direct interaction between cardiomyocytes and stem cells (Condorelli G, et al. 2001, Reinecke H, et al. 2000, Tomita S, et al. 2002), intramyocardial pressure, and electrical stimulation.

We reported that direct cell-cell interaction is a key mechanism in the differentiation of bone marrow stromal cells derived from transgenic mice expressing green fluorescent protein (GFP mouse) into cardiac lineage in a co-culture system (Fukuhara S, et al. 2003). Bone marrow stromal cells showed synchronous contraction with cardiomyocytes from day 2. Immunostaining showed myosin-heavy chain from day 1, connexin 43 and atria-nucleotide peptide from day 2, and troponin I from day 4. Studies have demonstrated the ability of human circulating bone marrow cells to differentiate into cardiomyocytes (Badorff C, et al. 2003). In 2002, a critical issue of cell fusion was reported (Terada N, et al. 2002). In this study, embryonic stem cells and bone marrow cells were derived from a GFP mouse. A proliferating colony expressing GFP had DNA from the embryonic stem cells. Because of the small rate of fusion, this phenomenon could not be fully explained. The co-cultivation of human stem cells with cells from other animal species cannot proceed until the possibility of trans-species gene transfer is completely eliminated. Several reports investigated this fusion issue, however, those results were scattered (Balsam LB, et al. 2004, Murry CE, et al. 2004). Oh H, et al. reported that adult heart derived cardiac progenitor cells expressing stem cell antigen-1 differentiate into cardiomyocytes roughly equally, with and without fusion to

host cells (Oh H, et al. 2003). If the fusion occurs as a physiological process, it would be worth to investigate the fate and the role of fusioned cells in vivo.

Many unresolved issues remain in the progress towards future clinical applications of cell-based therapies. Unanswered questions include optimal cell population, cell density, and mechanism of effect responsible for the improvement of cardiac dysfunction. If we can resolve these issues then we might be able to match certain disease processes to the best cell therapy for treatment.

As progress continues, adverse effects will be identified. Arrhythmia may be a critical issue. In addition, we still do not know how the proliferation and differentiation of transplanted cells are regulated and the effects of environment. So far there have been no reports in humans of malignant tumor formation or ectopic differentiation such as bone formation. Although there are many reports supporting cell transplantation using animals, we still do not know how transplanted cells behave in humans. No efficient cell tracking method has been developed.

Whether the benefits of cell-based therapy or/and those of gene therapy or angiogenic protein therapy in the future remains to be seen by well-designed study.

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CHAPTER 2

DEVELOPMENT OF MYOCARDIAL SHEETS AND THEIR CELL SOURCES

Cell Sheet Technology for Myocardial Tissue Engineering

Tatsuya Shimizu, Hidekazu Sekine, Yuki Isoi, Masayuki Yamato, Akihiko Kikuchi, and Teruo Okano

Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

Summary. Myocardial tissue regeneration including isolated cell transplantation and recruitment of bone marrow stem cells via cytokines have now emerged as one of the most promising treatments for patients suffering from severe heart failure. As therapy advances, the challenge to engineer three-dimensional (3-D) myocardial tissue grafts has also started. Tissue engineering has currently been based on the technology using 3-D biodegradable scaffolds as alternatives for extracellular matrix (ECM). However, insufficient cell migration into the scaffolds and inflammatory reaction due to scaffold biodegradation remain problems to be solved. By contrast, we have proposed novel tissue engineering technology layering cell sheets to construct 3-D functional tissues without any artificial biodegradable scaffolds. Electrical and morphological communications are established between layered cardiomyocyte sheets, resulting in simultaneous beating 3-D myocardial tissues both *in vitro* and *in vivo*. Layered cardiomyocyte sheets *in vivo* present long survival and functional improvement in accordance with host growth. For vascularization problems, multi-step transplantation of layered cardiomyocyte sheets overcame the thickness limitation of bio-engineered constructs. Cell sheet technology should have enormous potential for engineering clinically applicable myocardial tissues.

Key words. Myocardial tissue engineering, Cell sheet, Vascularization

Myocardial tissue engineering

Recently, alternative treatments for heart transplantation have been requested to repair damaged heart tissue, because the utility of heart transplantation is limited by donor shortage. Isolated cell transplantation is now considered to be one of the most effective treatments for impaired heart tissue (Field and Reinlib 2000). Autologous myoblast transplantation has been performed clinically and the contraction and viability of grafted myoblasts have been confirmed (Menasche P, et al. 2001). Autologous bone marrow cell transplantation has also been clinically applied and its effectiveness has been reported (Galinanes M, et al. 2004). As another therapeutic strategy, stem cell recruitment from bone marrow, via glanulocyte-colony stimulating factor (G-CSF) administration, has been clinically performed (Takano H, et al. 2003).
Although myocardial tissue regeneration therapy has been realized, it is difficult to control shape, size and location of the grafted cells in direct injection of dissociated cells. Additionally, neither isolated cell transplantation nor G-CSF treatment is applicable for replacing congenital defects. To overcome these problems, the challenge of fabricating 3-D myocardial tissue grafts by tissue engineering technology has now begun (Mann and West 2001).

Scaffold-based tissue engineering

Tissue engineering has currently been based on the Langer and Vacanti's concepts that 3-D biodegradable scaffolds are useful as alternatives for extracellular matrix (ECM) and that cells seeded on the scaffold reform their native structure according to its biodegradation (Langer and Vacanti 1993). This context has been also used in myocardial tissue engineering. The popular approach is to seed cells into prefabricated polymer scaffolds (Fig.1**a**). Papadaki M, et al engineered 3-D cardiac constructs by using poly (glycolic acid) (PGA) scaffolds processed into porous meshes (Papadaki M, et al. 2001). They improved cell attachment by utilizing a rotating bioreactor. Leor et al reported that bioengineered cardiac grafts, using porous alginate scaffolds, attenuated left ventricular dilatation and heart function deterioration in myocardial infarction model (Leor J, et al. 2000). Furthermore, Li et al demonstrated that transplantation of tissue-engineered cardiac grafts using biodegradable gelatin sponges replaced myocardial scar and right ventricular outflow track defect (Li RK, et al. 1999, Sakai T, et al. 2001). Recently, decellularized tissues have been used as alternatives of prefabricating scaffolds (Fig.1**b**). As another

approach, polymerization after mixing cells and soluble ECM alternatives has been reported (Fig.1**c**). Zimmermann et al engineered 3-D myocardial tissue by gelling the mixture of cardiomyocytes and collagen solution (Zimmermann WH, et al. 2002). They measured isometric contractile force of the constructs as heart tissue model and confirmed their contractile function *in vivo* (Zimmermann WH, et al. 2002).

In the technology using biodegradable scaffolds, insufficient cell migration into the scaffolds and inflammatory reaction due to scaffold biodegradation remain problems to be solved. Furthermore, the scaffolds change into ECM, resulting in cell-sparse tissue formation. On the other hand, in native myocardial tissue, cells are considerably dense and electrically communicated each other. Therefore, new methodology to minimize the scaffolds has been now investigated.

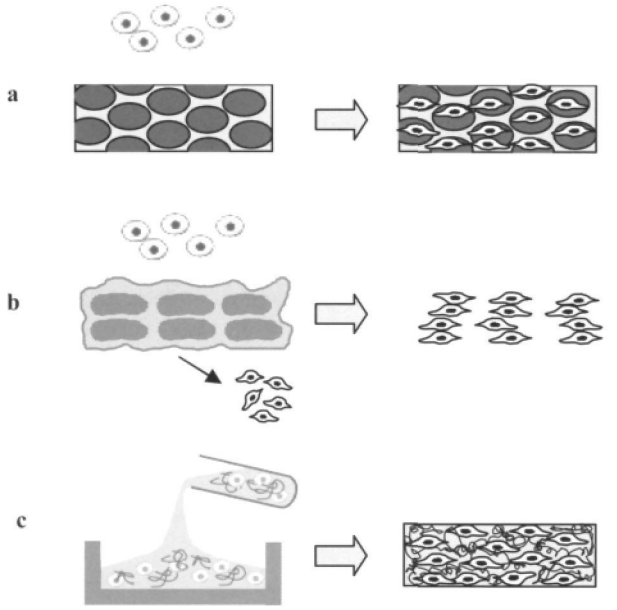

Fig. 1a, b, c. Tissue engineering methodologies. **a** Isolated cells are poured into a pre-fabricated porous scaffold. The scaffolds are biodegrated and ECM occupies the space within the cells, leading to 3-D tissues. **b** Existing tissue is decellularized once and utilized as a biological scaffold. **c** Mixture of isolated cells and biodegradable molecules are poured into an appropriate mold and then the molecules are polymerized. The construct is regenerated into tissues.

Cell sheet-based tissue engineering

In contrast to scaffold-based tissue engineering, we have proposed novel tissue engineering methodology that is to construct 3-D functional tissues by layering 2-D cell sheets without any biodegradable alternatives (Shimizu T, et al. 2003). To obtain intact cell sheets, we have exploited temperature-responsive culture dishes, from which cultured cells detach as a contiguous cell sheet simply by reducing temperature. This culture surface is covalently grafted with temperature-responsive polymer, poly (*N*-isopropylacrylamide) (PIPAAm) by electron beam (Okano T, et al. 1993). The surface is hydrophobic and cell-adhesive under culture condition at 37°C. By lowering temperature below 32°C, the surface changes reversibly to hydrophilic and is not cell-adhesive due to rapid hydration and swelling of the grafted PIPAAm chains. When cells are cultured confluently, they connect to each other via cell-to-cell junction proteins and ECM (Fig.2**a**). With enzymatic digestions including trypsinization, these proteins are all disrupted and the cells detach separately (Fig.2**b**). In the case of culturing the cells on PIPAAm-grafted dishes, cell-to-cell connections are maintained and cells are harvested as a contiguous cell sheet by decreasing temperature (Fig.2**c**). Furthermore, adhesive proteins underneath cell sheets are also preserved and they play a role as an adhesive agent in cell sheet transfer onto other culture surfaces or other cell sheets. Cell sheets are composed of confluent cells and small amount of biological ECM. Therefore 3-D cell-dense tissues are engineered by layering these viable cell sheets (Fig.3).

Pulsatile myocardial tissue engineered by layering cell sheets

In native heart tissue, cardiomyocytes are tightly interconnected with gap junctions and pulsate simultaneously. Therefore, it is critical whether electrical and morphological communications are established between layered cardiomyocyte sheets. To examine this point, two neonatal rat cardiomyocyte sheets, which were harvested from PIPAAm-grafted dishes, were overlaid. Two electrodes were set at monolayer parts of both cell sheets. As a result, electrical potentials of the two sheets were completely synchronized. Furthermore, electrical stimulation to the single-layer region of one sheet was transmitted to the other and the two cell sheets pulsated simultaneously. Histological analysis showed that bilayer cardiomyocyte sheets contacted intimately. Cell-to-cell connections including desmosomes and intercalated disks were observed by transmission electron

microscopy. These data indicate that both electrical and morphological communications are established between layered cardiomyocyte sheets (Shimizu T, et al. 2002). When 4 cardiomyocyte sheets were layered on frame-like collagen membranes *in vitro*, the constructs started to pulsate spontaneously in macroscopic view.

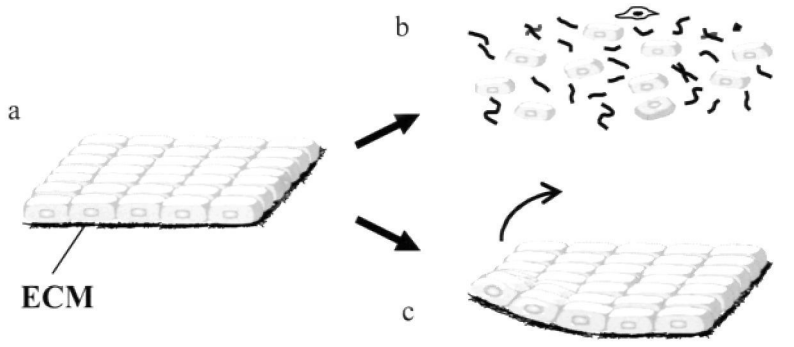

Fig. 2a, b, c. Cell sheet harvest from temperature-responsive culture surface. **a** When cells are confluently cultured, the cells attach on culture surface via ECM and connect to each other by cell-to-cell junctions. **b** In the case using protease digestion, cell-to-cell connections and ECM are disrupted and cells detach isolatedly. **c** When cells are cultured PIPAAm-grafted surface, cell-to-cell connections are completely preserved and the cells detach as a contiguous cell sheet. ECM retains underneath the cell sheet.

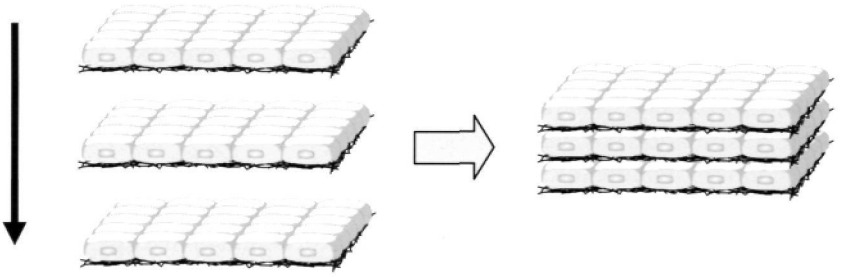

Fig. 3. Cell sheets harvested from temperature-responsive culture dishes are stacked, resulting in cell-dense 3-D tissue.

Next, to examine *in vivo* survival of layered cardiomyocyte sheets, triple-layer constructs were transplanted into dorsal subcutaneous tissues of nude rats. Surface electrograms originating from transplanted constructs were detected independently from host electrocardiograms 2 weeks after the operation. When transplantation sites were opened, macroscopic simultaneous beating of the grafted myocardial tissue was observed at the earliest period, 3days after the procedure (Shimizu T, et al. 2002). Furthermore, the grafts survived up to at least 1 year. Histological analysis demonstrated that enough neovascularization was organized in a few days. Cross-sectional views revealed stratified cell-dense myocardial tissues, well-differentiated sarcomeres and diffuse gap junctions. In time-course examination, the constructs developed morphologically and functionally in accordance with host rat growth. Size, thickness, conduction velocity and contractile force of transplanted myocardial tissue grafts increases as host rats grew. It is considered that both the increases of graft volume and contractile force are due to cardiomyocyte elongation and hypertrophy.

Thus, the basic technology using cell sheets for myocardial tissue engineering has been established.

Vascularization within engineered myocardial tissue

Sufficient supply of oxygen and nutrients is essential for functionally beating heart tissue. Therefore, vascular reconstruction within engineered tissues is one of the most critical issues in order to sythesise thicker and more functional myocardial tissue. Previous studies have revealed that the thickness limit of bio-engineered tissues is 50-200μm (Neumann T, et al. 2003). Although, in our studies, multiple neovascularization arose in transplanted cardiac grafts in a few days, primary insufficient oxygen and nutrient permeation also limited the number of transplanted cardiomyocyte sheets (3-4 sheets). Therefore, new additional methodology to accelerate vascular formation has been pursued.

Generally, administration of growth factors or gene-modification of cardiomyocytes may improve neovascularization. Co-culture with cells, which have the potential to differentiate into endothelial cells, may also improve primary vascular formation. In cell sheet technology, it has been reported that a single layer of endothelial cell sheet enhances the capillary formation *in vivo* (Soejima K, et al. 1998). Therefore, insertion of an endothelial cell sheet between cardiomyocyte sheets may promote neovascularization. We are now examining the effect of isolated endothelial progenitor cell insertion and endothelial cell sheet insertion within layered cardiomyocyte sheets.

As another approach for vascularized tissue reconstruction, we repeated transplantation of triple-layer myocardial tissue grafts after waiting neovascularization to enable sufficient nutrient supply. The two grafts overlaid in rat dorsal subcutaneous tissues and were completely synchronized at 1 week. Electrical stimulation to the first graft was transmitted to the second one. Azan staining showed that two myocardial tissue grafts connected to each other intimately and the whole transplanted tissues survived with well-organized vasculature without necrosis 1 month after the procedure. Furthermore, 10-time stacking of myocardial tissue grafts realized about 1mm-thick simultaneously beating grafts. So multi-step transplantation of layered cell sheets has overcome vascularization problems in tissue engineering.

Future views

It has been demonstrated that cell sheet-based technology has enormous potential in myocardial tissue engineering. However, there are some critical problems to be solved. The first one is myocardial cell sourcing. Further advance in stem cell biology for cardiomyocytes will be needed to realize clinical application of bioengineered myocardial tissues. The second issue is vascular reconstruction within the constructs. We successfully engineered cell-dense thick myocardial tissue with vascular network at least *in vivo*. The next challenge is to reconstruct vascularized myocardial tissue *in vitro*. Concerning *in vitro* thick tissue fabrication, not only microvasculars but also connectable vasculars should be accompanied with the constructs for surgical anastomoses. Interdisciplinary research and development will be needed to overcome these obstacles and to engineer clinically applicable myocardial tissues.

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Myocardial Regeneration Therapy with Tissue Implantation of Autologous Myoblast Sheets for Severe Impaired Heart Failure

Yoshiki Sawa, Imuran Memon and Hikaru Matsuda

Dept of Surgery, Osaka University Graduate School of Medicine

Summary. With regards to the therapy for end-stage failing hearts, the heart transplantation is the only effective treatment but is still limited by donor shortage and chronic rejection. Therefore, a novel strategy for myocardial regeneration is desired. Cell transplantation, a new approach for restoring impaired hearts still has several problems. Especially, autologous skeletal myoblasts (Skm) transplantation (Ctx) by injection has been clinically applied. However, its optimal effect and stable delivery methods are undetermined. Tissue implantation provides stable cell delivery with maintained inter-cellular communication and extracellular matrix. We hypothesize that the Skm tissue cardiomyoplasty might be more advantageous in regenerating the impaired heart. LAD ligated hearts (2 weeks) received Skm Ctx either by injection (MI; n=9) or by implanting two engineered monolayer myoblasts sheets (MS; n=9) or non-cellular therapy (Control=C; n=10). After 8 weeks, higher improvement of ejection fraction (%) was measured in MS compared to that of the other groups. Histological comparison revealed greater cellularity and abundant widespread neo-capillaries with a noticeably uniformly thickened wall in MS only. These results demonstrated that engineered skeletal myoblasts sheets regenerated the impaired myocardium, suggesting a promising therapy for severe heart failure.

Key Words. Myocardial regeneration therapy, Autologous myoblast, Bone marrow, Tissue engineering, Cell sheet

Introduction

The ventricular assist systems and heart transplantation are the only means available for dealing with terminal stage heart failure that is too severe to respond to medical treatment using beta-blockers or ACE inhibitors. We have reported data showing the clinical usefulness of this type of therapy in Japan. However, these replacement type therapies for severe heart failure involve many unresolved problems, such as donor shortage, immunosuppression and complications. These problems are hampering the application of replacement type therapies to many patients with severe heart failure.

In recent years, a great deal of attention has focused on myocardial regeneration as a new means of treating heart failure. Isolating myocardial stem cells and attempts of myocardial repair by progenitor cells from bone marrow have become the latest topics of intense discussion. Meanwhile, success at improving cardiac function by cell transplantation has been reported among the new attempts to treat heart failure, and clinical trials of the transplantation of myoblasts have started. However, the use of cell transplantation as a new approach for restoring impaired hearts still has several limitations. Therefore, a novel strategy for myocardial regeneration is desired.

Skeletal myoblasts, being autologous with feasible and safe clinical application experience (Pagani FD, et al. 2003), maintains a muscle regenerative quality with growing power to millions and acts as the most suitable autologous cell type for clinical application. Several research laboratories have reported the improved heart performance with myoblasts transplantation (Pouzet B, et al. 2001). However, the needle injection method disrupts the micro-intercellular communication, restricts the cell survival and growth and thus, appears deleterious to cell transplantation therapy (Okano T, et al. 1993).

Tissue engineering for tissue regeneration fulfills our criteria to repair the heart and discards such drawbacks (Ozawa T, et al. 2002). Utilization of scaffolds for tissue engineering is supportive but subject to issues regarding biocompatibility, biodegradability, cytotoxicity and surface adhesive molecule loss (Leor J, et al. 2000). Eliminating such disadvantageous hindrances and avoiding scaffolds use, we engineered cardiomyocyte sheets (Miyagawa et al. 2004). These cell sheets showed preserved cellular communication junctions,

endogenous extracellular matrix (ECM) and integrative adhesive agents (Shimizu T, et al. 2002). Non-ligature implantation of these sheets to infarct myocardium showed their integration with impaired myocardium and improved cardiac performance (Miyagawa et al. 2004). For clinical application, use of skeletal myoblasts sheets averts ethical and cell source issues.

In this paper, we report on our attempt to perform myocardial regeneration based on tissue engineering technology using autologous myoblast sheets, which was commenced in order to deal with severe heart failure, and with the goal of establishing a regeneration type therapy for severe heart failure.

Methods

One hundred and seventy seven Lewis strain female and male rats (180-220g; 6-10 week old; Seac Yoshitomi Ltd, Fukuoka, Japan) were used for this study. Humane animal care was used, in compliance with the "Principles of laboratory Animal care" formulated by the National Society for Medical Research and the " Guide for the care and Use of Laboratory Animals" prepared by the institute of Animal Resource and published by the National Institute of Health (NIH Publication No. 86-23, revised 1985. Under ketamine and xylazine anesthesia, acute myocardial infarction was induced in the rats by ligation of the left anterior descending coronary artery (LAD) for 14 days.

Myoblast Isolation

The tibialis anterior muscle was removed and placed in ice-cold normal saline solution. Superficial connective tissues and tendons were carefully removed. The isolated muscle was minced for 5-10 minutes and slurry muscle was treated with 1% of collagenase in Dulbecco's Modified Eagle Medium (DMEM) at 37° for 25-30 minutes. To remove the fibroblasts contamination, percoll density centrifugation was carried out. The cells were cultured in growth medium containing 80% DMEM and 20% fetal bovine serum (FBS) with 1% of antibiotics (5,000 IU/ml of penicillin and 5,000 μg/ml of streptomycin). Cultures contained 70-80% myoblasts as checked by desmin in vitro staining on the 7th day.

Myoblast-Sheet Tissue Engineering

Special thermoresponsive polymer-coated dishes were used for cell-sheet engineering. Specific procedures for preparation of these cell-culture dishes are described elsewhere (Shimizu T, et al. 2003). Briefly, temperature responsive polymer, poly-Nisopropylacrylamide (PIPAAm), was coated on a culture dish. These polymers are hydrophobic at 37° C and become hydrophilic when placed at temperatures lower than 20° C. The grafted polymers provided an adhesive base (hydrophobic) for cells at 37°C while released (hydrophilic) at < 20°C. Purified myoblasts were cultured for 7 days. Next day, the cell suspension, with a density of $5x10^6$, was then plated in the thermoresponsive cell culture dish at 37° C incubator for 1-2 days. Microscopically, myoblasts were seen attached to the surface of the culture dish within the squared shaped boundary of adhesive polymer. To release the confluent cells as a cell sheet, culture dishes were placed in another incubator, and set at 20° C for less than an hour. Spontaneous detachment of interactive myoblasts as square-shaped sheets occurred. The size of the sheet ranged to $1.00\pm0.05cm^2$ in area and $50\pm10.0\mu m$ in thickness. Shrunken, corner overlapped sheets were obtained in this manner. The sheets were then allowed to float in another dish to unfold the overlapped corners. Finally two sheets were piled to make a thicker sheet. A collagen sheet (33 mm thick) is overlapped on the cell-sheet surface to support delivery of the myoblast-sheets to the infarcted myocardium. The sheets were carefully handled from the corners by using sharp forceps (Fig. 1) and were directly placed over the scar area. Microscopic observation showed monolayer cell sheets in the culture dish but appeared as multi cell layers (2-3), due to shrinkage, after detachment. Maximum care was taken to maintain the width and prevent folding of the cellular sheet.

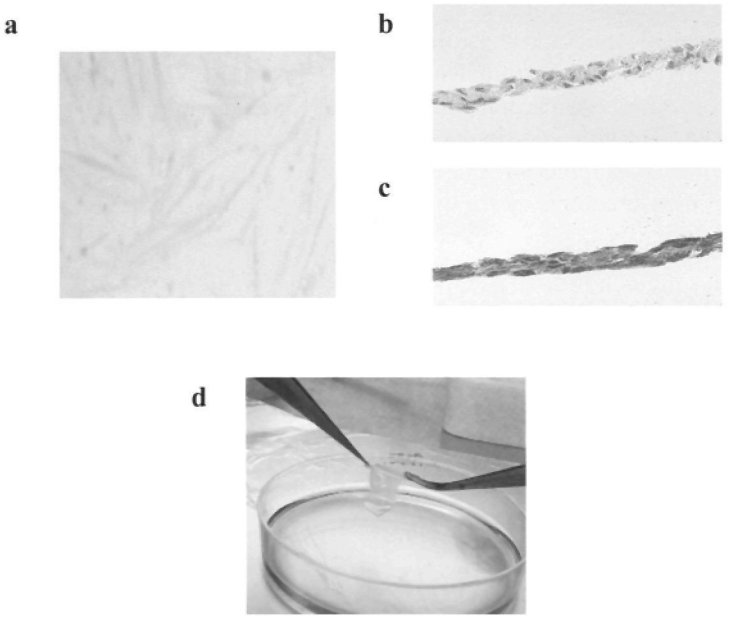

Fig. 1 . Myoblast-sheet construction and implantation **a** Desmin stained cultured myoblasts. (**b, c**) HE and MT with a thickness or 50μm. **d** The area of the myoblasts-sheet: 1.00±0.05 cm^2 (indicated by black arrow; appears as milky white shrunken contiguous cell-sheet). Red arrow indicates the collagen sheet (appears visibly transparent) which is overlapped on the cell-sheet surface for cell sheet delivery support.

Assessment of Cardiac Performance

After anesthesia heart function was monitored at baseline (2 weeks after LAD ligation) and at 2, 4 and 8 weeks after the cell transplantation. Echocardiography was performed using an ultrasound machine (Sonos 5500, Agilent Technologies, USA) equipped with a annular-array transducer operating at 12Mhz. Parasternal short and long axis views in B and M imaging

Modes were performed. The heart was imaged in 2-D mode in the short axis views at the position coinciding with the largest left ventricle (LV) diameter, and then the systolic and diastolic diameters were determined. Global cardiac parameters such as LV dimension at end-diastole (LVDd), LV dimension at end-systole (LVDs), cardiac output (CO) and ejection fraction (EF) were determined. The left ventricular end-systolic dimension was measured at the time of the most anterior systolic motion of the LV posterior wall while diastolic measurements were made at the time of apparent maximal LV diastolic dimension.

Myoblasts transplantation and histology

The recipient rats were anesthetized and a thoracotomy was performed between the fourth and fifth intercostal space. Depending on the mode of therapy, the rats were divided in 3 groups: MS, myoblast sheet implantation ($5x10^6$ cells × 2 sheets, n=29); MI, myoblasts suspension (1×10^7 cells, n=29); and C group, non-cellular collagen sheet implantation, (n =30).

Statistical Analysis

All data are presented as mean ± SD. To assess the significance, data were analyzed between the groups by using a 2-way repeated-measures analysis of variance (ANOVA) test. If a significant F ratio was obtained, further analysis was carried out with a post hoc test. A value of $P<0.05$ was considered statically significant.

Results

Effect of Myoblast

Transplantation on Cardiac Performance

Myocardial infarction resulted in <20% acute mortality rate within 24 hours of operation. Ventricular remodeling weeks after LAD ligation characteristically resulted both in global cavity enlargement and cardiac pump dysfunction as measured by echocardiography at baseline. Engineered-sheet implants in MS resulted in a significantly reduced LV chamber diameter and improvement in cardiac performance after the treatment, as compared with the other two groups (Fig. 2).

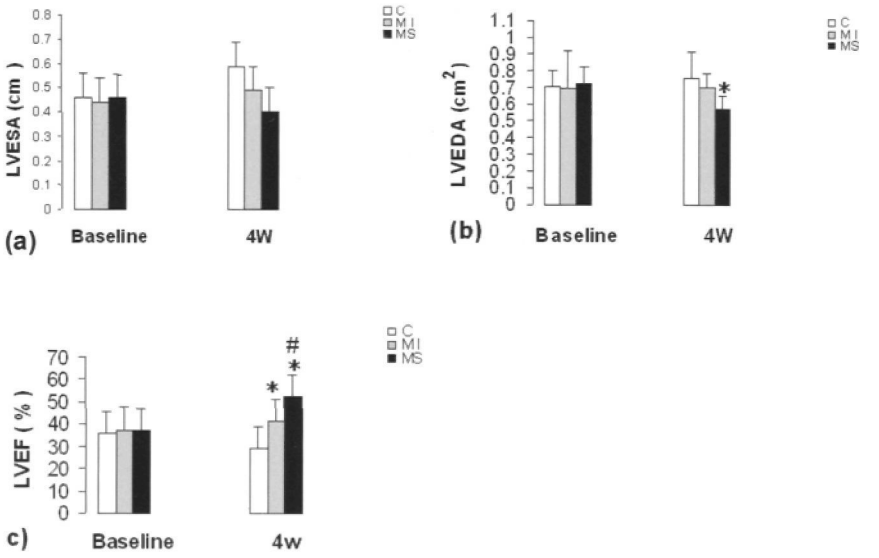

Fig. 2. Echocardiography measurement of cardiac performance before and after the cell therapy. **a** As measured by left ventricular end-systolic area (LVESA), MS showed attenuation and reduction of LV chamber dilatation as compared to the MI and C groups. **b** MS also showed significantly reduced left ventricular end-diastolic area (LVEDA) at 2, 4 and 8 weeks after the cell-sheet therapy. **c** Left ventricular ejection fraction (LVEF) was significantly higher in Myoblast sheet implant (MS) group and these improvements were preserved for 8 weeks. Myoblast injection (MI) showed initial attenuation of further LV chamber dilatation while control **c** group showed progressive LV chamber dilatation; *P<0.05 vs Control; #P<0.05 MS vs MI.

Histological findings

Stainings of cell sheets revealed that the engineered myoblasts sheets were highly cellularized lacking collagenous contents (Fig. 1). Histological stainings in MS revealed that the anterior wall was richly cellularized occupying the whole scar area and was uniform in thickness. MS showed preservation of thickened anterior wall until 8 weeks after the sheet implantation. MI cell recipient hearts also showed rounded small sized dispersed cells with surrounding collagen content. The infarct hearts in C were

thin walled, devoid of cells, and carried dense collagenous scars in the anterior wall area.

In view of histological finding by Masson' s trichrome staining(Fig.3), non-cellular collagenous sheet implantation, control group, also showed complete scar formation after 2 weeks of baseline measurements. Two weeks after the myoblast injection therapy, the scar area was dispersely replaced by cellular contents. Two weeks after the myoblast sheet implantation therapy, MS group showed greater replacement of scar area with higher cellularity. Cells appeared more or less round or oval in shape, resembling hematopoietic stem cells morphology.

Moreover, we evaluated the increased vascular density in the infarct myocardium after cell transplantation (Fig. 4). Representative immunohistologic staining by von Willebrand antibody shows neo-vascularization in Myoblasts-sheets, Myoblast injectionand Control groups at low magnification.

After 2, 4 and 8 weeks of cell transplantation, serial sections that were immunostained by directing against the fast and slow MHC isoform, indicated the survived multinuclei elongated structures. Our bservations showed positive staining of skeletal myofibers for fast MHC while a small number of skeletal myofibers also stained positive for slow MHC isoforms. We could also detect co-expression of fast and slow MHC in skeletal myofibers. The same sections stained negative with Troponin T, specific for cardiac myofibers, reconfirming their skeletal type identity (figure not shown).

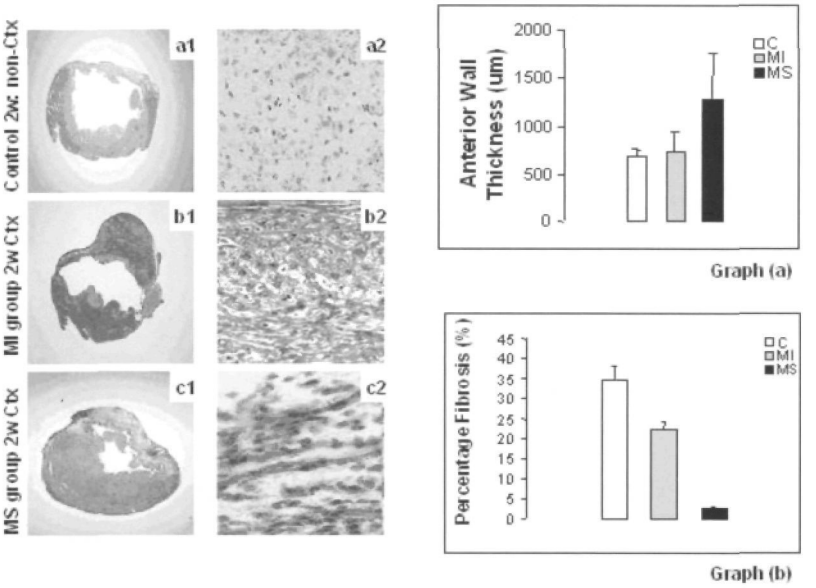

Fig. 3. Histological finding by Masson's trichrome staining. **a1, a2** Non-cellular collagenous sheet implantation, control group, also showed complete scar formation after 2 weeks of baseline measurements. **b1, b2** Two weeks after the myoblast injection therapy, the scar area was dispersely replaced by cellular contents. **c1, c2** Two weeks after the myoblast sheet implantation therapy, MS group showed greater replacement of scar area with higher cellularity. **b2, c2** Cells appeared more or less round or oval in shape, resembling hematopoietic stem cells morphology. **Graph (a)** shows a significant thicker, uniform anterior wall in MS group, while the other two groups show a thin anterior wall. Magnification; ×10 (**a1, b1, c1**); ×400 (**a2, b2, c2**). **Graph (b)** shows the percentage fibrosis comparison between 3 groups. The percentage of fibrosis was significantly reduced in MS group *$P<0.05$.

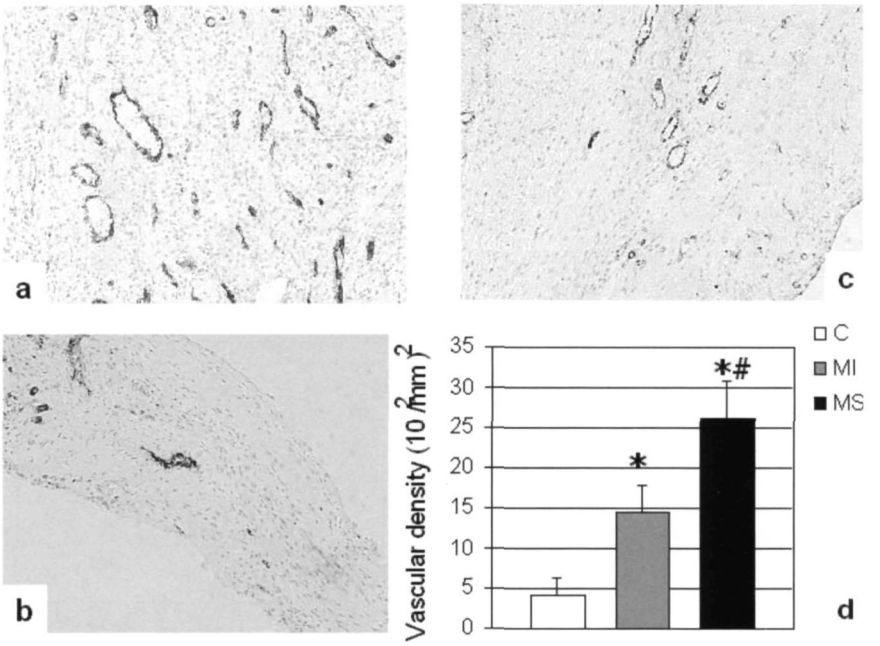

Fig. 4. Increased vascular density in the infarct myocardium after cell transplantation. Representative immunohistologic staining by von Willebrand antibody. **a,b,c** shows neo-vascularization in Myoblasts-sheets (MS), Myoblast injection (MI) and Control **c** groups at low magnification (×100). **d** Shows a significantly higher number of vessels in the MS group as compared to MI and C groups; *P<0.05 vs control; #P<0.05 MS vs MI.

Discussion

In this study we aimed to determine whether engineered autologous myoblast sheets regenerates the infarcted myocardial wall to attenuate the cardiac remodeling for improving the cardiac performance.

The concept of regenerative medicine and tissue engineering is to deliver healthy cells to the damaged or diseased tissues of the body and stimulate their repair and regeneration for restoration of the desired organ's function (Lanza RP, Langer R and Vacatni J. 2003). Tissue engineering, based on the use of

scaffolds rescues the damaged tissue through tissue regeneration. However, Zimmermann et al. (Zimmermann WH, et al. 2002) and Kofidis et al. (Kofidis T, et al. 2002) have reported several potential drawbacks accompanied with scaffold tissue constructs, such as the absence of evenly distributed vascularization and perfusion, non-homogenous compact tissue construct, non-homogenous cell distribution and viability in the tissue, limitation of cells function due to impaired nutrient availability and defective tissue geometry. Pondering the consequences, fate and objectives of our transplanted cells, we engineered a tissue without scaffolds. Geometry and size of our myoblast sheets were compatible to provide stable and even cell delivery to the whole infarct myocardial area. Beneficial for cell transplantation, as reported by Shimizu et al., the cell-sheet tissue maintains intact membrane proteins and adhesive proteins, incorporates ECM molecules and secretes growth factors due to favorable cell to cell cross-talk and prevents cellular micro-environment disruption due to enzymatic reactions (trypsin or dispase) for cell detachment from cell culture dishes (Kushida A. et al. 1999).

After implantation, our results demonstrated a uniform regeneration in the anterior wall, which played a primary role in re-remodeling the geometry of the LV chamber by probably providing a new structural and cellular mask. The reason for the attenuation of remodeling also appears to be the efficient delivery of sheet which overlaps the scar area and its borders in the form of a cellular bridge. A well maintained microenvironment, then, supports all necessary primary cellular activities. Considering the MI group, myoblasts settle in the cavities surrounded by the fibrous scar and begin the basic and delayed establishment of their microenvironment, which proves fatal for their primary survival. But even with an outstanding tissue construction and implantation, one should still expect the death of transplanted cells due to possible odds between the natural in vivo and artificially constructed in vitro tissue environment.

Our engrafted myoblasts continued to express fast skeletal myosin heavy chain isomers and they did not transdifferentiate into cardiomyocytes after grafting. Transplanted skeletal myoblasts showed a change to slow twitch myofibers, expressed slow myosin heavy chain isomers, and thus showed their ability to handle cardiac-type load (figure not shown). Our findings are in agreement with the reports by Murray et al. (Reinecke H, et al. 2000). We did not detect arrhythmias in any of the animals that received transplanted myoblasts. These results are in agreement with the report that fewer

transplanted myoblasts is a safe, non-arrhythmogenic therapy (Reinecke H et al, 2002).

Moreover, myoblasts also act as the natural supplier of hepatocyte growth factor (HGF) and prove to be an angiogenic and anti-fibrotic factor by utilizing it for its own growth and recruiting other cells (Tatsumi R, et al. 1998). Similar to SDF-1, the stable release of HGF by MS, as compared to MI, might have an advantageous role for a damaged heart. In addition, HGF facilitates $CD34^+$ cell molititym CXCR4 expression and SDF-1 mediated directional migration (Kollet O, et al. 2003).

Color kinesis results from our past study showed no recovery in regional systolic function after the fibroblast sheet implantation (Miyagawa et al. 2004). Taylor et al. also showed the failure in improvement of systolic function by fibroblasts transplantation compared with myoblasts transplantation. Mesh implantation only supports the preservation of LV geometry without improvement in its regional systolic function. These results indicate that a fibroblast-sheet or mesh should be used for a control group.

We conclude that tissue cardiomyoplasty is superior to cellular cardiomyoplasty, regenerates the infarcted myocardial wall to attenuate the cardiac remodeling and improves cardiac performance.

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min stained cultured myoblasts. (b and c) HE and MT with a thickness or 50μm. (d) The area of the myoblasts-sheet: 1.00±0.05 cm^2 (indicated by black arrow; appears as milky white shrunken contiguous cell-sheet) Red arrow indicates the collagen sheet (appears visibly transparent) which is overlapped on the cell-sheet surface for cell sheet delivery support (d) Masson's trichrome staining shows non-collagenous nature of myoblasts sheet with thickness of 50±10.0μm.

Cardiovascular Cell Differentiation from ES Cells

Jun Yamashita

Laboratory of Stem Cell Differentiation,Stem Cell Research Center, Institute for Frontier Medical Sciences, Kyoto University.53 Shogoin Kawahara-cho, Sakyo, Kyoto 606-8507, Japan.

Summary. Pluripotent embryonic stem (ES) cells are potent materials for both regenerative therapeutic approaches and developmental research. Recently, a novel ES cell differentiation system combined with 2-dimensional culture and flowcytometry assisted cell sorting (FACS) has been developed. In this system, cells in the cardiovascular system, that is, endothelial, mural, blood cells and cardiomyocytes can be systematically induced from common progenitor Flk1 (vascular endothelial growth factor receptor-2)-expressing cells. This system can constructively reproduce various stages of cardiovascular development in vitro, such as cell differentiation, diversification, and higher structure formation, providing novel possibilities to elucidate the cellular and molecular mechanisms of cardiovascular development. Cardiovascular cell induction from primate ES cells reveals primate-specific developmental mechanisms. ES cells will also contribute to regenerative medicine not only as a cellular source for transplantation but also for discovery of novel genes and drugs for regeneration. In this review, the significance of ES cell study in basic science and clinical medicine of cardiovascular field is discussed.

Key Words. Embryonic stem cells, Cardiomyocytes, Endothelial cells, differentiation, Regenerative medicine

Embryonic stem (ES) cells are pluripotent stem cells established from the inner cell mass of the embryo at the blastocyst stage. They maintain self-renewality and are capable of differentiating into a wide range of cells both in vitro and in vivo. As ES cells can contribute to many different tissues, including the germ line, in chimeric embryos, they enable the introduction of various genetic alterations into mouse species such as knock-out mice, with a large contribution to biological development. ES cells also possess the potential to give rise to a variety of cells in vitro. Now many trials are being carried out to draw-out their differentiation potentials in vitro in order to develop novel cell-based regenerative medicine for human disease and injury. Recent establishment of human ES cells (Thomson JA, et al. 1998) is facilitating ES cell research for regeneration. Cardiovascular regeneration is one of the most important targets of regenerative medicine, because coronary ischemic artery disease is still the predominant cause of premature death. Induction of cardiomyocytes and endothelial cells has been already achieved in both mouse and human ES cells. ES cells are, thus, a promising cell source for cardiovascular regeneration as well as somatic stem cells in bone marrow and so on.

Another indispensable role of ES cells is as a potent tool for dissecting the differentiation process of mammalian cells in vitro. The in vitro reproduction of cell differentiation and tissue development using ES cells provides us novel possibilities to elucidate their cellular and molecular mechanisms. In particular, human ES cell study could be a unique method to investigate the cellular and molecular mechanisms of human development to which knockout animal approach is not available. ES cell study would provide a novel methodological basis for developmental biology and regeneration therapy.

Induction of ES cells to vascular cells

During normal development of the embryo, the majority of vascular cells are considered to be derived from the mesoderm. Shortly after gastrulation, the mesoderm forms as a distinct layer between the endoderm and ectoderm. Expression of vascular endothelial growth factor receptor-2 (VEGF-R2), also known as Flk1 in mice, is an indicator of the lateral plate

mesoderm. Flk1 is also designated as the earliest functional differentiation marker for blood and endothelial cells. Flk1 deficient mice failed to develop blood and endothelial cells (Shalaby F, et al. 1995), and both vascular and hematopoietic lineages were shown to be differentiated from $Flk1^+$ cells in embryo or ES cells (Yamashita J, et al. 2000).

Until now, embryoid bodies (EBs) which form as aggregates of ES cells were often used to induce various cell types including endothelial cells , neural cells , cardiomyocytes , and blood cells and so on . Spontaneous differentiation of ES cells occurs in EBs by the interaction of cells within EBs, partially or locally mimicking the body plan or positional information in the embryo. Developing EBs contain various cell types including endothelial cells and mural cells (pericytes in capillary vessels and vascular smooth muscle cells in arteries and veins) and often form blood vessel-like structures (Feraud O, et al. 2001 and Vittet D, et al. 1996). This system is often used to evaluate the overall potentials of ES cell differentiation. Though the EB method is convenient for inducing differentiation, it has several weak-points in dissecting cellular and molecular mechanisms during differentiation such as: **i)** Difficulty to dissect the differentiation mechanisms by highlighting cells and signals of interest among the complicated cellular interaction in EBs. **ii)** Difficulty to directly observe differentiating cells at the cellular level by microscopy. **iii)** Inability to conduct single cell analysis of differentiation. **iv)** Difficulty in dissociating cell aggregates and obtaining single cell suspensions of induced cells. These drawbacks have been hampering further analyses of cell differentiation process and its molecular mechanisms using ES cells.

To overcome these disadvantages in EB cultures, a novel ES cell differentiation system under 2-dimentional culture conditions has been developed. This system is amenable for easy monitoring or manipulation of cells and the analysis of differentiation at the cellular level. $Flk1^+$ cells are induced in a monolayer culture of ES cells on type-IV collagen-coated dishes in the absence of LIF (leukemia inhibitory factor) for 4 days. Presumptive $Flk1^+$ lateral plate mesoderm cells are purified by FACS (flow-cytometry assisted cell sorting) using anti-Flk1 antibody. Vascular cells are then induced by several culture conditions using purified $Flk1^+$ cells as starting material. By this system, all cellular components of blood vessels,

that is, endothelial cells, mural cells, and blood cells can be differentiated from the common progenitor $Flk1^+$ cells, and vascular structures form both in vitro and in vivo (Yamashita J, et al. 2000 and Yurugi-Kobayashi T, et al. 2003).

Differentiation of endothelial cells from ES cells

In EBs, gene expression of Flk1 is observed from differentiation day 3 and becomes prominent at day 4. Detection of Flk1 protein by immunofluorescent staining occur one day later than mRNA detection. Endothelial cell markers, such as CD31, VE (vascular endothelial)-cadherin, and tie-2 are detected approximately one or two days after Flk1 expression. Vascular-like structures with $CD31^+$/VE-$cadherin^+$ cells are observed within EBs at day 11 (Vittet D, et al. 1996). When EBs are cultured in type-I collagen gel, sprouting of vascular-like structures with endothelial cells and mural cells are observed (Feraud O, et al. 2001). Differentiation of vascular cells in EBs may largely recapitulate the vascular development process in the embryo.

To trace the vascular differentiation process at the cellular level, especially at the single cell level, 2-dimentional vascular differentiation is required. $Flk1^+$ cells are induced on type-IV collagen-coated dishes at day 4 of differentiation, which is almost the same time course of $Flk1^+$ cell induction in EBs. When purified $Flk1^+$ cells are re-cultured on type-IV collagen-coated dishes with serum and VEGF (vascular endothelial growth factor), sheet-like structures of $CD31^+$/VE-$cadherin^+$ endothelial cells appear. During the differentiation of purified $Flk1^+$ cells, most of $Flk1^+$ cells lose their expression of Flk1 one day after the re-culture. Cells that retain Flk1 expression start to co-express other endothelial cell markers, such as CD31 and VE-cadherin. Another endothelial cell marker, CD34 is observed one day later than CD31 and VE-cadherin expression (Hirashima M, et al. 1999). On the contrary, cells that have lost Flk1 expression then become positive for mural cell markers, smooth muscle actin (SMA), platelet-derived growth factor (PDGF) receptor-beta, and so on. $Flk1^+$ cells cultured on type-IV collagen with serum and VEGF show selective differentiation to vascular cells, endothelial cells and mural cells (Fig.1). Single

cell culture of $Flk1^+$ cells demonstrate that $Flk1^+$ cells possess the bipotency to give rise to both vascular cells at the single cell level, and that $Flk1^+$ cells suffice as vascular progenitor cells (Yamashita J, et al. 2000).

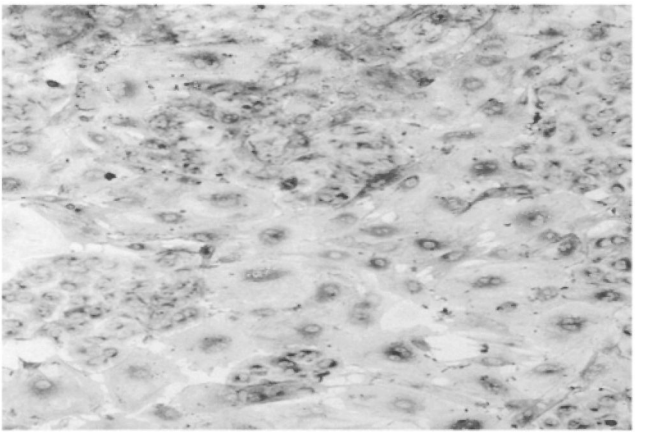

Fig. 1. Cardiovascular cell differentiation from Flk1+ cellsVascular cell differentiation from Flk1+ cells. Purified Flk1+ cells were cultured on typeIV collagen-coated dishes with VEGF (50ng/mL) and serum for 3-4days. Cells were then stained with endothelial cell marker, CD31 and mural cell marker, a-smooth muscle actin. CD31+ endothelial cells (*small, dark*) and SMA+ mural cells (*large, light*) selectively appeared from Flk1+ cells in this culture condition.

For endothelial cell differentiation from $Flk1^+$ cells on collagen, VEGF is essential. In the absence of VEGF, no endothelial cells appear from $Flk1^+$ cells in serum-free condition. Other angiogenic factors, such as basic FGF (fibroblast growth factor) show less effect on endothelial induction than VEGF. Among VEGF isoforms, $VEGF_{165}$ is more efficient than $VEGF_{121}$, and Flt1 (VEGF receptor-1) specific ligand, but PlGF (placental growth factor) has no effect on endothelial induction (Yamashita J, et al. 2000). Moreover, VEGF-E, specific Flk1 ligand, shows a prominent and a sustained effect on endothelial differentiation (Hirashima M, et al. 2003), indicating that Flk1 and neuropilin (a co-receptor of Flk1 for only

$VEGF_{165}$), but not Flt1, are involved in endothelial cell differentiation from $Flk1^+$ cells.

Endothelial maturation is also critical in the application of induced cells for transplantation. Transplanted ES cell-derived vascular cells can contribute to in vivo angiogenesis both in the embryo (Yamashita J, et al. 2000) and in tumor-bearing adult mice. VE-cadherin$^+$ early endothelial cells but not $Flk1^+$ mesoderm cells can specifically contribute to newly formed tumor vessels, and efficiently increase tumor blood flow (Yurugi-Kobayashi T, et al. 2003). This result suggests that the adjustment of maturation stage between transplanted endothelial cells and recipient tissues should be important for the specificity and efficacy of the cell integration, and successful schemes of cell transplantation therapy for vascular regeneration.

Several ES cell-derived endothelial cell lines have been reported. Induced endothelial cells are immortalized by polyoma middle T or selected by tie-1 promoter-driven puromycin resistance gene expression. They showed common properties of endothelial cells and the latter line was demonstrated to be integrated into in vivo angiogenesis. Development of specified endothelial cell lines in various differentiation stages should be useful not only for research on vascular biology, but also for practical application to vascular regeneration, especially for the industrialization of vascular regeneration by generation of transplantable cell products.

Differentiation of mural cells from ES cells

The origin and differentiation process of mural cells are more obscure than those of endothelial cells. Vascular smooth muscle cells (VSMCs) in a vascular tree rooted at the departure of the aorta from the heart and the vessels of the face and forebrain originate from neural crest-derived ectodermal cells. VSMCs of coronary arteries are differentiated from proepicardial cells, a common progenitor for epicardium and coronary VSMCs . The remaining mural cells are hypothesized to arise from mesenchymal cells, mainly derived from mesoderm (Darland D'Amore 1999). In ES cell study, although EB methods have revealed several factors and genes involved in mural cell differentiation such as desmin, serum re-

sponse factor, myocardin , retinoic acid and db-cyclic-AMP , the origin and differentiation process of mural cells have not been demonstrated.

Vascular development system from $Flk1^+$ cells was the first report that revealed an origin and differentiation pathway for mural cells using ES cells. SMA^+ mural cells arise from $Flk1^+$ cells, even from a single cell. Small amounts of mural cells appear even in serum-free condition, and the appearance of mural cells is strongly enhanced by the addition of serum or PDGF-BB, but not PDGF-AA (Yamashita J, et al. 2000). This is consistent with PDGF-B or PDGF receptor-β knock-out mice studies that show PDGF-B/receptor-β signaling is not essential for de novo differentiation of mural cells but required for pericyte proliferation and recruitment to the vascular wall (Hellstrom M, et al. 1999).

The origins of mural cells are not restricted to $Flk1^+$ cells. Mural cells can arise from $Flk1^-$ cell populations, such as $Flk1^-$/PDGF receptor-α^+ para-axitial mesoderm cells (unpublished data). Transdifferentiation of endothelial cells to mural cells is also suggested in embryonic development (DeRuiter MC, et al. 1997), ES cell differentiation (Ema M, et al. 2003) and an ES cell-derived endothelial cell line (Sone M, et al. 2003). These results indicate that mural cells may be a default fate of mesoderm cell differentiation. What machinery, including epigenetic mechanisms, forms such default pathways should be elucidated.

Endothelial cell specification during ES cell differentiation

Recently, various molecular markers for specified endothelial cells, that is, arterial, venous, and lymphatic endothelial cells have been reported. Receptor tyrosine kinase, EphB4 and its ligand, ephrin-B2 were reported as the first venous and arterial endothelial cell markers, respectively (Wang HU, et al. 1998). Since this report, various artery-, vein-, and lymphatic-specific genes have been documented (**table 1**). These findings enable us to study endothelial specification at the cellular level, without prior knowledge of the vessel location. Nevertheless, induction of specified endothelial cells from ES cells has not been reported so far. We recently found culture conditions for the induction of ephrin-$B2^+$ arterial, ephrin-$B2^-$ venous, and prox-1^+ lymphatic endothelial cells from $Flk1^+$ cells

(unpublished data). Induction of arterial endothelial cells was blocked by the inhibition of notch signaling (unpublished data), being compatible with previous reports that notch signaling is essential for the induction of the arterial endothelium in zebrafish (Lawson and Weinstein 2002). Constructive induction of specified endothelial cells using ES cells should be a new approach to elucidate cellular and molecular process of endothelial diversification.

Table 1. Markers of arterial-, venous-, and lymphatic-endothelial cells in the mouse(Yamashita in press)

Artery	Vein	Lymphatics
ephrin-B2	Eph-B4	VEGF-R3 (flt-4)
Delta-like 4		L YVE-1
Bmx		podoplanin
Alk-1		prox-1
Notch-1, 4		
Jagged-1		

Differentiation of cardiomyocytes from ES cells

Cardiomyocyte differentiation in EBs has been well recognized from the early stages of ES cell research due to easy detectability of the differentiated cells with spontaneous beating. Indeed, most of the cardiomyocyte differentiation studies using ES cells employ the EB method. Spontaneously beating cells are observed around 8 days after EB formation (Hidaka K, et al. 2003). In spite of easy induction, efficiency of cardiomyocyte differentiation is relatively low (approximately 5% of total cells)(Hidaka M,

et al. 2003), and it is difficult to trace their differentiation process in the EB method. As no cardiac-specific cell surface markers for induced cardiomyocytes are currently available, marker genes such as GFP and LacZ driven by various cardiac-specific gene promoters are usually used for cardiomyocyte detection and purification (i.e. α-myosin heavy chain (MHC) , myosin light chain-2v , Nkx2.5 gene and so on). However, since all these markers are observed only in the later stages of cardiomyocyte differentiation, it is difficult to detect cardiac progenitors in the early differentiation stage. These methodological limitations have hampered further development of research for cardiomyocyte differentiation using ES cells.

In the embryo, cardiomyocytes arise from anterior lateral plate mesoderm with the presence of underlying endodermal layer. ES cell-derived Flk1+ cells represent lateral plate mesoderm, inferring cardiomyocytes could be induced from Flk1+ cell population besides vascular cells. Therefore, we decided to attempt cardiomyocytes induction from Flk1+ cells.

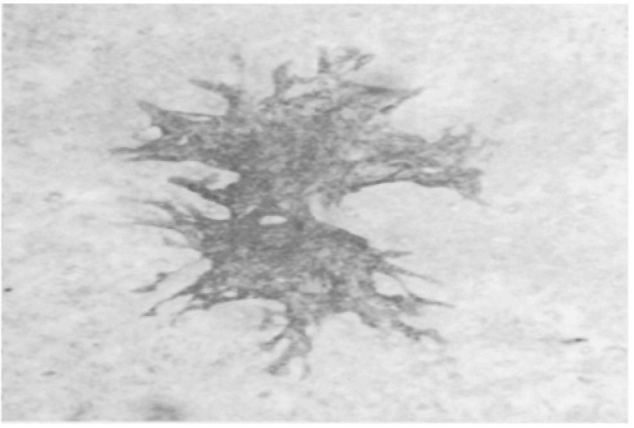

Fig. 2. Cardiomyocyte induction from Flk1+ cells on OP9 stroma cells. When Flk1+ cells were cultured on OP9 stroma cells, spontaneously beating cardiomyocytes appeared after 4-5 days culture. Beating colonies were positive for cardiomyocyte markers, such as ventricular myosin (*dark*).

We sorted and purified Flk1+ cells from ES cells, and examined various conditions to induce cardiomyocytes on 2-dimentional culture. When Flk1$^+$ cells were cultured on stroma cells such as OP9, spontaneously beating cells appeared from 4 or 5 days of Flk1$^+$ cell culture (Fig.2). Induction efficiency evaluated by α-MHC promoter-driven GFP expression revealed that approximately 10-18% of Flk1+ cells became GFP+ cardiomyocytes (unpublished data). Moreover, both cardiomyocytes and ECs were induced from a single Flk1+ cell on the stroma cells (unpublished data). This 2-dimensional, single cell-based cardiomyocyte induction method should provide novel possibilities to research cardiac development and regeneration.

ES cell-derived Flk1$^+$ cells can thus be differentiated into all the cardiovascular cellular components. In this system, it is possible to systematically induce and observe a various range of cardiovascular developmental process including cell diversification from common progenitors, maturation and specification(Fig.3)

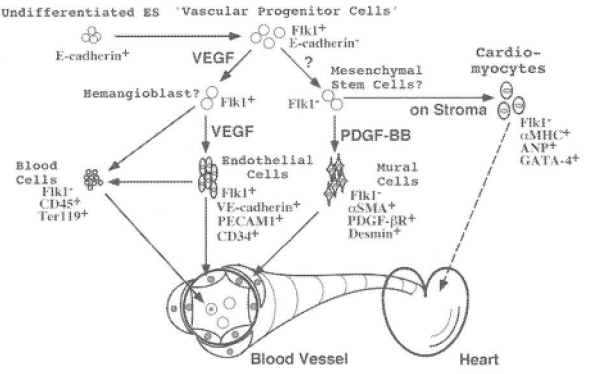

Fig. 3. Cardiovascular differentiation from Flk1$^+$ cells.ES cell-derived Flk1+ cells are common progenitors for cardiovascular system. Flk1$^+$ cells differentiate into endothelial cells by VEGF. In the absence of VEGF, Flk1$^+$ cells lose Flk1 expression and differentiate into mural cells mainly by PDGF-B signaling. Induced vascular cells can form vascular structures both in vitro and in vivo. Flk1+ cells give rise to cardiomyocytes when cultured on stroma cells. This system may reproduce the early process of cardiovascular development in vitro.

Cardiovascular cell differentiation in primate ES cells

Monkey and human ES cells were derived in 1995 and 1998, respectively, by Thomson JA, et al (Shalaby F, et al. 1995). Cardiomyocytes were induced from primate ES cells using EBs . Two-dimensional induction of cardiomyocytes from human ES cells on visceral-endoderm-like cells were also reported (Mummery C, et al. 2002). As for endothelial cells, from both monkey (rhesus monkey) and human ES cells, EB-derived endothelial cells have been reported. They showed various endothelial cell features including endothelial cell marker expression and tube-like structure formation both in vitro and in vivo with tumor or implanted polymer scaffold. Induction on 2-dimentional culture from both monkey (cynomolgus monkey)(Sone M, et al. 2003) and human ES cells (Gerecht-Nir S, et al. 2003) were also reported. Primate ES cells give rise to VEGF-R2 (Flk1)$^+$ cells on type-IV collagen or OP9 stroma cells. VEGF-R2$^+$ cells then differentiate into both endothelial cells and mural cells, and form vascular-like structures in 3-dimentional culture. These overall features of primate vascular cell differentiation resemble those observed in mouse ES cells.

Several differences, however, exist in primate ES cells, such as surface antigens, leukemia inhibitory factor-independence, and a long doubling time. Their differentiation kinetics are also different. In contrast to mouse ES cells, undifferentiated monkey ES cells express VEGF-R2. Four days after the induction of differentiation, VEGF-R2 expression disappears, but is re-expressed at 8 days of differentiation. VEGF-R2$^+$ cells at day 8 correspond to mesodermal progenitor cells that are observed at day 4 of mouse ES cell differentiation. Thus, VEGF-R2 shows a different expression pattern during monkey ES cell differentiation, and monkey ES cells seem to differentiate twice as slow as mouse ES cells. Our recent data suggests that human ES cells also show almost the same differentiation kinetics with little difference from monkey ES cells (unpublished data). Primate ES cell study is not a simple analogy of mouse studies. Many differences of differentiation machinery between mouse and primates must exist. Primate ES cell study is indispensable to exploring the primate-specific mechanisms that mouse ES cell study fails to reveal.

Perspectives

ES cell differentiation combined with 2-dimensional culture and FACS, and progeny induction from a single progenitor cell may lead to novel methods of investigating developmental biology. This method can be expanded into other cell types beyond cardiovascular cells, and would contribute to elucidate molecular and cellular mechanisms of cell differentiation. Identification of functional genes for differentiation without depending on knock-out mouse studies is one of the most expected roles of ES cell study. Significance of ES cells in developmental biology would significantly increase especially in human research.

ES cells are, of course, important for regenerative medicine. Cells of various lineages and maturation stages should be applied to cell transplantation therapy. A great advantage of ES cells in the application to regeneration medicine is that ES cell-derived cells can be made as cell products and be commercially available and should bring industrialization of this new field. Besides the direct use of induced cells, ES cell study can provide many seeds of new regeneration methods for discovery of novel genes or drugs. ES cells, therefore, possess great potentials and are indispensable also in regenerative medicine. ES cells research can develop a new field integrating basic life science with industrialized medicine.

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CHAPTER 3

HYBRID TISSUES

Preparation and Recellularization of Tissue Engineered Bioscaffold for Heart Valve Replacement

Toshia Fujisato[1], Kenji Minatoya[2], Sachiko Yamazaki[2], Yin Meng[4], Kazuo Niwaya[2], Akio Kishida[3], Takeshi Nakatani[4], and Soichiro Kitamura[2]

[1]Department of Regenerative Medicine & Tissue Engineering, [2]Cardiovascular Surgery, [3]Biomedical Engineering, and [4]Organ Transplantation, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

Summary. Tissue engineered grafts based on polymeric or acellular xenogeneric matrices have been widely studied, and found to have greater durability and functionality with growth potential and less immunogenicity than current bioprostheses. On the other hand, there are still several problems to be solved such as degradation control of biodegradable polymeric scaffolds and unwanted transfer of unknown animal related infectious diseases. In this chapter, our novel tissue processing of decellularization named PowerGraft by ultrahigh pressure treatment for safe tissue transplantation is reported. Porcine heart valves were isolated under sterile conditions and treated by cold isostatic pressing (CIP) at 4°C for disruption of donor cells. The cell debris was then washed out in PBS under microwave irradiation at 4°C. The tissues were completely cell free when they were treated by a CIP of 980 MPa (10,000 atm) for 10 min. There was no porcine endogeneous retrovirus (PERV) detected in the treated tissue. There were no significant changes in biomechanical properties of breaking strength and elastic modulus. From the in vitro incubation test, the tissues were disinfected when CIP was applied to the tissues contaminated by normal bacteria floras. The endothelial cells were well seeded on the acellular bioscaffold by the roller and circulation culture systems sequentially. This PowerGraft processing may provide a more durable and safe bioscaffold for tissue transplantation.

Key words. Scaffold, Acellular tissue, PERV, High pressure, Microwave

Introduction

The artificial heart valve is one of the most clinically used artificial devices applied to about 300,000 patients per year worldwide, whereas it has still several shortcomings should be solved. The mechanical valve made of the pyrolytic carbon has good durability that might be longer than the patient's life time, however it has poor biocompatibility due to blood coagulation and patients must take an anti-coagulant drug under strict regulations throughout the rest of their lives. This drug, warfarin, is teratogenic and the female patient who wants to have a baby can not receive a mechanical valve. The xenograft valve made of the chemically crosslinked porcine valve or bovine pericardium in order to minimize the host's immune reaction has good biocompatibility, and hemodynamics, and is resistant to infections. The use of the xenograft valve is on the increase since it is superior to the mechanical valve in the quality of life, because it does not require any administration of an anti-coagulant drug. However, the durability of the xenograft is shorter than the mechanical valve, being about 15 to 20 years in elderly and 5 to 10 years in younger patients, due to calcification of the glutaraldehyde-fixed animal tissue. It is recommended that the xenograft should be used for the elderly patient over 65 years old in the guidelines of the American Heart Association and the American Association of Thoracic Surgeons.

Thanks to the establishment of tissue banks in this decade, some patients have had their defective tissues (heart valve, blood vessel, skin, and bone) replaced with cryopreserved donated tissue from a cadaver, rather than the current imperfect artificial devices.

The cryopreserved allograft valve, referred as the homograft valve, is clinically available in many countries and has been reported to have good clinical results. The homograft valve has the advantages of better biocompatibility compared to the mechanical valve, in durability to the xenograft valve, and in resistance to infections the both valves. However, the limitation on homograft valve availability might never be improved even in the

future. The Ross operation, in which the dysfunctional aortic valve is replaced by the patient's own autologous pulmonary valve and the homograft valve is implanted at the compromised pulmonary position, has been reported to have good clinical results especially in pediatric patients. The autologous tissue does not evoke an immune rejection and becomes bigger in size depending on the patient's growth. Since the other mechanical, xenograft, and homograft valve remains as an exogenous material in the patient's body and never grows, the pediatric recipients must have multiple operations through their lives.

To overcome these shortcomings in the current mechanical and biological heart valves, many research groups have been developing tissue engineered (TE) heart valves with properties similar to autologous valve tissue. Since the TE valves might be substituted by the host cells and tissues after the transplantation, the recipients can enjoy their good biocompatibility, durability, and growth potential.

TE heart valve

For the recovery of defected tissues, substitutional scaffolds must be implanted for tissue regeneration. There are two approaches that allow the scaffold materials to realize the TE tissues. One approach is using artificial biodegradable polymeric materials such as polylactic acid, polyglycolic acid, and polycaprolacton. Prof. Shin-oka and his group have reported successful clinical experiences of about 50 patients implanted with TE blood vessels made of the polyglycolic acid seeded with the patients' autologous bone marrow cells (see the chapter by Shin-oka T, this volume). However, the biodegradable polymeric materials are generally stiffer than the native biological tissues and do not easily take the same shape and structure as the biological tissues. Especially for aortic heart valve replacement, the scaffold requires flexible mechanical properties and strict degradation control for sufficient strength against the blood pressure.

The other approach is using acellular tissues for the scaffold as described in this chapter in which the cells and antigen molecules are removed to diminish the host tissue reaction. The acellular scaffold may have the same structure and composition as the natural tissue and be regu-

lated by interaction with the host tissue cells. CryoLife, Inc. (Kennesaw, GA) is the first company that provided acellular heart valves and blood vessels both from allogeneic and xenogeneic tissues. This company obtained a patent for the decellularization process using the gentle enzymatic treatment named SynerGraft® technology in 1994 and put the decellularized porcine heart valves (SynerGraft® Heart Valve, Model 700) on the market of Europe in 2001. It was reported in 2001 that they were successfully repopulated in a few months after the transplantation (Elkins et al. 2001). Whereas the multicenter clinical outcomes in Europe and Australia for the reconstruction of the right ventricular outflow tract in pediatric patients from 2001 to 2002 showed that only 7 of 19 valves remained implanted and clinically functional at the last follow-up, with 4 deaths (Simon et al. 2003 and R. Chard et al. 2004). On the other hand, the multicenter registry of the decellularized allograft both of pulmonary and aortic valves (CryoValve® SG) in USA from 2000 to 2003 demonstrated excellent clinical performance with more than 92% patient survival after 2 years transplantation (Clarke et al. 2004). Factors on the failure in the xenogeneic decellularized valves were not clear but were presumed to be mainly the result of the processing methods and remaining xenogeneic cell debris inside the tissue. Prof. Konertz and his group in Germany have also started clinical trials of the porcine pulmonary heart valves decellularized by the sodium-deoxycholate named AutoTissue technology in Ross procedure since 2002 (Dohmen PM, et al. 2002). They have reported that the pulmonary grafts named Matrix P showed excellent postoperative results with only 1 death in more than 120 patients and no functional failure and calcification in the grafts (Konertz WF. 2004). Prof. Haverich and his group in Germany have started clinical study too on decellularized allograft valves seeded by the patients' endothelial progenitor cells from 2002 and reported successful results (Teebken OE, et al. 2003 and S. Cebotari et al. 2004). They have been using the detergent Triton® X-100 (Bader A, et al. 1998) or the enzyme trypsin as the agent for the decellularization. In addition, there are several research groups developing acellular heart valves such as Prof. Ingham (Booth C, et al. 2002) in England and Prof. Stock (Schenke-Layland K, et al. 2003) in Germany.

We have been developing acelluar scaffolds for heart valve, blood vessel and trachea made of porcine tissue and their patients' autologous recellularization in vitro for the custom-made tissue transplantation since 2000 (Fig. 1). The scaffold with autologous cells may be replaced by the host tissue by the remodeling process regulated by the surrounding cells through digestion of the scaffold matrices and production of the autologous extracellular matrices. After the remodeling had been completed, the implanted tissue may be identical with an original autologous tissue and may have growth potential. Also, the recellularized grafts may enhance the functional performance such as anti-coagulation and anti-calcification in the early stage of the postoperation.

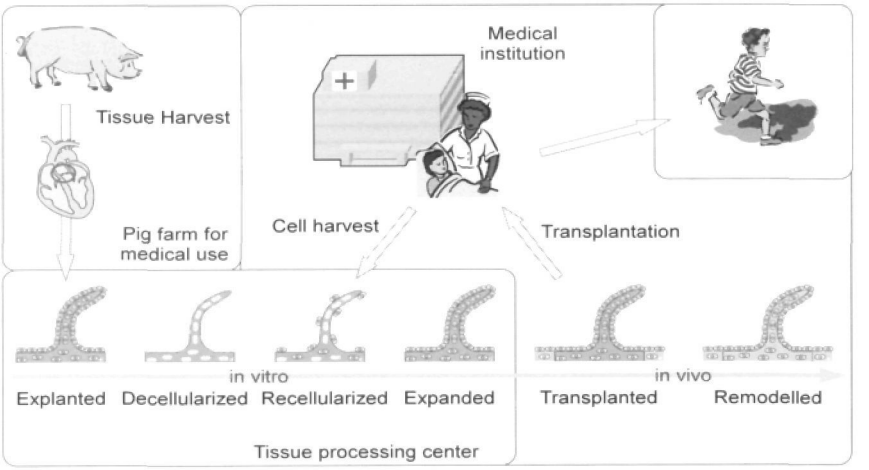

Fig. 1. Custom-made tissue transplantation.

Power Graft technology

All of the above groups are using detergents and/or enzymes as decellularization media such as Triton® X-100, sodium dodecyl sulfate, deoxycholate, trypsine, DNase, and RNase. We have started to investigate decellularization of porcine heart valves using Triton® X-100 and found that the cells in the cusps were unstained by H-E staining after 6 hrs treatment,

however cells still stained deep inside the basal tissue of cusps even after 24 hrs incubation regardless of the detergent concentrations of 0.5%, 1% and 2.5% (Fig.2). Under scanning electron microscopy, gaps between endothelial cells were observed after more than 3 hrs of treatment, however residues of the endothelial cells on the basal membrane were still attached. Since the detergents are generally cytotoxic and it takes time for their removal before the transplantation and cell seeding, it may lead to denature of biological properties and contamination in the process. Recent BSE (Bovine Spongiform Encephalopathy) and vCJD (variant Creutzfeldt-Jakob disease) issues have been affecting tissue transplantation from the point of view of safety. Especially if the scaffolds are prepared from animal tissues, the animal cell components must be removed completely for the prevention of unknown transfer of animal related infectious diseases. In addition, if the tissue source is porcine, the removal of porcine endogenous retroviruses (PERVs) that have ability of infection to the human cells in vitro must be validated (Magre S, et al. 2003). However, it is not easy to remove the cell components completely in the decellularization process by the detergent and proteinase as described above because of the limited permeability of the agents.

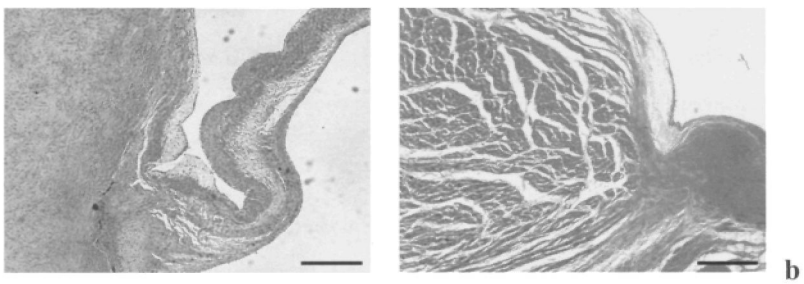

Fig. 2a. b. Decellularization of porcine heart valve basal tissue by detergent. **a** Native. **b** Treated by 1% Triton® X-100 for 24 hrs. Scale bars are 500 μm.

We have introduced a novel decellularization process to create a safe tissue scaffold by the combination of ultrahigh pressure treatment of the cold isostatic pressing (CIP) and washing under the microwave irradiation named PowerGraft technology. During CIP, when fluid pressure is added to the material enclosed in a pressure vessel, the whole surface of the ma-

terial will receive the pressure evenly which is equal to the fluid pressure and then compressed without flattening. This CIP technology has already been established in the food industry and Meijiya Food Co., LTD, Japanese Jam factory, has already commercialized the world first food processed by the CIP in 1990. It has been reported that the functional proteins are denatured by pressing at about 300 MPa and the most of the viruses like Human Immunodeficiency Virus are inactivated at more than 600 MPa (Hayashi R. 2002).

The porcine aortic and pulmonary valves, aorta, and trachea were isolated from 6 month-old Clawn miniature pigs (Japan Farm Co. Ltd, Kagoshima, Japan) weighing about 15 kg under sterile conditions. The harvested tissues were washed and packed in sterile bags filled with PBS. The packed tissues were treated by ultrahigh pressure at 4 °C using a CIP apparatus (Kobe steel LTD, Kobe, Japan). They were then washed by PBS under microwave irradiation at 4 °C (Azumaya Medical Devices Inc., Tokyo, Japan) for accelerated removal of the residues of the broken cells from the CIP treated tissues. H-E staining of the cusps of porcine aortic heart valve showed that the tissues were completely cell free when the CIP of 970 MPa was applied for 10 min and washed under microwave irradiation for 5 days. The pulmonary valve, aortic tissue, and trachea were also completely cell free even in the cartilage tissue of the trachea (Fig.3). We have chosen the Clawn miniature pig as a donor animal since its size adapts to human tissues well and its genome has been well studied in order to develop a human gene induced transgenic animal for organ transplantation. There was no PERV products detected in a PCR assay from the aortic and tracheal tissues processed by the CIP, whereas it was still detected in the tissue treated by Triton® X-100 after 24-hr incubation (Fig. 4). Tissues pre-contaminated by the normal bacteria floras were decontaminated when treated at more than 485 MPa. There were no significant changes in biomechanical properties in terms of the breaking strength and elastic modulus of the leaflets treated at 970 MPa for 10 min. This was supported by elastica-van Gieson staining, which showed collagen and elastin fibers were well maintained in the bioscaffold tissue decellularized by the CIP. The effect of microwave irradiation is the same as the appliance of conventional microwave oven using the vibration of water molecules at 2,450

MHz. The principle of accelerating the washing time by microwave irradiation is still unclear, but it is presumed that the high-speed motion of water molecules and enhances the permeation of the tissues. It is not necessary to use the microwave for washing after the CIP treatment, however it makes washing time about one tenth of that compared to conventional incubation.

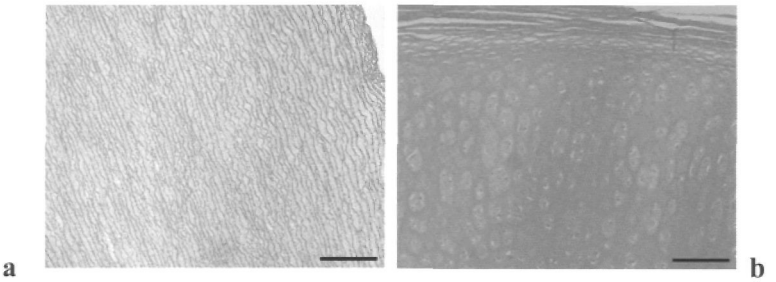

Fig. 3a. b. Decellularization by Power Graft technology using CIP at 980 MPa for 10 min. **a** Aorta. **b** Trachea. Scale bars are 100 μm.

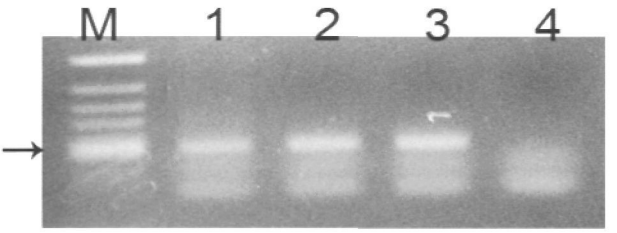

Fig. 4. PERV assay on the decellularized heart valves. (M: Marker, 1: Native, 2,3: 1% Triton® X-100 for 24 hrs, 4: PowerGraft of 980 MPa for 10 min)

Recellularization of Power Graft in vitro

The native heart valve tissue is fully covered with endothelial cells and is occupied mainly by smooth muscle cells. As described above, the incorporation of autologous cells to the acellular scaffold may maintain physiological activity and prevent calcification at the graft site during the early

stage. Endothelial cell seeding on the acellular scaffold was studied. Generally, the cell seeding onto the culture dish is achieved by a static incubation of the cell suspension in about a few hours. It is not difficult to seed the cells on the inner surface of a tubular body like blood vessels by the rolling of the body at a low speed continuously or intermittently in the cell suspension. Since the heart valve has relatively complicated three dimensional cusp surfaces, it is not easy to seed the cells uniformly and completely on them by a simple roller culture. Some research groups have reported pulsatile circulatory bioreactors and/or multi-rotation axes bioreactors for cell seeding and expansion on the heart valve scaffolds (Zeltinger J, et al. 2001, Laube and Matthaus 2001, and Hildebrand DK, et al. 2004). Prof. Umezu and his group have reported a combined bioreactor with multi-axes and a circulation culture for decellularization and endothelial cell seeding sequentially (Iwasaki K. 2004). We have developed a simple double axes roller culture system using a bottle roller for the cell seeding on the valve scaffold and a circulation culture system using a blood pump and gas exchanger for their expansion. Porcine endothelial cells were isolated from the femoral artery of a future recipient by collagenase digestion. After 3 weeks in vitro expansion of the cells, the endothelial cells were suspended and seeded by the roller culture system for 4 hrs. The cells were then expanded in the circulation culture system for 5 days. The autologous endothelial cells were well seeded onto the three dimensional surfaces of the PowerGraft heart valve by the culture systems (Fig. 5).

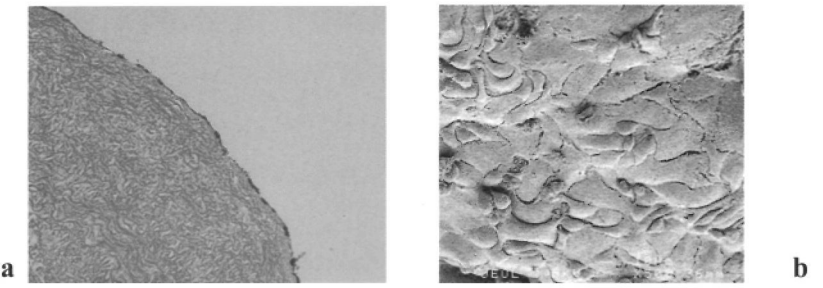

Fig. 5a b. Endothelial cells seeded on the PowerGraft heart valve by the roller and circulation culture systems. **a** H-E. **b** SEM.

Preliminary Animal study of Power Graft

Some research groups have already put their TE heart valves to clinical trials. The allogeneic transplantation study of the PowerGraft aorta and pulmonary heart valve was continued in a porcine model as a pre-clinical study. The morphological and histological changes after the implantation were evaluated in the aorta model without cell seeding, because it seemed the strength and calcification of the acellular tissue in the artery was more critical than the pulmonary tissue. The decellularized porcine aorta was implanted at the descending aorta in the Clawn miniature pig through a left thoracotomy. Surgery was carried out with single clamp technique and the animals were sacrificed at 4 weeks and 12 weeks after the implantation. The explanted grafts were examined histologically and immunohistologically. There was no dilatation and no aneurysmal change and the explanted grafts showed no macroscopic abnormality including their anastomoses. The inner surface was smooth and had no thrombus formation. Cell infiltration was identified at 4 weeks dominantly on the outer side and intimal thickening was observed at 12 weeks. The luminal surfaces of the aorta were completely covered with endothelial cells at 4 weeks after the implantation (Fig. 6). These results are very encouraging to produce durable and safe TE heart valves.

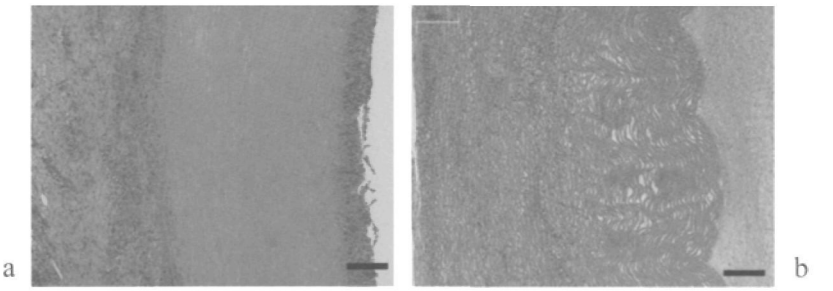

Fig. 6a. b. Explanted aortas decellularized by PowerGraft technology without cell seeding after the allogeneic transplantation in the miniature pig model. **a** 4 weeks and **b** 12 weeks after the transplantation. Left side is the outer side of the implanted graft. Scale bars are 200 μm.

Conclusion

There have been a lot of medical devices developed that still require innovation in many areas and are unable to give growth activity to the current artificial devices. In the heart valves, limitations on homograft valve availability require the need for a better clinical option for the patient and surgeon especially with respect to the pediatric patients because of the limited outcomes of current artificial heart valves. We are developing custom-made tissue transplantation in which patient's autologous cells are seeded on and in the appropriate scaffold for defective tissues of heart valves, blood vessels, pericardium, trachea, esophagus, and dura mater. Our novel decellularization method of PowerGraft was developed to produce a safe bioscaffold by ultrahigh pressure treatment of the CIP and washing under microwave irradiation. Porcine cells and PERV were removed completely from the animal tissues in a short period by the CIP of 980 MPa without changing the biomechanical properties. These findings suggest the tissues treated with CIP can be used as a safe bioscaffold, even if based on xenogenic tissues that have risks of unknown animal related diseases. We are currently studying autologous cell seeding on and in the scaffold prepared by PowerGraft technology and their applications in animal experiments. These acellular tissues are going to be put into clinical study in the near future. The TE heart valves might be substituted for the current artificial heart valves in the future.

Acknowledgement

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Biotube Technology for a Novel Tissue-Engineered Blood Vessels

Hatsue Ishibashi-Ueda[1] and Yasuhide Nakayama[2]

[1]Department of Pathology, National Cardiovascular Center Hospital, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan
[2] Department of Bioengineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

Summary. Autologous tubular tissues as small caliber vascular prostheses were created in vivo using tissue engineering. We named them "Biotubes". The six kinds of polymeric rods made of polyethylene (PE), polyfluoroacetate (PFA), poly-methyl methacrylate (PMMA), segmented polyurethane (PU), polyvinyl chloride (PVC) and silicone (Si) as a mold were embedded in the dorsal skin of six of New Zealand White rabbits. Biotubes were formed after 1 month by fibrous tissue encapsulation around the polymeric implant except PFA. None of the Biotubes were ruptured when a hydrostatic pressure was applied up to 200 mmHg. The wall thickness of the Biotubes ranged from 50 to 200 μm depending on the implant materials in the order PFA<PVC<PMMA <PU<PE. The tissue mostly consisted of fibroblasts and collagen-rich extracellular matrices. The tissue created by Si rod was relatively firm and inelastic and the one created by PMMA was relatively soft. For PMMA, PE and PVC the stiffness parameter (β value; one of the indexes for compliance) of the Biotubes was similar to those of the human coronary, femoral and carotid arteries, respectively. Biotubes, autologous tubular tissues, can be applied for use as small caliber vessels and are ideal prostheses because of avoidance of immunological rejection.

Keywords. Graft prosthesis, Tissue engineering, Autologous transplant, Small caliber vessel

Introduction

In vivo, the encapsulation of foreign materials by own fibrous tissue has been well documented as a biological reaction of self-defense system since the 1930's. Peirce et al. attempted to utilize capsular tissues as artificial vascular vessels (Knott I, et al. 1973, Peirce EC II, 1953). Sparks et al. examined the clinical application of grafts consisting of a combination of capsular tissues and Dacron tubes in the latter half of the 1960's (Hallin RW and Sweetman WR 1976, Sparks CH, 1972). Recently, Campbell et al. studied capsular tubular tissues obtained by implantation of silicone rods into animal peritoneal cavities(Campbell JH, et al. 1999). Tubular tissues possessed a wall with several layers of myofibroblasts and collagen-rich extracellular matrices covered with a single layer of peritoneal mesothelium. By inverting the tubular tissues, mesothelial cells became internal lining cells within tubes like endothelial cells. Autotransplantation of these tubes as grafts resulted in high patency for several months, suggesting the possible application of capsular tissue prosthesis for arteries.

The patency rate of small caliber artificial grafts is much worse than medium to large diameter artificial grafts because of thrombosis in the early stage and of neointimal hyperplasia in the chronic stage. Among the many factors determining the patency of small caliber artificial grafts, the compliance mismatch between the native artery and grafts has been discussed as a major detrimental factor of graft failure (Abott WH, et al. 1987, Kinley CE and MarbleAE 1980, Pevec EC II, et al. 1992, Stewart SF and Lyman DJ 1992).

In this study, the mechanical properties of the tubular tissues were investigated for small caliber blood vessels. Various polymeric rods were implanted in subcutaneum of the dorsal skin of rabbits. Then, own tubular tissues by encapsulation were obtained and we called them "Biotubes". The mechanical properties including pressure resistance, pulse followability and compliance of the Biotubes were measured after histological analysis of their components. The designs of the matrix including luminal surface, mechanics and shape of the Biotubes were discussed.

Methods and Results

Preparation of Biotubes

Nine New Zealand White rabbits, weighing 2.0 to 2.5 kg, according to the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication, No. 56-23, revised 1985) were used as hosts. Six kinds of polymers with rod shape (length; 20 mm, diameter; 3 mm) were placed in the subcutaneous layer of dorsum of rabbits. The polymer materials were polyethylene (PE), poly-fluoroacetate (PFA), poly-methyl methacrylate (PMMA), segmented polyurethane (PU), polyvinyl chloride (PVC) and silicone (Si). The coating thickness was 50 μm. At 1 month after insertion, rods were found to be encapsulated by a membranous tissue in the subcutaneum of rabbits (Fig. 1a, 1b). After 3 months, all implants were still covered with membranous capsular tissues. We called these autologous capsular tissues "Biotubes".

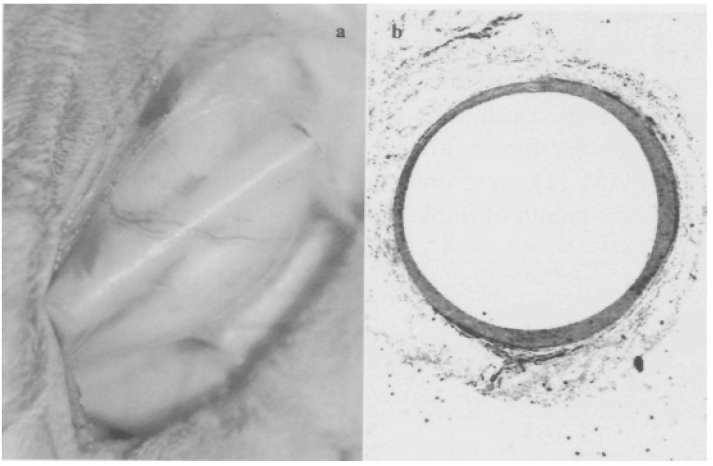

Fig. 1. (a): " Biotube", which was formed by implantation of the PMMA for 1 month in rabbit dorsal skin. (b): A photomicrograph of a cross section of the PMMA tube obtained after 3-months implantation

Histological Examination

The explanted Biotubes were fixed in 10 % buffered formalin solution and embedded in paraffin. Tissue cross sections were stained with hematoxylin and eosin for histological evaluation (Fig. 1b, 2). The wall thickness of the Biotubes was approximately 70 μm one month after implantation of Si, PVC, and PFA rods. In cases of PU or PMMA implants the wall showed 100-150 μm in thickness (Fig. 2). The capsules by the PE implant revealed the thickest wall (approximately 200 μm). For PMMA, PVC, and PE, the wall thickness increased by 1.5 to 2 fold after 3-month (Fig 3). The Biotube walls around the PFA showed sparse collagen with fibroblasts. Regarding the Si rod, the Biotube wall was thin but contained collagen fibers with sparse fibroblast. The walls around PMMA, PU and PVC rods were of a moderate thickness and contained relatively thick collagen fibers. The Biotubes that formed around the PU, PE and PVC bases showed numerous inflammatory cell infiltrations such as lymphocytes. Especially in the tubular tissues of PE and PU, foreign body giant cells were also observed. The capsular wall of PE rod showed 200μm in thickness but almost no regular mesh structure of collagen fibers.

Immunohistochemistry was also performed to identify the muscular component of the Biotubes. Vimentin, a mesenchymal tissue marker, was positive for all tubular tissues around the various rods after 1- and 3-month implantation. α-smooth muscle actin was intensely positive for all tubular tissues after 3 months. Desmin as a cytoskeleton of matured muscle was negative in all tubular tissues after 1 and 3 months. A small number of macrophages (RAM 11), were observed in the tubular tissues of PU, PFA and PMMA after 3-month of implantation.

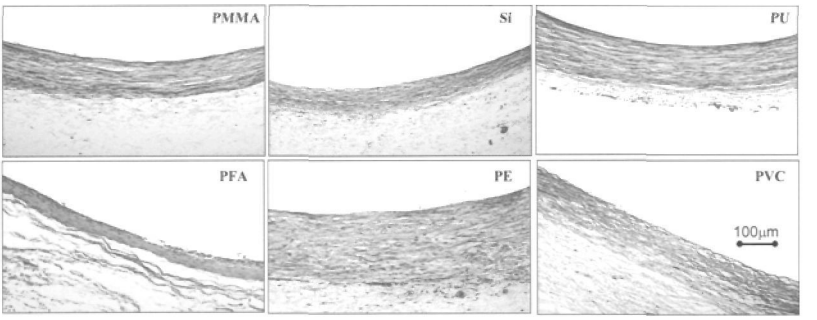

Fig. 2. Photomicrographs of cross sections of the Biotubes, They were formed by 3 months implantation of six kinds of polymer rods in the rabbit skin (hematoxylin and eosin stain)

Mechanical Properties of the Biotubes

The luminal pressure-diameter relationship was determined using an apparatus designed by Takamizawa and Hayashi (Takamizawa K and Hayashi K 1987). Changes in the outer diameter of the Biotubes were measured. None of the Biotubes ruptured even after 200 mmHg of inner pressure after 1 month of implantation. The Biotubes around Si rod became slightly dilated when exposed to water at low pressure but did not change significantly with high pressure (about 20 mmHg or higher). In contrast, the Biotube formed around the PMMA rod became dilated at low-pressure ranges and gradually increased with pressure up to a high range, indicating "J"-shaped curves. The dilatation rate of the outer diameter at a water pressure of 200 mmHg was about 5 % for Si and about 25 % for PMMA.

Repeated water pressure loadings, range of 0 to 200mmHg, were investigated in the PMMA Biotube rods. The external diameter of the PMMA Biotube was about 2.7 mm before loading and dilated to about 3 mm after loading at several 10 mmHg water pressure and thereafter continuously dilated slowly with an increase in inner load reaching about 3.2 mm at 200 mmHg. Changes in the outer diameter luminal pressure were basically the same with repeated pressure loadings in the lumen (Fig 3).

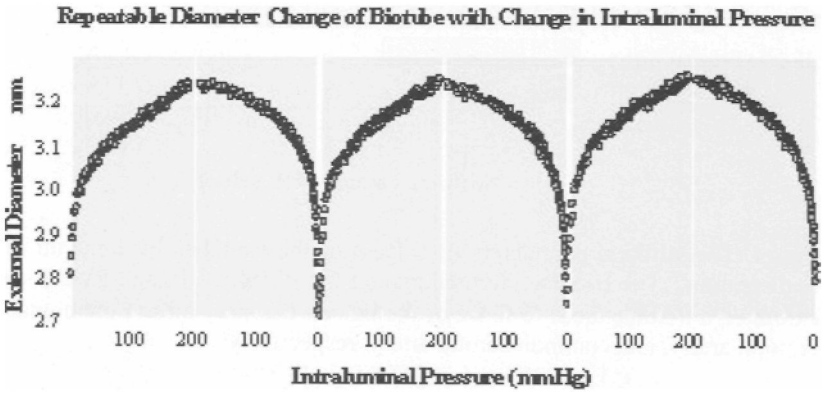

Fig. 3. External diameter changes in loading and removing of water pressure in range of 0 to 200 mmHg to the lumen of the Biotube around the PMMA rod after 3 months of implantation

The compliance of the tube was determined by the stiffness parameter (β) as defined by Hayashi et al., (Hayashi K, et al. 1980, Hayashi K and Nakamura T 1985) which is described according to the following equation:

$$\ln(P/Ps) = \beta(D/Ds-1)$$

where P, Ps, D and Ds denote luminal pressure, standard pressure (100 mmHg in this study), external diameter and diameter at the pressure Ps, respectively. The relationship between logarithmic value of the relative pressure and relative outer diameter was obtained from the relationship between outer diameter and luminal pressure. After 1-month of implantation, the highest β value was obtained from the Si Biotube and the β value decreased in the order of PMMA, PE and PVC (Fig 4). The Biotube around the PMMA rod exhibited the β value resembling that of the human coronary artery whilst the β values of the Biotubes formed around PE and PVC bases were similar to those of the human femoral and common carotid arteries, respectively.

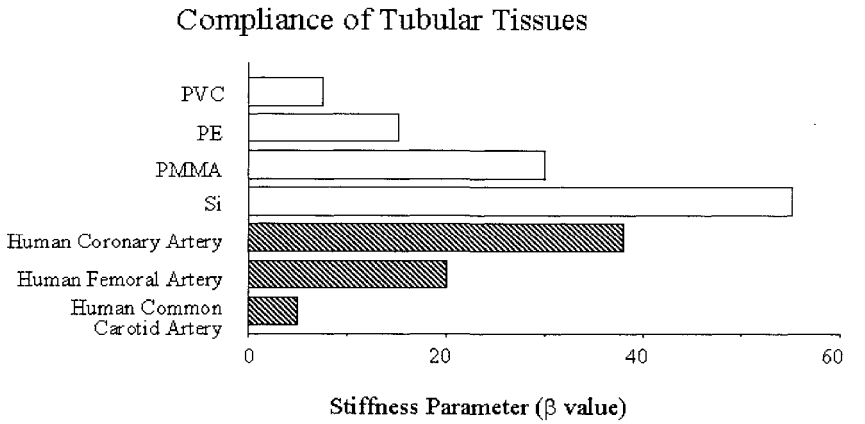

Fig. 4. The stiffness parameters (β values) of the Biotubes by 1 month of implantation. The Biotubes formed around the PMMA, PE, and PVC rods exhibited a β value close to those of the human coronary artery, the human femoral artery, and common carotid artery, respectively

Discussion

Considering immune responses among tissue or organ transplants, grafts are better if they consist simply of own self tissues. For example, autografts such as a great saphenous vein, an internal thoracic artery, and a radial artery are used for conventional aorto-coronary bypass operations.

However, it is sometimes difficult to obtain a sufficient amount of grafts or healthy grafts due to the patient's limited supply of vessels.

Larger caliber grafts vessels, with more than 5 mm in diameter, are usually composed of artificial materials such as Dacron fabrics or expanded poly-tetrafluoroethylene (ePTFE). Small caliber artificial grafts, however, may occlude within a short period after implantation by thrombosis. Therefore, the development of hybrid type artificial blood vessels combining artificial and biological materials is expected. The layered hybrid vessels resembling biological vascular walls have been developed in vitro. (Ishibashi K and Matsuda T 1994, Matsuda T and Miwa H 1995, Miwa H and Matsuda T 1994). The invented hybrid vessels with 4 mm in diameter showed high patency after 1-year of autotransplantation in a canine carotid artery. Other hybrid grafts prepared by seeding and culturing cells from the own great saphenous vein in a polylactic acid tubular sponge, have successfully been used in reconstruction of the pulmonary artery in humans (Hibino N, et al. 2003, Shin'oka T, et al. 2001, Shin'oka T, et al. 1998). Recently, it has been proposed that the preparation of various hybrid artificial vessels could be achieved by in vitro tissue engineering techniques using stem cells, including endothelial progenitor cells (Asahara T and Isner JM 2002, Isner JM, et al. 2001) and ES cells (Nishikawa S 1997, Yamashita et al. 2000), as a cell source (Kaushal S, et al. 2001, Shirota T, et al. 2003).

For patency of artificial blood vessels, particularly those with a small caliber (5 mm or less in diameter), the following factors are required. 1) Resistance to blood pressure, 2) antithrombotic properties to avoid thrombotic occlusion in the early stage of implantation, and 3) mechanical compatibility including compliance matching and pulse follow-ability to avoid occlusion in the chronic stage. We aimed to develop small caliber artificial blood vessels for a clinical application that have high patency in combination of the tubular tissue preparation technique and in vivo tissue engineering (Nakayama Y, et al. 2004). Biotubes consist of own self cells and extracellular matrices, which is similar to autotransplantation. Therefore, immunological rejections to the tubular tissue may be avoided. Moreover, the tissue may grow after transplantation in the body. In this study, various polymeric rods as a mold were implanted in the skin to form own tissue tubes. The materials used for the polymeric rods were PMMA, PU, PVC, PE and Si, all of which are hydrophilic, and PFA, which is water-repellent, and all are currently used as biomaterials. None of the Biotubes ruptured with 200-mmHg inner pressures except for tissue created by PFA. The walls of the Biotube by PFA showed sparse collagen fibers with numerous inflammatory cells, without capsularization (Fig. 2). In the tube that formed around PU, inflammatory cell infiltration was noted. Although PU

is used as a tissue compatible material for artificial hearts (Zdrahala RJ and Zdrahala IJ 1999), when transplanted as an artificial vessel, granulation is often recognized (Seifalian AM, et al. 2003, Sonoda H, et al. 2003). PU is considered to have a strong tissue response. A dense collagen mesh structure was formed around Si. The wall thickness of the Biotubes after 1-month of implantation decreased in the order of PE>PU>PMMA> PVC>Si>PFA and increased with transplantation period apart from PFA, PU, and Si.

The Si base tubular tissue as a graft strengthened with Dacron has been attempted for arterial bypass in the lower limbs clinically (Hallin RW and Sweetman WR 1976, Sparks CH, 1972). The tubular tissue showed vascular function at an early stage of the transplantation. However, the grafts were occluded within a short period in most cases because of lack of endothelialization. It has recently been reported that mesothelial cells were arranged on the luminal surface of tubular tissue obtained using Si bases (Campbell JH, et al. 1999). The patency rate was high, about 70 %, after 2-month of transplantation in animal experiments.

The stiffness parameter (β) is one of the indexes for compliance of blood vessels and indicates the mechanical property under physiological blood pressure (Hayashi K, et al. 1980, Hayashi K and Nakamura T 1985). Lower values in the β value indicate the material is soft and flexible. Within the polymers used in this study, the β value decreased in the order of Si>PMMA>PE>PVC. The Biotubes that formed around Si were relatively firm and inflexible, while the Biotubes that formed around PMMA were elastic within a low-pressure range and less extensible at a high-pressure range, showing a mechanical property similar to that of biological arteries. The relationship between intraluminal pressure and external diameter showed a 'J'-shaped curve, similar to genuine arteries. The Biotube obtained after one month by PMMA, PE and PVC exhibited compliance similar to that of the human coronary artery, human femoral artery and human carotid artery, respectively. Selection of specific rod materials and embedding period allow the design of artificial blood vessels with matching mechanical properties to that of genuine vessels. This matching is expected to prevent intimal hyperplasia causing luminal occlusion in the chronic stage. Biotubes are expected to grow with the patient blood vessels and are ideal vascular grafts. Furthermore, it is possible to design specific mechanics and it is easy to match the host's vascular shapes. We are planning to establish the Biotube preparation method that combines surface design as described above and demonstrate the usefulness of Biotubes as small caliber artificial blood vessels by animal transplantation experiments. These results will be reported in near future.

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Clinical Application of Tissue-Engineered Blood Vessels

Goki Matsumura, Toshiharu Shin'oka

Cardiovascular Surgery, The Heart Institute of Japan, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, Japan, 162-8666

Summary. Materials commonly used to repair complex cardiac defects in cardiovascular surgery lack growth potential and have other unwanted side-effects. We designed and tested a biodegradable scaffold seeded with bone marrow cells (BMCs) that avoids these problems.

We aspirated BMCs from human subjects during surgery and isolated mononuclear bone marrow cells (MN-BMCs) using the Ficoll technique. These cells were seeded onto a tubular or sheet-shaped scaffold and implanted as an autograft. Post-operative evaluations in human subjects were performed using computed tomography, magnetic resonance imaging and cardiac catheterization.

Since September 2001, we have performed 43 procedures in 42 patients using tissue-engineered materials. There was one mortality case for heart failure at 2 months after the operation. One case required re-operation for an R-L shunt in the atrium after a lateral tunnel procedure at 2 months after the operation. Stenosis of the tissue engineered graft was observed in 2 patients who underwent a total cavo-pulomonary connection (TCPC). Mild stenosis was observed in 2 patients after a pulmonary artery stenosis repair, and stenosis of a superior vena cava repair was observed in one case. The other surgical interventions had excellent outcomes.

These results provide direct evidence that the utilization of BMCs enable the establishment of tissue-engineered vascular autografts (TEVAs), and are especially useful for children who require biocompatibility and growth potential materials in cardiovascular surgery.

Key words. Tissue engineering, Biodegradable scaffold, Autologous bone marrow cells, Cardiovascular surgery

Introduction

Pediatric cardiovascular surgeons often encounter patients requiring surgical intervention utilizing foreign materials to repair complex lesions. However, the materials that are commonly used lack growth potential and long-term results have revealed several material-related failures, such as stenosis, thromboembolization, calcium deposition, and risk of infection (Kirklin J and Barratt-Boyes B 1993) To solve these problems, in particular for children who require the implantation of a dynamic material with growth potential, we sought to develop optimal filling materials with biocompatibility and growth potential.

Previously, we reported the advantages of tissue-engineered vascular autografts (TEVAs) in animal models (Watanabe M, et al. 2003, Shin'oka T, et al. 1995, Shin'oka T, et al. 1996, Shin'oka T, et al. 1997) and in human clinical applications (Shin'oka T, et al. 2001, Isomatsu T, et al. 2003, Naito Y, et al. 2003, Matsumura G, et al. 2003) utilizing autologous cells and biodegradable scaffolds. The key benefits from utilizing such scaffolds are that they degrade in vivo, thereby avoiding the long-term presence of foreign materials (Langer R, et al. 1993), and the seeded cells proliferate and differentiate to construct new tissue. Previously reported procedures (Matsumura G, et al. 2003, Naito Y, et al, 2003, Shin'oka T, et al. 2001), which used harvested vessel walls and required cell isolation, are associated with a risk of infection during cultivation. Other drawbacks to these procedures include the possibility that sufficient amounts of cells for seeding onto the scaffolds may not be obtained, that cell culture may require substantial time, which is not available in emergency cases, and, finally, that they require the use of serum from other species in the culture medium, which has associated risks that significantly reduce the merit of the procedures.

Recent studies have demonstrated the existence of bone marrow-derived endothelial progenitor cells that contribute to vasculogenesis and angiogenesis (Asahara T, et al. 1999, Shintani S, et al. 2001, Takahashi T, et al. 1999) and the successful endotheliazation of artificial grafts using BMCs. (Noishiki Y, et al. 1996) Recently, we provided evidence that BMCs as a cell source for seeding onto a biodegradable scaffold is useful, and that seeded cells contribute to the histogenesis of TEVAs (Matsumura G, et al. 2003). Therefore, we applied this technique in clinical trials and good results have been obtained up to new. Here, we show the latest protocol and results in "tissue-engineering blood vessels" utilizing autologous MN-BMCs.

Protocol

Materials and Methods

Biodegradable Scaffolds

A copolymer of lactic acid and ε-caprolactone (P(CL/LA)) was synthesized by ring-opening polymerization. This copolymer is a polyester with a molar composition of lactic acid and ε-caprolactone at a 50:50 ratio, and is degraded by hydrolysis after a few months in vivo. The matrix is over 80% porous with a pore diameter of 20 to 50μm. We used a woven fabric made with poly-L-lactic acid (PLLA) or poly-glycolic acid (PGA) for reinforcement of the porous matrix, which is degraded by hydrolysis in approximately 2 years and 8 weeks. Using these polymers, we fabricated a hybrid tubular scaffold or sheet by pouring a solution of P(CL/LA) onto the PLLA or PGA woven fabric sheet, followed by freeze-drying under vacuum.

Preparation of BMCs and TEVAs

Under general anesthesia, we began cardiac surgery and, at the same time, aspirated approximately 1~6ml/kg of BMCs from the anterior superior iliac spine through a puncture needle into a syringe containing heparin. Aspirated BMCs were passed through a nylon cell strainer to remove fat and bone fractions, and then the MN-BMCs were isolated using the Ficoll technique. After the MN-BMCs were obtained, the cells were seeded onto a tubular- or sheet- shaped scaffold and macerated in the patient's serum to preserve the seeded cells. The scaffold was stored at 37°C in 100%humidity and a 5%CO_2 atmosphere in the incubator until use. TEVAs were viable for several hours under these conditions.

Clinical Trials

Patients Profiles

From September 2001 to March 2004, 42 patients underwent cardiac surgery using tissue-engineered tubular– or sheet-shaped autografts prepared with seeded BMCs. The study was carried out according to the principles of the Declaration of Helsinki. The ethics committee at Tokyo Women's

Medical University approved the study protocol. The patient's parents and/or patients were thoroughly informed and consented to the procedure.

The patient sample consisted of 17 boys, 1 man, 22 girls and 2 women ranging from 1 to 24 years of age (mean 7.36±6.40 years). Forty patients had a previous history of surgery ranging from 1 to 4 times (mean 2.08± 1.00), and 31 of this 40 patients had palliative operations 1 to 4 times (mean 1.58±0.79 times).

Surgical Procedures

We performed 43 procedures in 42 patients using the protocol for this clinical trial. We performed a TCPC in 26 (extracardiac TCPC in 23, lateral tunnel method in 3 patients), pulmonary artery patch angioplasty in 12 patients, right ventricular outflow tract reconstruction in 4 patients and superior vena cava patch repair in 1 patient utilizing either tubular- or sheet-shaped biodegradable scaffolds.

Outcomes and Evaluations after Surgery

Mean hospital stays and follow up periods after surgery were 56.1±33.7 days (ranging from 24 to 158 days), and 42.1±23.4 days (ranging from 18 to 130 days), respectively. There was one mortality case due to heart failure at 2 months after surgery. And there was one morbidity case requiring reoperation as an R-L shunt was detected by echocardiography and cine-angiography in the atrium at 2 months after a lateral tunnel intervention. Two patients showed slight and local stenosis of the tubular graft, which were utilized for extracardiac TCPC. Pressure studies showed 3 to 5 mmHg gradients between the stenotic sites in these cases. Percutaneous transluminal balloon angioplasty is scheduled in the future. In one patient, a post-operative catheterization study showed stenosis at the site of the superior vena cava repaired with a tissue-engineered patch. We performed percutaneous transluminal balloon angioplasty at 5 months after surgery, and the stenosis was successfully released. In this case, we had performed pulmonary artery angioplasty with a T-shaped tissue-engineered patch, simultaneously. Post-operative pulmonary arteriograms performed at 10 months after the operation showed successful repair (Fig.1). Computerized tomography was performed in all patients who underwent surgery with tissue engineered materials. Fig. 2 shows the patency of a 12mm TEVA at 10 months after the hepatic vein was rerouted to the right pulmonary artery. The TEVAs were patent under low-blood-flow circumstances.

All other surgical repairs were done without subsequent occlusion. No other complications such as thromboembolization, infection, calcification

or rupture of the TEVAs occurred. No other patient required further surgical or catheter intervention.

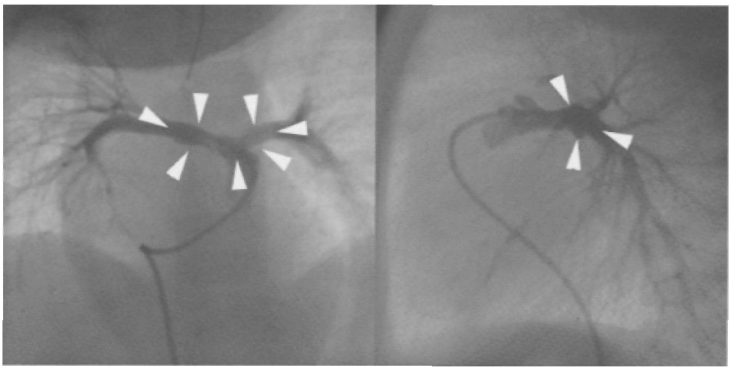

Fig. 1. Post operative pulmonary arteriogram obtained at five months after a pulmonary artery angioplasty with a T-shaped TEVA on the main trunk and both branches of the pulmonary artery. White arrowheads indicate the site where the TEVA was implanted.

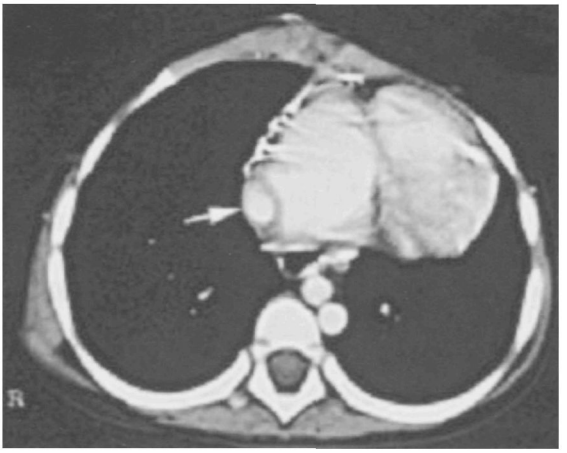

Fig. 2. Computerized tomography of a patient who underwent hepatic vein rerouting to the left pulmonary artery with a TEVA (12 mm in a diameter). White arrow indicates the TEVA.

Discussion

In this study, we demonstrated the feasibility of using TEVAs constructed with seeded BMCs in cardiovascular surgery. Utilizing TEVAs would be a good choice for patients who are candidates for surgical interventions that require use of synthetic materials for peripheral or stenotic cardiovascular lesions in low-pressure circumstances. Because TEVAs are a biocompatible auto "living-tissue", we believe that they could contribute to the patients' well-being as they appear to reduce the incidence of complications caused by implantation of incompatible materials. Furthermore, they lead to a reduced likelihood of further surgery, because of their potential for growth or remodelling to an appropriate size and shape, reduced incidence of calcification, absence of the risk of rejection as autologous cells are used, and have a minimal risk of infection. In addition, they may lead to better vessel patency with less risk of thromboembolization allowing the patient freedom from long-term anticoagulation therapies.

In contrast, foreign materials often cause unwanted side-effects and have the potential to lead to subsequent surgery as these materials often cause stenosis or obstruction and/or calcification. Furthermore, such prostheses, occasionally, cause discrepancies in the sizes of the material as children grow. This results in an undesirable hemodynamic load for the heart and other organs, which requires several interventions or reoperations. Consequently, utilizing TEVAs is an ideal strategy in cardiovascular surgery that offers several theoretical advantages for patients.

In our series, twelve of 42 patients required further surgery with tissue-engineered materials due to the failure of the material that was used in a previous operation. In 7 of the 12 patients, material failure occurred in spite of utilizing autologous pericardium, which is often used to repair complex lesions. Since we believe that autologous tissue is more desirable than synthetic materials, we often use autologous pericardium for repairs. However, for the patients who require one or more operations, we could not obtain sufficient amount of autologous pericardium for reconstruction of the vascular defects. Therefore, for those patients who require substitute materials, our tissue engineered materials are useful, as they can be made available at any time. These observations underscore the dire need of cardiac surgeons for ideal biocompatible materials for use in reconstruction surgery, especially in pediatric cardiac surgery.

Although we obtained excellent surgical outcomes in our patients, it is impossible to document tissue-generation and the contribution of seeded-BMCs in our patients. Therefore, we performed simultaneous animal studies, which showed that the seeded BMCs home in, adhere, proliferate and

differentiate in the scaffold to construct components of a new vessel wall within a few months (Matsumura G, et al. 2003). Therefore, we believe that seeding BMCs onto a scaffold and implanting these seeded scaffolds into the patients is essential for tissue engineering vascular structures.

From September 2001, we switched to using BMCs as the cell source in tissue engineering for clinical applications as Noishiki (Noishiki Y, et al. 1996) reported that BMCs implanted onto the surface of an artificial graft leads to endothelialization in a dog model. In addition, it has been reported that multi-potential cells exist in the bone marrow that have the potential to differentiate into several types of cells and organs in vivo (McKay R. 2000). Furthermore, previous studies have shown the existence of endothelial progenitor cells derived from bone marrow that contribute to vasculogenesis and angiogenesis (Asahara T, et al. 1997) and have great potential in endothelialization (Shi Q, et al. 1998, Bhattacharya V, et al. 2000). Recently, we have demonstrated the contribution of seeded and circulating bone marrow cells in TEVAs in a dog model (Matsumura G, et al. 2003). Together, the results of these reports and our experimental results suggest that seeding BMCs onto the scaffold would result in a valuable tool for constructing tissue engineered materials.

Using autologous cells for tissue-engineering offers several advantages in that the patients do not need a donor and there is no fear of rejection. When using BMCs, we were able to obtain sufficient cells on the day of surgery, and, as a consequence, patients do not need extra hospitalization for vein-harvesting, as suggested in previous protocols (Shin'oka T, et al, 2000, Naito Y, et al. 2003, Matsumura G, et al. 2003). While patients made a preliminary hospital visit to harvest vein walls, according to the previous methods, in about half of the cases, sufficient cells were not obtained by culturing. After changing the protocol, we used BMCs as the cell source for these patients. Using BMCs as the cell source removes considerations of cell culture and reduces the risk of contamination. In addition, according to the latest protocol, we use auto-serum to preserve the bone marrow seeded scaffold until the day of surgery. Removing the exposure of the donor cells to other species serum and other commercial available reagents (except Ficoll solution) leads to increased safety for the patients and improved cost-effectiveness of the procedure.

While postoperative examinations revealed good surgical results, it is very difficult to evaluate the histogenesis of tissue-engineered materials in humans. However, we were able to evaluate the post-operative surgical results by catheterization, computerized tomography and magnetic resonance imaging. To our knowledge no unwanted calcification or microcalcification is found in our patients to date. In an experimental model, Bhattacharya (Bhattacharya V, et al. 2000) suggested that this undesirable side

effect was avoided by utilizing selected CD34-positive cells, although a long term careful follow up is recommended.

In our series, anticoagulation therapies have been administered to all patients. During the early postoperative period, heparin was continuously injected, and the anticoagulation time was controlled to between 180 and 230 seconds. After extubation, we administered 5 mg/kg of aspirin and Warfarin for 3 months to protect patients from thromboembolic complications. We assumed that auto-tissue does not require anticoagulants after the endothelialization of the graft; therefore, patients should be free from medication from that time on. This would improve the patient's quality of life, especially for young women who want to become pregnant and need to be free from anticoagulation therapy such as Warfarin.

We believe that the therapy described here is an ideal strategy in cardiac surgery that contributes to the patients' well-being, since TEVAs reduce the instance of complications caused by incompatible materials and lead to a reduced likelihood of further surgery. From our experimental models, our tissue-engineering techniques seem to generate vessel walls within a few months from our experiment models. However, this therapy is in its infancy, and there are several problems with the procedures that need to be resolved. More ideal biodegradable scaffolds and better procedures for cell preparation and tissue generation should be investigated. Furthermore, there are many questions that remain as to how randomly seeded bone marrow–derived cells proliferate, differentiate, and arrange themselves in an appropriate fashion to constitute a new tissue. In addition, which types of cells contribute to the generation of different types of newly formed tissues? Finding answers to these questions would permit us to obtain better tissue engineered materials. Finally, we feel that autologous tissue-engineered materials will offer several advantages for the children around the world who require cardiac surgery.

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CHAPTER 4

NEW ASPECTS OF ANGIOGENESIS

Vascular Regeneration and Remodeling by Circulating Progenitor Cells

Masataka Sata and Ryozo Nagai

Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Summary. Atherosclerosis is responsible for more than half of all deaths in western countries. Numerous studies have reported that exuberant accumulation of smooth muscle cells play a principal role in the pathogenesis of vascular diseases. It has been assumed that smooth muscle cells derived from the adjacent medial layer migrate, proliferate and synthesize extracellular matrix. Although much effort has been devoted, targeting migration and proliferation of medial smooth muscle cells, no effective therapy to prevent occlusive vascular remodeling has been established. Recently, we reported that bone marrow cells substantially contribute to the pathogenesis of vascular diseases, in models of post-angioplasty restenosis, graft vasculopathy and hyperlipidemia-induced atherosclerosis. It was suggested that bone marrow cells may have the potential to give rise to vascular progenitor cells that home in the damaged vessels and differentiate into smooth muscle cells or endothelial cells, thereby contributing to vascular repair, remodeling, and lesion formation. This article overviews recent findings on circulating vascular precursors and describes potential therapeutic strategies for vascular diseases, targeting mobilization, homing, differentiation and proliferation of circulating progenitor cells.

Key words. Smooth muscle cell, Atherosclerosis, Progenitor, Bone Marrow, Endothelial cell

Smooth muscle cells and vascular remodeling

Exuberant accumulation of smooth muscle cells (SMCs) plays a principal role in the pathogenesis of vascular diseases (Ross R, 1999). In atherosclerotic plaques, SMCs proliferate and synthesise extra cellular matrixes, thereby contributing to lesion formation. Percutaneous coronary interventions (PCIs) have been widely adopted for treatment of coronary atherosclerosis. However, a significant number of these procedures fail due to post-angioplasty restenosis. Although the increasing use of new devices for dilatation of stenosed arteries has lowered the incidence of acute complications, restenosis still limits the long-term outcome of percutaneous interventions. SMC hyperplasia is a major cause of post coronary bypass surgery occlusion, too. Furthermore, graft vasculopathy, a leading cause of graft failure and retransplantation after the first postoperative year, also results from SMC hyperplasia. Therefore, much effort has been devoted to understanding the molecular pathways that regulates SMCs hyperplasia to prevent vascular diseases (Ross R, 1999). However, the pathogenesis remains largely unknown and, consequently, no effective therapy has been established.

There is a widely accepted view that atherosclerotic lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall. In brief, upon endothelial injury, inflammatory cells infiltrate and secrete various cytokines. Cytokine triggers dedifferentiation of adjacent medial SMCs which serve to regulate vascular tone and blood flow under physiological conditions (Ross R, 1999). Dedifferentiated SMCs are characterized by a large cell body containing synthetic and secretary organelles (Ross R, 1999). It has been hypothesized that dedifferentiated SMCs migrate into the subendothelial space, proliferate, and synthesize extracellular matrix. Similarly, it has been assumed that all of the neointimal cells in post-angioplasty restenosis and graft vasculopathy are derived from adjacent medical smooth muscle cells. Thus, numerous pharmacological and gene therapies have been proposed targeting dedifferentiation, migration, proliferation of medial smooth muscle cells. However, there are several phenomena that could not be explained in accordance with this hypothesis

(Sata M, 2003). Evidence in support of this hypothesis was provided in several models of vascular diseases (Saiura A, et al. 2001; Sata M. 2003; Sata M, et al. 2002; Tanaka K, et al. 2003) (Fig. 1).

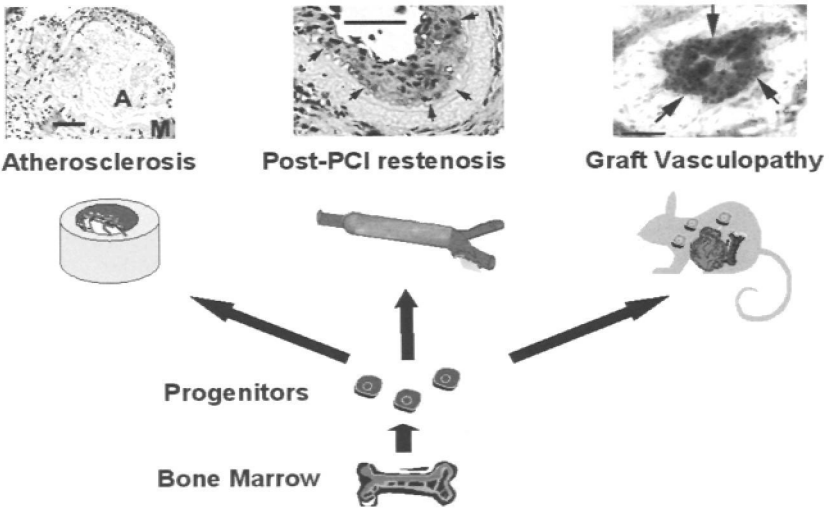

Fig. 1. Bone marrow derived vascular progenitors contribute to vascular remodeling in hyperlipidemia-induced atherosclerosis, post percutaneous coronary intervention (PCI) restenosis, and graft vasculopathy (Sata M, et al. 2002). Reproduced with permission from Nature Publishing Company

Contribution of bone marrow cells to transplant-associated arteriosclerosis

The contribution of bone marrow cells was first investigated in graft vasculopathy, a robust form of atherosclerosis which develops in transplanted organs rapidly, leading to failure of the allograft (Sata M, 2003). In this study, heterotopic cardiac transplantation was performed between wild-type mice and ROSA26 mice (Saiura A, et al. 2001; Sata M, et al. 2002), which are knock-in mice ubiquitously expressing LacZ (LacZ-mice). Four weeks after transplantation, the allografts were harvested and stained with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). The lumen of large epicardial coronary arteries, their smaller branches and arterioles were found to have narrowed owing

to concentric neointimal hyperplasia. The majority of the neointima was composed of recipient cells expressing LacZ (Saiura A, et al. 2001; Sata M, et al. 2002). It was also observed that some of the medial SMCs as well as endothelial cells (ECs) had been replaced by recipient cells. Immunofluorescence studies revealed that LacZ-positive cells in the neointima expressed various markers for SMCs, including myosin heavy chain, calponin, h-caldesmon, and α-smooth muscle actin (α-SMA) (Sata M, et al. 2002). Conversely, when LacZ-positive hearts were transplanted into wild-type mice, LacZ-negative neointima developed on the LacZ-positive coronary arteries. Furthermore, in situ hybridization of the allografts from female to male mice revealed that most of the cells corresponded to cells from the recipients (Saiura A, et al. 2001). These results indicated that the majority of the neointimal cells derived from the recipient cells, but not from the medial cells of donor origin. Consistent with these observations, others independently reported that recipient cells are a major source of graft vasculopathy in the aortic transplantation model (Hillebrands J, et al. 2001; Hu Y, et al. 2002; Shimizu K, et al. 2001). Moreover, it was reported that most of the neointimal cells and ECs were derived
from recipients in human transplant-associated arteriosclerosis after renal transplantation (Grimm P C, et al. 2001).

To identify the potential source of recipient cells that contribute to allograft vasculopathy, bone marrow transplantation (BMT) was performed form LacZ mice to wild-type mice ($BMT^{LacZ \rightarrow wild}$ mice). After four to eight weeks, wild-type hearts were transplanted into the $BMT^{LacZ \rightarrow wild}$ mice. Four weeks after cardiac transplantation, most of the neointimal cells were found to be LacZ-positive. Similarly, when wild-type hearts were transplanted into wild-type mice whose bone marrow had been reconstituted with that of transgenic mice that ubiquitously expressed enhanced green fluorescent protein (GFP mice) ($BMT^{GFP \rightarrow wild}$ mice) (Sata M, et al. 2002), it was observed that GFP-positive cells accumulated on the luminal side of the graft coronary arteries under a fluorescence illuminator. Immunofluorescence study revealed that some of the GFP-positive cells in graft vasculopathy expressed α-SMA. These results indicate that recipient bone marrow cells may substantially contribute to neointimal formation in transplanted grafts.

Contribution of bone marrow cells to vascular remodeling after mechanical injury

Bone marrow cells can also contribute to the pathogenesis of lesion formation after mechanical vascular injury (Sata M, et al. 2002; Tanaka K, et al. 2003). The bone marrow of wild-type mice were replaced with that of LacZ-mice ($BMT^{LacZ \rightarrow wild}$ mice). It was found that transplanted LacZ bone marrow cells had settled in bone marrow, spleen, and thymus, whereas no LacZ-positive cell was detected in uninjured femoral arteries of $BMT^{LacZ \rightarrow wild}$ mice. Four to eight weeks after BMT, a large wire was inserted into the femoral artery of $BMT^{LacZ \rightarrow wild}$ mice, using a new method of vascular injury that resembles balloon angioplasty (Sata M, et al. 2000). This injury led to complete endothelial denudation and marked enlargement of the lumen (Sata M, et al. 2003, Sata M, et al. 2001, Sata M, et al. 2000). The cellularity of the medial layer decreased owing to acute onset of SMC apoptosis (Sata M, et al. 2000). One week after the injury, the artery remained dilated. X-gal staining revealed that LacZ-positive cells attached to the luminal side of the injured vessels. LacZ-positive cells did not express a marker for SMCs (α-SMA) or that for endothelial cells (ECs, CD31). The dilated lumen gradually narrowed due to neointimal hyperplasia, which was primarily composed of SMCs. A significant amount of neointimal and medial cells were LacZ-positive as determined by X-gal staining (Sata M, et al. 2002; Tanaka K, et al. 2003). Immunofluorescence double staining documented that some bone marrow-derived LacZ positive cells in the neointimal lesions expressed α-SMA or CD31 (Tanaka K, et al. 2003). These results indicate that bone marrow cells may give rise to vascular cells, thereby contributing to arterial remodeling after wire-mediated endovascular injury.

Bone marrow-derived SMC-like cells in atherosclerotic plaques

To evaluate the potential source of SMCs observed in atherosclerotic plaques (Ross R, 1993), the bone marrow of ApoE -/- mice was replaced with that of GFP-mice ($BMT^{GFP \rightarrow ApoE-/-}$ mice) or ROSA26-mice ($BMT^{LacZ \rightarrow ApoE-/-}$ mice) (Sata M, et al. 2002). The recipient mice were fed a western-type diet for eight weeks, starting at four weeks after BMT.

GFP-positive cells accumulated in atherosclerotic plaques developing in the aorta of $BMT^{GFP \rightarrow ApoE-/-}$ mice. Immunofluorescence study on the cross sections revealed that some of the GFP-positive cells expressed markers for smooth muscle cells (α-SMA). Similarly, in the atherosclerotic lesions of the $BMT^{LacZ \rightarrow ApoE-/-}$ mice, some of the LacZ-positive cells expressed α-SMA. Furthermore, immunogold-labeling for LacZ identified bone marrow-derived SMC-like cells having muscle fibers in the atherosclerotic plaques of $BMT^{LacZ \rightarrow ApoE-/-}$ mice. The SMC-like cells displayed typical "synthetic" phenotype. These results suggest that some of the SMC-like cells observed in hyperlipidemia-induced atherosclerotic plaques may originate from bone marrow.

New mechanism for SMC accumulation in vascular lesions

Coronary angioplasty causes vessel wall injury and induces SMCs proliferation with subsequent abundant production of extracellular matrix. Transplant-associated arteriosclerosis is also considered a consequence of an immunological attack against the allograft by the recipient (Billingham ME, 1987). Similarly, various atherogenic substances, such as oxidized LDL, homocysteine, angiotensin II and lipopolysaccharides, have been reported to induce vascular cell apoptosis, presumably initiating the earliest phase of lesion development in atherosclerosis (Tricot O, et al. 2000). Therefore, neointima formation appears to be similar to the healing process in response to vascular injuries. In addition to the conventional assumption that damaged tissues are repaired by individual parenchymal cells, an accumulating body of evidence indicates that there exist somatic stem cells that are mobilized to remote organs, differentiate into required lineages and participate in organ repair and regeneration (Asahara T, et al. 1997; Hill J M, et al. 2003). Bone marrow might be an additional source of vascular cells that contribute to vascular repair and pathological remodeling in models of post-angioplasty restenosis, transplant-associated arteriosclerosis and hyperlipidemia-induced atherosclerosis.

Cell fusion as a possible mechanism of bone marrow "differentiation" into SMC

Recent evidence suggests that somatic stem cells or adult stem cells remain even in the adult body. Many animal experiments documented that adult stem cells can transdifferentiate into other lineages. In gender-mismatched human bone marrow transplantation, it was reported that donor-derived hematopoietic stem cells participated in organ regeneration. On the other hand, recent papers suggest that adult stem cells adopt tissue-specific phenotype by cell fusion in vitro (Terada N, et al. 2002) and in vivo (Wang X, et al. 2003) but not by transdifferentiation. Consistent with this notion, previous reports documented polyploidization of vascular SMCs in response to mechanical and humoral stimuli (Campbell J H, et al. 1991). Thus, it is possible that cell fusion can account for, at least in part, the accumulation of bone marrow-derived SMC-like cells in vascular lesions , although spontaneous cell fusion between recipient and donor-derived cells seems to be a rare event in a murine model of cardiac transplantation (Saiura A, et al. 2003).

Implications for regenerative medicine

There is an accumulating body of evidence that in the adult bone marrow there are somatic stem cells that can differentiate into various lineages (Blau H M, et al. 2001). Our results also suggest that we may be able to construct biological artificial arteries using bone marrow-derived vascular progenitors (Fig. 2)(Kaushal S, et al. 2001). Given the pluripotency of adult stem cells, they may potentially differentiate into unfavorable cell-types. In fact, there are a number of similarities between stem cells and cancer cells (Reya T, et al. 2001). Our findings constitute evidence that somatic stem cells do participate in pathological remodeling in remote organs (Saiura A, et al. 2001; Sata M, et al. 2002). For clinical use of adult stem cells, attention should be paid to potential adverse effects as well as beneficial aspects caused by the transplanted stem cells.

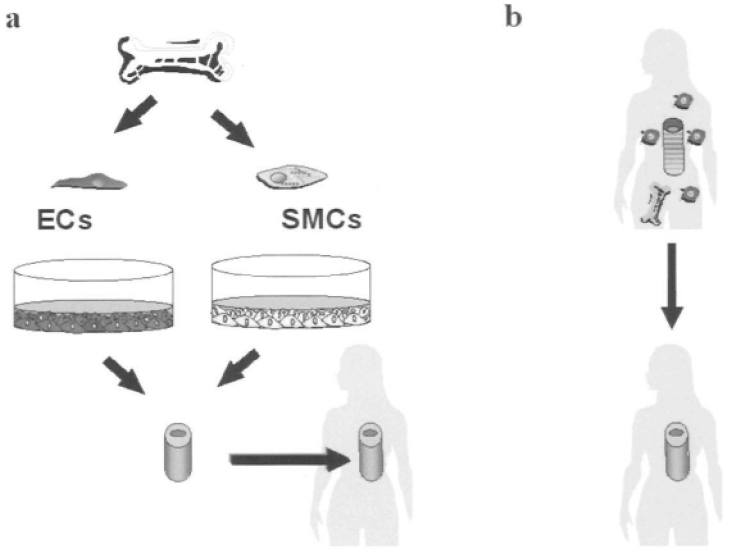

Fig. 2. Functional artery might be engineered with bone marrow cells or circulating vascular progenitors. **a** In vitro differentiation method. Bone marrow-derived endothelial cells (ECs) and smooth muscle cells (SMCs) are used to reconstitute the arterial wall. **b** Scaffold method. Circulating progenitors are attracted to the implanted scaffold to generate the arterial wall

Future Direction

Further studies are needed to identify and characterize putative vascular progenitor cells. It was observed that bone marrow-derived cells were negative for markers of SMCs and ECs when they attached to the luminal side of the artery one week after mechanical injury (Sata M, et al. 2002). It is likely that plastic immature cells may be mobilized to the injured vessels, where they differentiate in response to mechanical and humoral stimuli. Consistently, it was reported that blood cells contain progenitors that have the potential to differentiate into either ECs or SMCs in vitro according to the composition of the culture medium (Simper D, et al. 2002). Experiments that dissect molecular mechanisms by which progenitors are recruited and differentiate at the site of injury are warranted.

Conclusions

In summary, our findings indicate that bone marrow cells, including hematopoietic stem cells, contribute not only to the healing process of injured organs, but also to pathological remodeling. Our findings provide the basis for the development of new therapeutic strategies for vascular diseases, targeting mobilization, homing, differentiation and proliferation of bone marrow-derived vascular progenitor cells.

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Gene Therapy with Hepatocyte Growth Factor for Angiogenesis in Severe Pulmonary Vascular Disease

Masamichi Ono, Yoshiki Sawa, and Hikaru Matsuda

Division of Cardiovascular Surgery, Department of Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka, 565-0871, Japan

Summary. Severe pulmonary vascular disease, such as pulmonary hypertension (PH) which is characterized by proliferation of pulmonary artery smooth muscle cells and progression of pulmonary arterial fibrosis, is not curable without lung transplantation. Thus further development of alternative therapies is required. This article, using rat and piglet model of advanced pulmonary vascular diseases, shows that gene transfer with Hepatocyte Growth Factor (HGF) inhibited overgrowth of pulmonary artery smooth muscle cells and reduced lung expression level of endothelin-1 and TGF-beta, which prevented vascular medial hyperplasia and matrix accumulation of pulmonary arteries, as well as induced angiogenesis. HGF gene transfer also enhanced the ameliorating effect of PGIS in experimental PH. These results suggest that gene transfer with HGF may be found suitable in treating subjects with severe pulmonary vascular disease.

Key words. Gene therapy, Hepatocyte growth factor, Angiogenesis, Pulmonary hypertension, Lung transplantation

Introduction

In spite of recent advances in medical and surgical therapy, we find difficulties in the treatment of severe pulmonary vascular disease, such as

pulmonary hypertension (PH), pulmonary infarction, and pulmonary vascular hypoplasia associated with congenital heart disease. Histologically, the proliferation of pulmonary artery smooth muscle cells (PA-SMCs), the progression of pulmonary arterial fibrosis, and decrease of the number of pulmonary arteries, are key features of the disease (Runo JR, et al. 2003, Heath D, et al. 1958, Voekel N, et al. 1997), and it is impossible to reverse progression of pulmonary vascular changes by conventional therapy. Recently, clinical gene therapy using growth factors, such as VEGF, or HGF, has been successfully performed for ischemic coronary and peripheral artery diseases (Losordo DW, et al. 1998, Isner JM, et al. 1996). This is called therapeutic angiogenesis. These results delineate a possibility of clinical gene therapy for pulmonary vascular disease.

Hepatocyte Growth Factor (HGF), which was originally purified and cloned as a potent mitogen for hepatocytes (Nakamura T, et al. 1989), has mitogenic, motogenic, morphogenic, and antiapoptotic activities in various cell types (Zarnegar R, et al. 1995, Matsumoto K, et al. 1996). The pluripotent activities of HGF are mediated by a membrane-spanning tyrosine kinase receptor encoded by the c-met proto-oncogene (Botaro DP, et al. 1996). Physiologically, HGF acts as an organotrophic factor for protection from injury and the regeneration of various organs (Michaopoulos GK, et al. 1997, Mizuno S, et al. 1998, Nakamura T, et al. 2000). In the lung, biological and pulmotrophic roles for HGF have been well documented (Ohmichi H, et al. 1996). In response to acute and chronic lung injury, HGF plays a role in lung regeneration and protection (Yanagita K, et al. 1993, Yamada T, et al. 2000, Sakamai Y, et al. 2002, Yaekashiwa M, et al. 1937). In the present article, we reviewed our studies, which investigated the roles of HGF gene therapy under pathological conditions, using the model of monocrotaline-induced pulmonary hypertension of rats and that of pulmonary vascular hypoplasia by pulmonary artery banding of piglets.

HGF plasmid and Gene Transfection Vector using HVJ

To prepare an HGF expression vector, the cDNA for human HGF was inserted into the Not I site of the pUC-SRα expression vector plasmid. In this plasmid, expression of the HGF cDNA is regulated under the control

of the SRα promoter. As for the vector, we used HVJ (Hemmaggulutinating Virus of Japan; Sendai virus) liposome, or HVJ envelope vector, which has been recently developed by Kaneda et al (Kaneda Y, et al. 2002).

In this article, we performed two methods of gene transfection. One is trans-arterial method of gene transfection to the pulmonary vasculature, which was applied in accordance to the previous study (Ono M, et al. 2002). The other method is gene transfection to the liver, of which we previously reported the usefulness for the pulmonary vasculature using PGIS gene (Suhara H, et al.). Using this method, we tested the co-transfection of HGF and PGIS for the treatment of pulmonary hypertension.

Gene Therapy for Pulmonary Hypertension

Pulmonary hypertension (PH) is a progressive disease caused by a variety of pulmonary and/or cardiac disorders and characterized by an increase in pulmonary vascular resistance that leads to right ventricular failure. Recent studies delineate a central role for PA-SMC overgrowth in provoking the initial pathogenesis of PH, and several lines of evidence indicate that transforming growth factor-beta (TGF-beta) is responsible for lung fibrogenesis (Broekelmann TJ, et al. 1991); and endothelin-1 (ET-1) is a potent mediator for blood hypertension via enhancing the vasoconstriction of PA-SMCs (as pericytes)(Jasmin JF,et al. 2001). Such previous studies focused on molecular roles in the initial pathogenesis, but little information is available about the body's self-defense system, which should help block the PH-related pathological changes. Therefore, identification of natural ligands involved in an anti-pathogenic mechanism would shed light on how to develop a therapeutic strategy for advanced PH. Therefore, we used monocrotaline (MCT)-injected rats as an animal model to mimic human PH and investigated the roles of HGF under pathological conditions (Ono M, et al. Circ. In press, Ono M, et al. EJCS. In press).

Pulmonary vascular changes in MCT-induced PH

After MCT administration, the progression of PH was confirmed (Fig.1). The expression levels of lung TGF-beta and ET-1, and the total collagen

significantly increased (Fig.2**a**), but lung HGF, mRNA and protein decreased significantly after MCT administration (Fig.2**b**). The c-Met HGF receptor expression was evident in the parenchyma and endothelial cells in normal pulmonary arteries, whereas c-Met immunostaining was positive in almost all of the PA-SMCs of the pulmonary arteries in rats with MCT-induced PH (Fig.2**c**).

Trans-arterial Gene Transfection for Pulmonary Hypertension

To assess the effect of exogenous HGF, rats that had received MCT two weeks previously were performed trans-arterial gene transfection of the left lung, according to our previously published methods (Ono M, et al 2002). Two weeks after the HGF gene transfection, we found by histological examination a marked decrease in medial wall thickening in the HGF-transfected lung compared with the control (Fig.3**a**). Proliferating cells were significantly decreased after transfection with HGF. Furthermore, smooth muscle cell apoptosis, detected with TUNEL staining was significantly increased. Immunostaining using a TGF-beta antibody showed markedly decreased expression of TGF-beta in the HGF transfected lung. The tissue concentration of TGF-beta in the HGF-transfected lung was significantly lower than in the control (Fig.3**c**). Likewise, the total collagen content of the HGF-transfected lung was significantly lower than in the control. To assess the effect on hemodynamic change induced by the exogenous HGF, we evaluated the tissue concentration of ET-1, which in the HGF-transfected lung was significantly lower than in the control (Fig.4**a**). The pressure and weight ratio of the right ventricle to the left, were also significantly attenuated on day 14 after the transfection (Fig.4**b**).

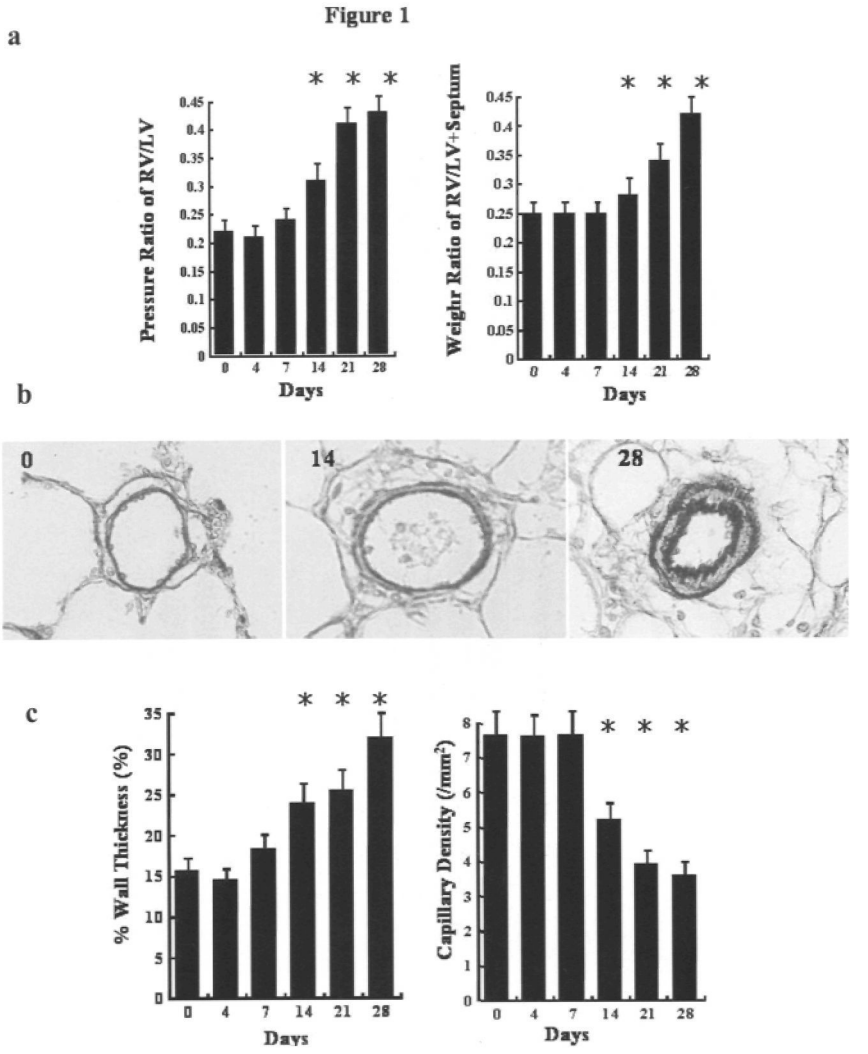

Fig. 1a, b, c. *Pulmonary Hypertension after MCT administration.* **a** Pressure and weight ratio of the right ventricle to the left. **b** Representative photomicrographs of peripheral pulmonary arteries before (0) and on days 14 and 28 after the MCT injection (Magnification×400). **c** Percent medial wall thickness of peripheral pulmonary arteries and capillary density. Each value represents the mean ± SEM. *P < 0.01 versus control (day 0).

Figure 2

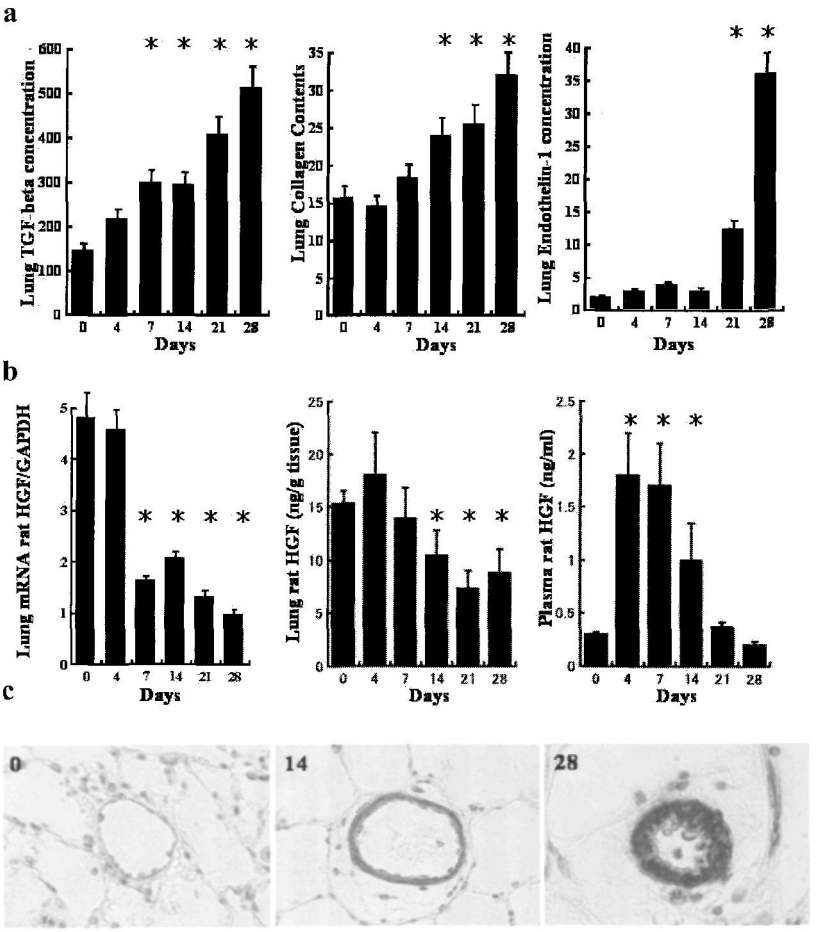

Fig. 2a, b, c. a TGF-beta concentration, total collagen content, and Endothelin-1 concentration of the lung. **b** Lung HGF mRNA, Lung HGF protein, and plasma HGF. (mean ± SEM). *P < 0.05 versus control (day 0). **c** Immunohistochemical staining for the c-Met/HGF receptor on days 0, 14, and 28 days after MCT administration (Magnification×400).

Figure 3

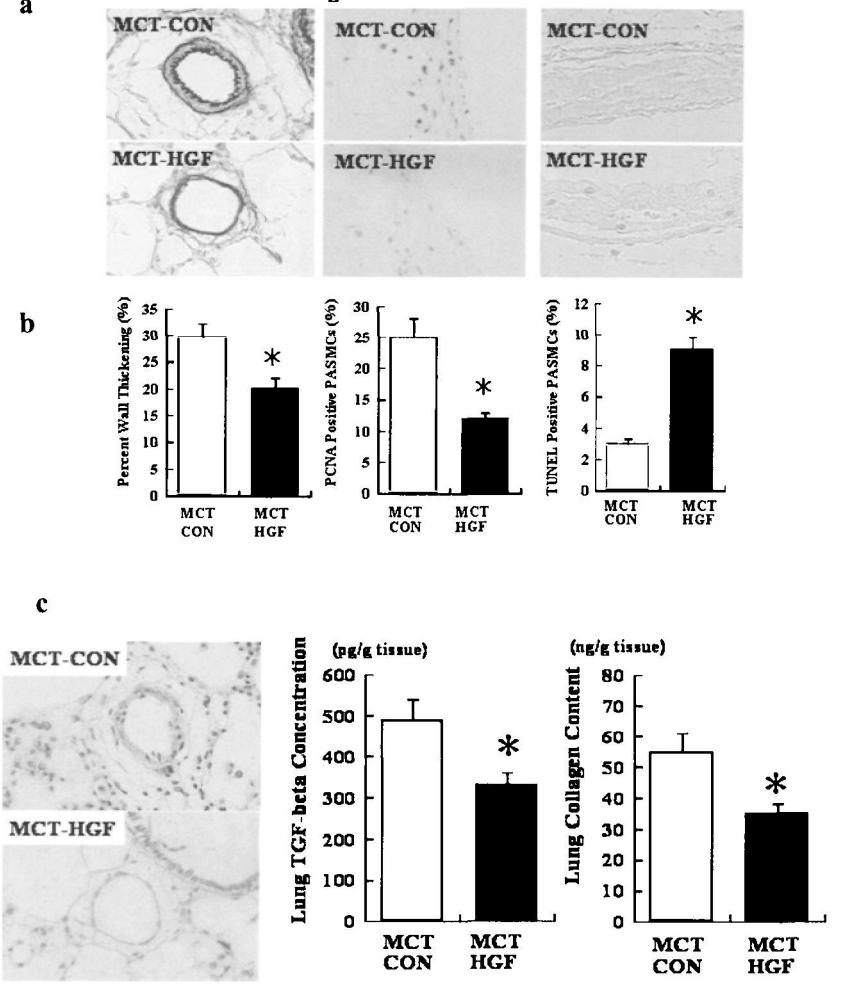

Fig. 3a, b, c. *Effects of transfection with the HGF gene.* **a** Elastica van Gieson PCNA, and TUNEL staining in HGF transfected lung (MCT-HGF) and the control (MCT-CON); (magnifications, x200). **b** Percent medial wall thickness of pulmonary arteries, percent PCNA-positive PA-SMCs, percent TUNEL-positive PA-SMCs on day 14 after gene transfection.(in mean ± SEM). * $P < 0.05$ versus control. **c** TGF-beta and total collagen content after transfection with HGF gene.

Figure 4

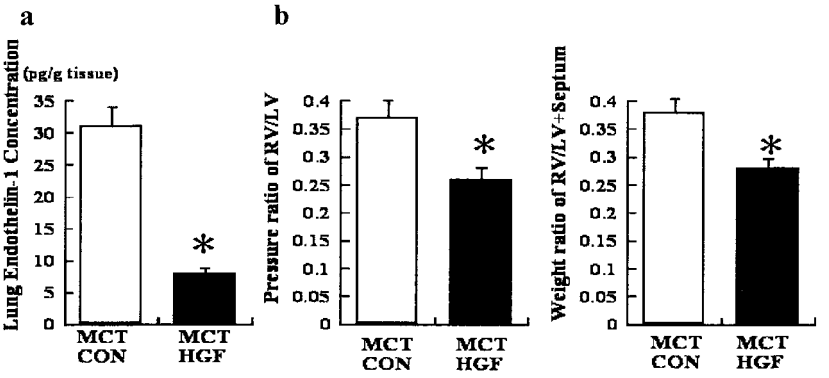

Fig. 4a, b. *Effects of HGF on Endothelin-1 concentration and pulmonary hypertension.* **a** Tissue level of Endothelin-1. **b** Pressure and weight ratio of right to left ventricle. Each value represents the mean ± SEM. *P < 0.05 versus control.

Co-Transfection of HGF and/or PGIS to the Liver for Pulmonary Hypertension

To assess the effect of co-transfection of HGF and PGIS, gene transfer was performed in the liver as DDS. The day before MCT injection rats underwent small midline laparotomy and received an injection of HVJ liposome containing HGF and/or PGIS gene into the left lobe of the liver according to our previous study (Suhara H, et al 2002). Histological changes in the small pulmonary arteries 28 days after MCT administration demonstrated that percent medial wall thickening of small pulmonary arteries was significantly lower in HGF transfected group, but not in the PGIS group. Co-transfection of HGF and PGIS group also had significant difference compared with the control group (Fig.5). To assess the progression of pulmonary hypertension, and the effect of HGF and/or PGIS gene transfection, we assessed pressure and weight ratio of the right ventricle to the left. Both of the indicators showed a significant decrease in the PGIS group and HGF/PGIS group compared with the Control group, but not in the HGF group. Furthermore, the indicators in the HGF/PGIS groups were significantly lower than PGIS group (Fig.6).

Figure 5

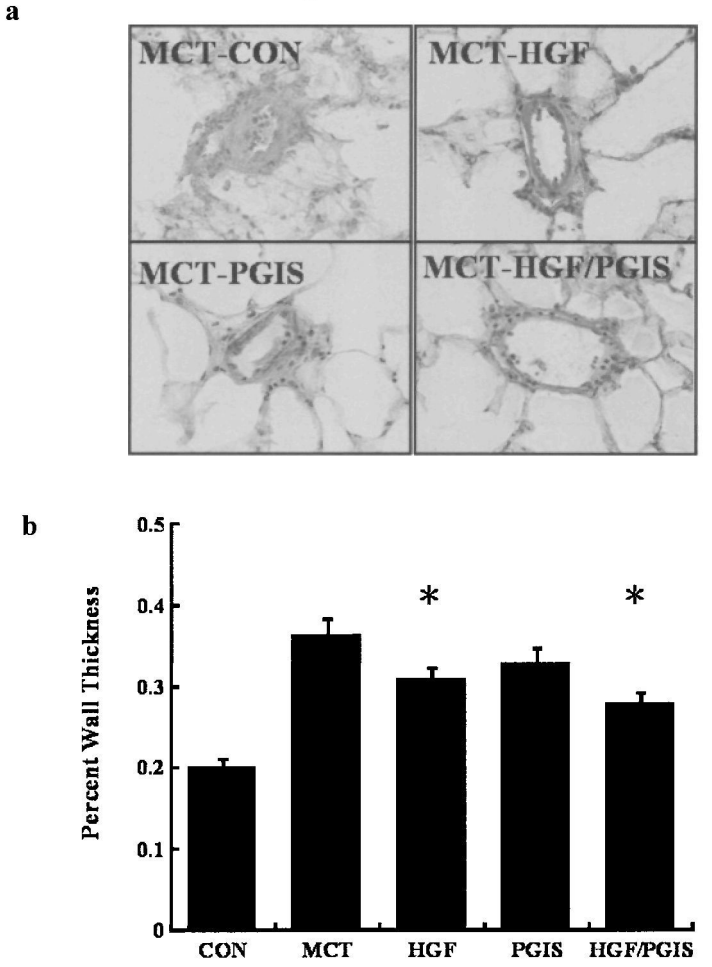

Fig. 5.a, b. *Histological Changes after gene transfection.* **a** Representative photomicrographs of peripheral pulmonary arteries on days 28 after gene transfection (Magnification×400). **b** Changes in percent medial wall thickness of peripheral pulmonary arteries. Each value represents the mean ± SEM. *P<0.05 versus MCT

Figure 6

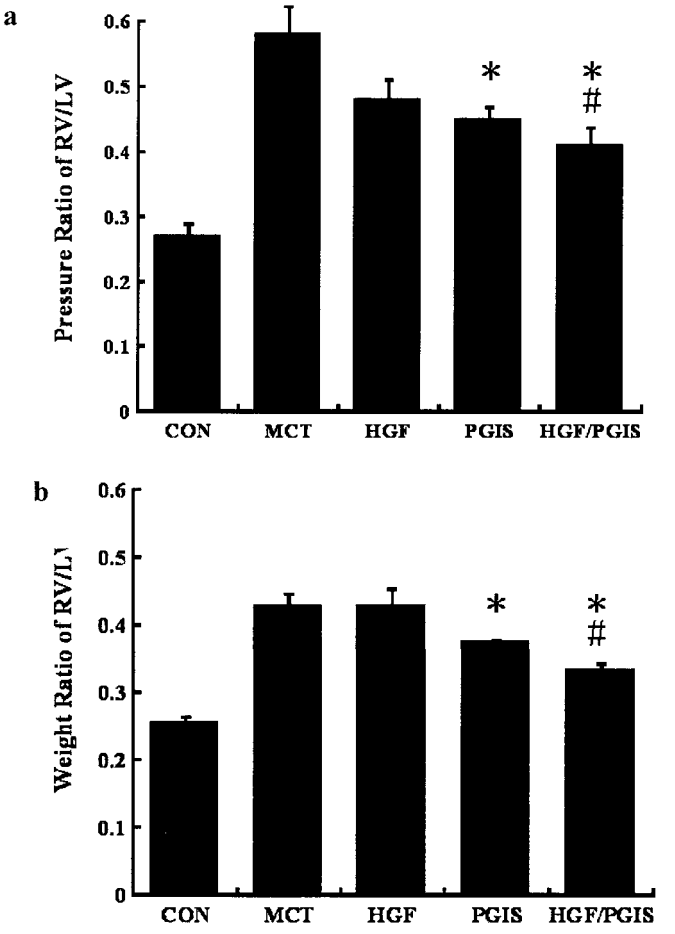

Fig. 6a, b. *Effects of gene transfection for pulmonary hypertension after MCT injection.* **a** Pressure and **b** weight ratio of the right ventricle to the left ventricle. Each value represents the mean ± SEM. *P < 0.01 versus MCT. #P < 0.01 versus HGF and PGIS.

Angiogenic Gene Therapy for Hypoplastic Pulmonary Vasulature

As for the angiogenic effect of HGF in pulmonary vasculalture, we previously demonstrated transarterial HGF gene transfection to the normal rat lung (Ono M, et al. 2002), and showed angiogenesis was obtained in the lung as well as in the heart and the limbs. The vessels in transfected lung were morphologically and functionally normal. Therefore, we investigated the role of HGF in pulmonary vascular hypoplasia in the piglet (Ono M, et al. JTCS, in press) model.

Pulmonary vascular changes in pulmonary arterial hypoplasia

Piglets weighing 5kg underwent left pulmonary artery banding (PAB). Seven days after PAB, selective left pulmonary arteriogram demonstrated pulmonary vascular hypoplasia of the left lung (Fig.7**a**). Histological evaluation shows pulmonary arteries along the terminal bronchioles were small after PAB (Fig.7**b**). Then, the HVJ envelope-plasmid complex (20 ml, containing 2mg of cDNA) was infused via the catheter, following the occlusion of the left PA flow by balloon occlusion.

Effect of HGF Gene Transfection for Pulmonary vascular hypoplasia

Seven days after gene transfection, left pulmonary angiogram demonstrated a marked increase in pulmonary vasculature of the left lung (Fig. 8**a**). Likewise, a histological feature after HGF gene transfection was an increase in the number of pulmonary arteries (Fig.8**b**). Furthermore, a marked increase in factor VIII-positive pulmonary arteries along a single terminal bronchiole was observed after HGF gene transfection (Fig.8**c**).

Figure 7

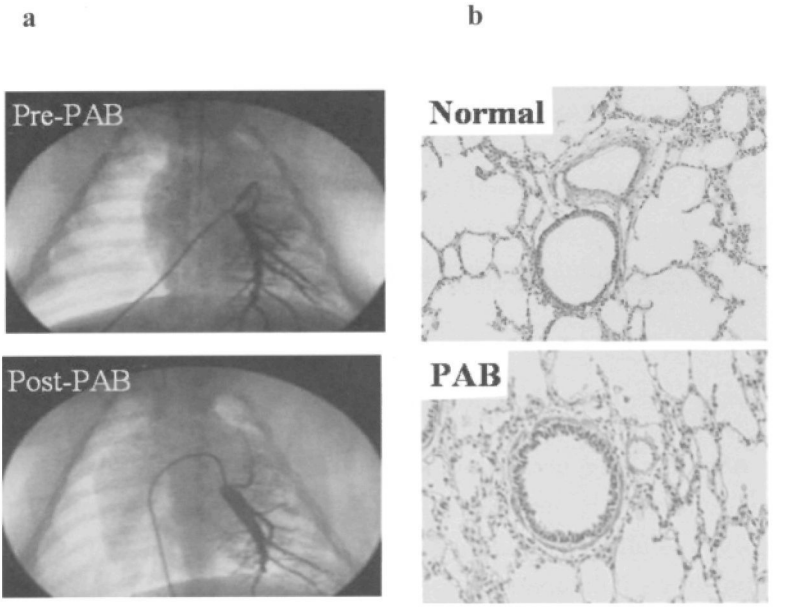

Fig. 7a, b. *Evaluation of pulmonary vascular hypoplasia by left pulmonary artery banding.* **a** Selective left pulmonary arteriogram seven days after left PA banding. **b** Pulmonary artery along the terminal bronchiole in the left lung (Magnifications × 100).

Figure 8

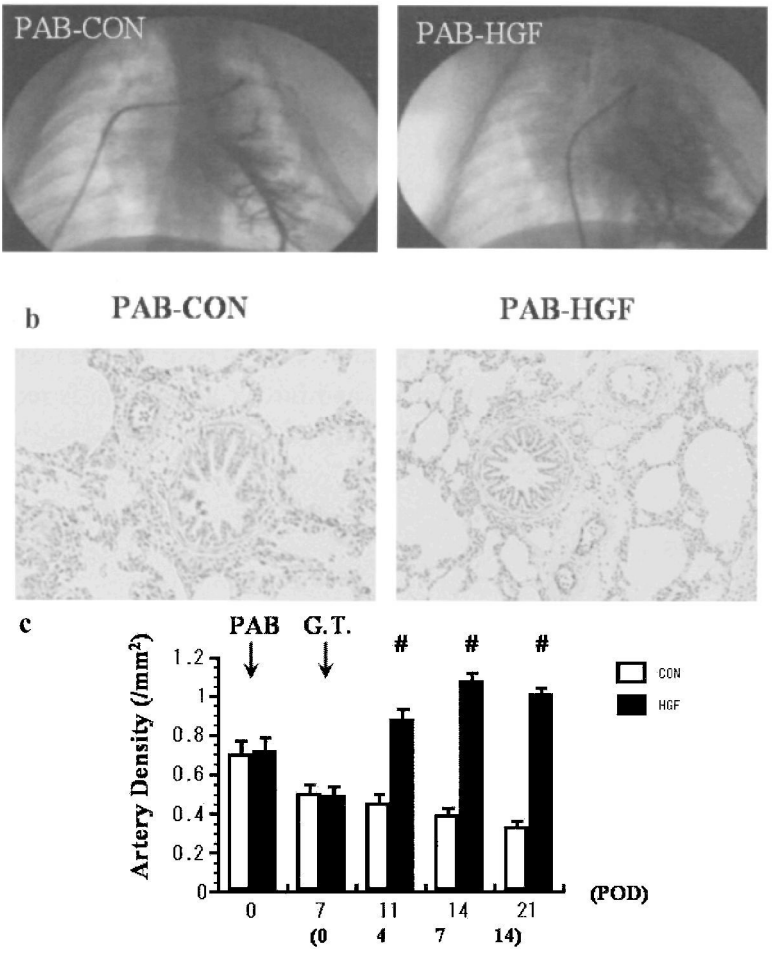

Fig. 8a, b, c. *Effect of HGF gene transfer after PAB.* **a** Selective left pulmonary arteriogram after gene transfection. **b** Pulmonary arteries along terminal bronchiole in the left lung (Magnifications × 100). **c** Number of Pulmonary artery along the terminal bronchiole. Each value represents the mean ± SEM, G.T.; Gene Transfection.

Conclusion

Throughout the present experiments, we delineated the roles of HGF gene therapy in severe pulmonary vascular disease to antagonize overgrowth of PA-SMCs as well as to suppress the lung expression of TGF-beta and ET-1, major fibrogenic and hypertensive mediators. HGF is angiogenic for endothelial cells and possibly morphogenic for alveolar or bronchial epithelial cells. Thus, HGF has been recognized as a regenerative factor thorough anti-fibrotic, pulmotrophic, and angiogenic effects in advanced pulmonary vascular disease. Collectively, many roles played by HGF could participate in lung reconstruction, even after the onset of pulmonary vascular disease. The potential therapeutic value of HGF for the treatment of patients with PH deserves attention, and further assessment is required for clinical application of gene therapy with HGF.

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Basic Fibroblast Growth Factor and Angiogenesis

Akira Marui[1], Kazuhiko Doi[2], Keiichi Tambara[1], Yutaka Sakakibara[1], Koji Ueyama[3], Atsushi Iwakura[4], Masaya Yamamoto[5], Tadashi Ikeda[1], Yasuhiko Tabata[5], and Masashi Komeda[1]

[1]Department of Cardiovascular Surgery, Kyoto University Graduate School of Medicine, 54 Kawara, Shogoin, Sakyo, Kyoto 606-8507, Japan
[2]Department of Cardiovascular Surgery, Takeda Hospital, 841-5 Higashi-Shiokoji, Shimogyo, Kyoto 600-8558, Japan
[3]Department of Cardiovascular Surgery, Otowa Hospital, 2 Otowachinji, Yamashina, Kyoto 607-8062, Japan
[4]Division of Cardiovascular Research, St. Elizabeth's Medical Center of Boston, 736 Cambridge Street, Boston, MA 02135, USA
[5]Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara, Shogoin, Sakyo, Kyoto 606-8507, Japan

Summary. Regenerative medicine using growth factors is an important therapeutic tool in patients with tissue ischemia or defects. Among such therapies, we have mainly addressed the sustained release of basic fibroblast growth factor (bFGF) as a safe and effective treatment. We developed a biodegradable hydrogel composed of acidic gelatin to enable bFGF to be released at the site of action for a sufficient time period. We experimentally applied this method in various animal models for cardiovascular research with satisfactory results as follows:1) Sustained release of bFGF from gelatin hydrogel is effective in ischemic heart disease, dilated cardiomyopathy, pulmonary hypertension, limb ischemia, and bone regeneration;2) Both sustained release of bFGF and bone marrow cell transplantation induce angiogenesis, but only bFGF produces arteriogenesis;3) bFGF increases efficacy of cell transplantation, such as cardiomyocyte or skeletal myoblast. Future study is warranted for safe and effective clinical applications of the bFGF controlled-release system.

Key words. Basic fibroblast growth factor, Angiogenesis, Controlled release, Cell transplantation

Introduction

bFGF is one of the potent mitogens regulating proteins that induces proliferation of a variety of cells, such as epithelial and mesenchymal cells, and promotes growth and regeneration of organs and tissues in vivo (Rifkin DB, et al. 1989). Because solution form of bFGF does not exert sufficient biological activities to induce expected results due to its short half-life, tissue regeneration is not successful in most cases. Recently we developed a biodegradable hydrogel composed of acidic gelatin to enable bFGF to be released at the site of action for a sufficient time period (Tabata Y, et al. 1994). Using the method, bFGF can be gradually released from gelatin hydrogel maintaining its biological activities along with hydrogel biodegradation, and the release period is controlled by the weight percentage of water in the hydrogel for up to 3 months. Moreover this allows bFGF to act only in situ without any systemic side-effect, which is considered to be one of the hurdles for gene therapy to overcome for its safe clinical use.

In this review, we briefly describe the effectiveness of sustained release of bFGF from gelatin hydrogel in various animal studies in the cardiovascular research. Then, we compared the effects of bFGF with bone marrow cell transplantation as angiogenic agents, and evaluated the effects of combining bFGF and cell transplantation. Finally, we refer to future prospects for regenerative medicine using this controlled-release system of growth factors.

Preparation of bFGF-incorporated Gelatin Hydrogel

We designed 2 types of preparations for gelatin hydrogel, 'sheet' and 'microsphere', as a release carrier of the growth factors. The gelatin hydrogel sheet and microsphere were made as described previously (Tabata Y, et al. 1998, 1994). Briefly, a gelatin with an isoelectric point value of 4.9 is isolated from bovine collagen by means of an alkaline process using $Ca(OH)_2$. Gelatin hydrogel is prepared glutaraldehyde cross-linking of the gelatin. The gelatin hydrogel microsphere was washed with acetone (4℃) and recovered by centrifugation. Both the gelatin hydrogel sheet and microsphers were freeze-dried, followed by impregnation with an aqueous solution containing human recombinant bFGF with an isoelectric point value of 9.6, and we obtained bFGF-incorporated gelatin hydrogel sheet or microsphers.

Animal Studies in Cardiovascular Area

Ischemic Heart Disease

Optimal delivery method of bFGF into the ischemic heart

We searched for the optimal method for delivery of bFGF into rat ischemic hearts. Myocardial infarction (MI) was created by ligating the left anterior descending artery (LAD) in Lewis rats. Four weeks later, bFGF was administered by 4 types of delivery methods in these rats with MI: free bFGF by central venous bolus injection (group I); free bFGF by intracoronary injection via the left ventricular (LV) apex during ascending aorta clamping (group II); free bFGF by intramyocardial injection into the infarct and peri-infarct area (group III); bFGF-incorporated gelatin hydrogel microspheres by intramyocardial injection (group IV). The activity of bFGF was monitored by ^{125}I radioiodination.

The remaining radioactivity (%) of bFGF in heart and blood samples was counted at 1, 24, and 72 hours later. Both group I and II showed little bFGF remaining in the heart withon 1 hour after administration of bFGF. In group III, the remaining bFGF rapidly decreased from $16.2 \pm 6.9\%$ (1hour) to $1.8 \pm 0.9\%$ (72 hours). In group IV, there were no significant changes in the bFGF remaining between 1 hour and 72 hours (35.7 ± 1.0 vs. $32.0 \pm 5.2\%$). No remnant bFGF in the blood was detected in all groups at any sampling points. Thus, intramyocardial injection of bFGF-incorporated gelatin hydrogel microspheres was the optimal route for administration of bFGF among the 4 methods (Sakakibara Y, et al. 2003).

Myocardial infarction

Pigs were divided into 2 groups 4 weeks after making MI by ligating the LAD. The control group had gelatin hydrogel alone, and the FGF group received bFGF-incorporated gelatin hydrogel at 5 perimyocardial infarcted areas per animal in the LV wall (40μg each, total 200μg). Four weeks after treatment, the FGF group had smaller LV diastolic diameter and higher LV end-systolic elastance than the control. Histology showed that many newly formed vessels were found in and around the scar tissue and the vascular density in the FGF group was significantly higher. In addition, the infarcted LV walls were less expanded and thicker in the FGF group. In conclusion, sustained release of bFGF improved LV function and inhibited LV

remodeling by angiogenesis in pigs with chronic MI (Sakakibara Y, et al. 2003).

"Bio-CABG": Revival of a classic concept by means of bFGF

In 1936, O'Shaughnessy introduced a technique, omentopexy, in which the greater omentum was brought through the left diaphragm and wrapped around the ischemic heart. We developed a new strategy for revascularization of severely diseased and tiny coronary arteries by 1) Utilization of a healthy large-bore donor artery, such as the gastroepiploic artery (GEA), located near the ischemic area, and 2) Stimulation of angiogenesis from this donor artery with simultaneous administration of bFGF into the ischemic myocardium. In other words, this strategy is a revival of the classic concept, except for utilization of modern biotechnology, bFGF (biologic coronary artery bypass grafting, or *Bio-CABG*).

Acute MI was created by ligating the major branch of the circumflex artery in rabbits and they were divided into four groups: no treatment (control group); the infarcted area was wrapped with the omentum (GEA group); a gelatin hydrogel sheet incorporating 100 μg bFGF was placed over the infarcted area (FGF group); the infarcted area was similarly treated with bFGF followed by omental wrapping (Bio-CABG group). Using echocardiography, the Bio-CABG group showed a better fractional area change than the other groups. Postmortem angiography showed that communication between the GEA and the coronary artery was created through a rich vascular bed in the Bio-CABG group (Fig.1). In histology, the infarct size was reduced and the number of capillaries increased to a greater extent only in the Bio-CABG group. These results suggested that vascular communication from the GEA to the coronary arteries can be achieved without surgical anastomosis through sustained release of bFGF and omental wrapping in this rabbit acute MI model (Ueyama K, et al. 2004).

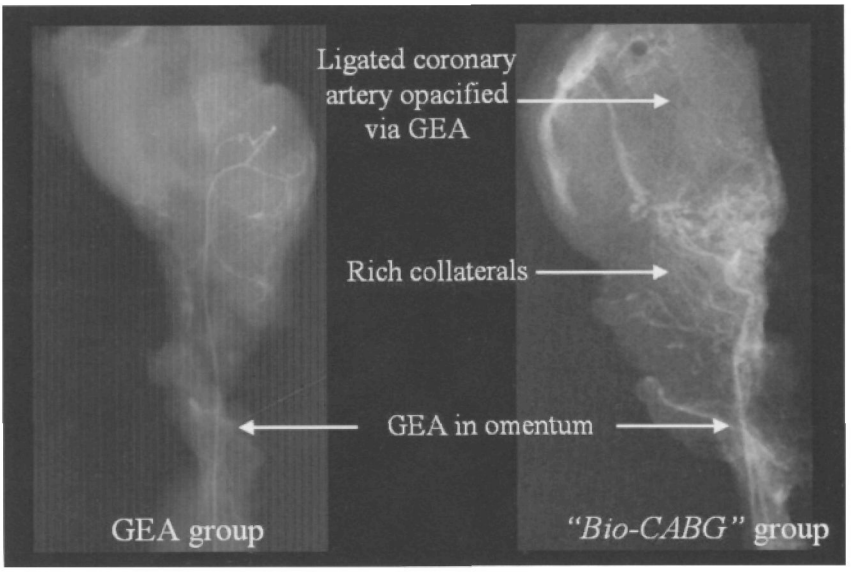

Fig. 1. Representative postmortem angiogram in groups with omental wrapping (GEA group) and omental wrapping with bFGF (Bio-CABG group) Left, in the GEA group, GEA in omentum is opacified, but no communication to coronary arteries is defined. Right, in the Bio-CABG group, numerous collaterals are created between GEA and occluded coronary arteries.

Dilated Cardiomyopathy

Dilated cardiomyopathy (DCM) is considered to be related to ischemia in myocardial microcirculation. Therefore, bFGF may exert beneficial effects for DCM. We assessed the hypothesis that epicardial administration of bFGF via gelatin hydrogel sheet improves LV function in a rat model of DCM. Experimental autoimmune myocarditis was induced by immunization with porcine cardiac myosin in Lewis rats. Six weeks later, DCM rats were treated with 100μg bFGF-incorporated gelatin hydrogel sheet onto the LV surface. Two weeks later, LV end-diastolic dimension showed no difference between the groups by echocardiography. However, the FGF group demonstrated a smaller LV end-systolic dimension and higher fractional shortening than the control. The tissue blood flow in the LV wall, assessed using laser Doppler perfusion image analyzer, was much higher in the FGF group than in the control at both 2 and 4 weeks. In histology, vascular density in the LV free wall was much higher in the FGF group

than in the control group. In conclusion, the sustained release of bFGF in the epicardium may improve LV function by inducing angiogenesis and increasing tissue blood flow in hearts with DCM (Tambara K, et al. 2003).

Pulmonary Hypertension

Wistar rats had a subcutaneous injection of monocrotaline (60mg/kg) and were simultaneously treated with an intraperitoneal injection of 100μg bFGF-incorporated gelatin hydrogel microspheres. Four weeks later, the right ventricular/femoral artery pressure ratio was significantly lower in rats treated with bFGF-incorporated gelatin hydrogel (FGF group) than in the control group (no treatment). The survival rate was significantly higher in the FGF group than the control. Thus, sustained release of bFGF may have inhibited the progress of pulmonary hypertension and reduced mortality. In future experiments, it should be investigated whether this treatment method can be applicable for advanced pulmonary hypertension.

Limb Ischemia

Hindlimb ischemia was created by ligating the right femoral artery of male Japanese white rabbits. Two weeks after surgery, the ischemic limb was treated with an intramuscular injection of bFGF-incorporated gelatin hydrogel microspheres (30μg or 100μg). Hindlimb blood flow was evaluated by the ratio (%) of ischemic (right) to normal (left) hindlimb blood flow measured by laser Doppler perfusion image analyzer. Four weeks after treatment, bFGF dose-dependently increased the blood compared to the control (no treatment). Histology showed that the bFGF also dose-dependently increased capillary density and arteriole density. This method was also effective in ischemic limbs of streptozotocin-induced diabetic rats. bFGF induced satisfactory angiogenesis in not only nondiabetic but also diabetic limb ischemia (Marui A, et al. 2003).

Bone Regeneration After Median Sternotomy with Removal of Bilateral Internal Thoracic Arteries

Previous studies have recommended the use of bilateral internal thoracic arteries (BITAs) for coronary bypass surgery associated with sternal wound complications, because BITAs provide the major blood supply to the sternum. Therefore, we examined whether or not the topical use of bFGF-incorporated gelatin hydrogel sheet would accelerate sternal healing after median sternotomy with the removal of BITAs.

Diabetic Wistar rats with blood glucose levels >400mg/dL and body weight loss >20g were established by a single intravenous injection of streptozotocin (55mg/kg). After median sternotomy and BITAs removal, 16 diabetic rats received either a gelatin sheet that incorporated bFGF (100μg/sheet) on the posterior table of the sternum (FGF group, n=9) or no gelatin sheet (control group, n=7). Peristernal blood flow, as measured by a noncontact laser Doppler 4 weeks after surgery, in the FGF group showed marked angiogenesis around the sternum in the FGF group. Deep sternal wound complications developed in 5 control rats but only in 1 rat in the FGF group (Fig.2). Bone mineral content, as assessed by dual-energy x-ray absorptometry, was significantly greater in the FGF group. Gelatin sheets that incorporate bFGF may offset sternal ischemia and accelerate sternal bone regeneration and healing, even in diabetic patients. We also confirmed the effects of this therapeutic modality on sternal healing in a large-animal model (dog) before performing a clinical trial (Iwakura A, et al. 2003, 2001).

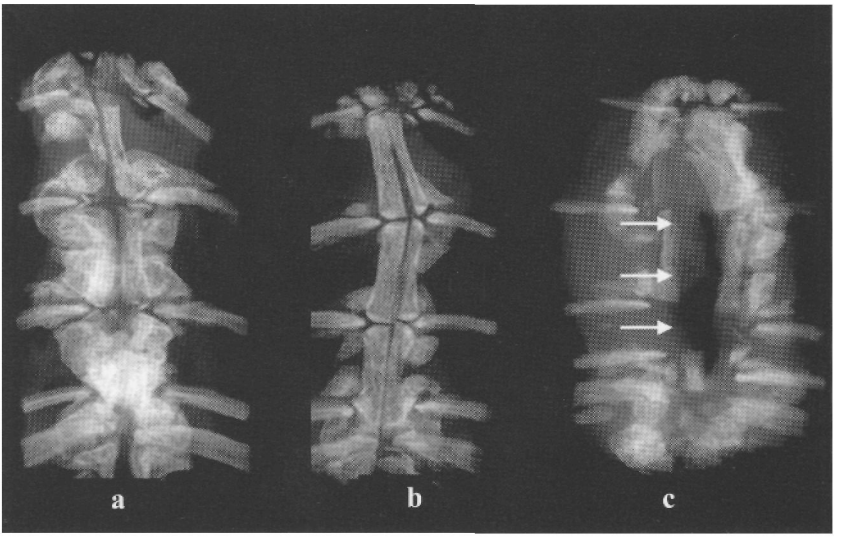

Fig. 2a, b, c. Radiograph shows sternal bone regeneration 4 weeks after surgical treatment in diabetic rats. **a** Almost complete sternal regeneration was seen in FGF group. **b** Slow/poor sternal regeneration was shown in control group. **c** Obvious sternal dehiscence due to deep sternal wound complication was observed in the control group (arrows).

bFGF and Cell Transplantation

Prevascularization with bFGF increases efficacy of cardiomyocyte transplantation

The area in and around the infarct regions is a poor environment for transplanted cells because certain requirements cannot be adequately supplied to the cells. We hypothesized that prevascularization with bFGF may increase the efficacy of cardiomyocyte transplantation by improving the poor condition of the ischemic area.

Rats with MI were randomized into 4 groups: a culture medium injection to the LV wall (control group), fetal cardiomyocyte transplantation (CM group), 100μ of bFGF-incorporated gelatin hydrogel microsphere (FGF group), and bFGF pretreatment sequentially, followed by cardiomyocyte transplantation (FGF/CM group). One week after the treatment, neovascularization was found in the scar tissue in the FGF and FGF/CM groups. Four weeks later, the CM, FGF, and FGF/CM groups showed better fractional shortening than the control. Histology showed that the more transplanted cells survived in the FGF/CM group than in the CM group (Fig.3). Thus prevascularization with bFGF-incorporated gelatin hydrogel microsphere increases efficacy of cardiomyocyte transplantation (Sakakibara Y, et al. 2002)

Sustained release of bFGF vs. bone marrow cell transplantation

We conducted a comparative study to investigate the difference in quantity and quality of angiogenesis between sustained-release administration of bFGF and bone marrow cell (BMC) transplantation using a pig MI model. MI was created in 18 swines by transcatheter embolization of the LAD. Four weeks later, they were divided into 3 groups; Control group was treated with an injection of saline alone, the BM group received a bone marrow-derived mononuclear cell transplantation (totally $1\text{-}2\times10^8$ cells), and FGF group received an injection of 200μg bFGF-incorporated gelatin hydrogel microspheres. Each agent was subepicardially or intramuscularly injected at five peri-infarct areas per animal. Four weeks after treatment, the BM and FGF groups had smaller LV end-diastolic dimensions, better ejection fraction, and better end-systolic elastance than the control. There was no significant difference between BM and FGF groups in any parameters. The plasma levels of BNP were highest in the control. Histology showed that the rich vascularity was found in peri-infarct areas in BM and

FGF groups. It should be noted that the number of arterioles (>100μm diameter) in the FGF group was higher than those in the control and BM groups. In an occasional histological section, we found cartilage formation derived from donor BMCs. These findings suggested a significant difference in angiogenic properties between bFGF administration and BMC transplantation: bFGF induces arteriogenesis, but BMCs do not (Sakakibara Y, et al. 2002).

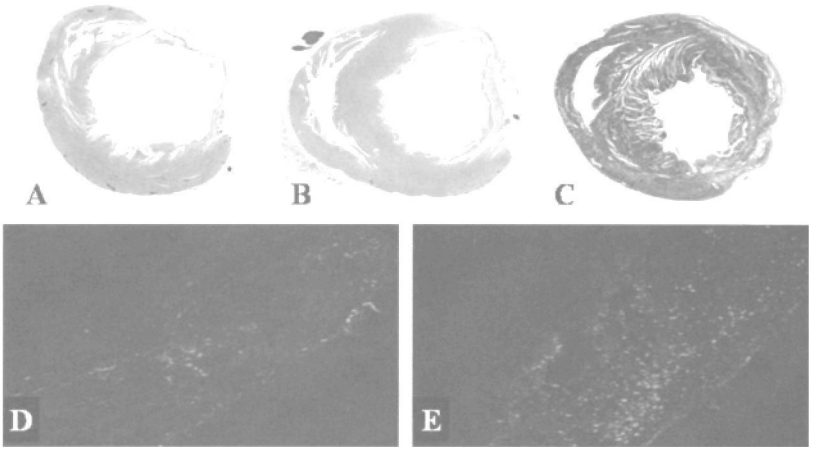

Fig. 3a, b, c, d, e. a-c Histologic findings 4 weeks after each treatment: **a** control group; **b** CM group; **c** FGF/CM group. (Hematoxylin and eosin staining, original magnification 1×.) **d, e** Fluorescent image of transplanted cells labeled with PKH26 red fluorescent dye (**d** peri-infarct area in the CM group; **e** middle of the myocardial infarction in the FGF/CM group). (Original magnification 40×)

Combination of bFGF and skeletal myoblast transplantation

We investigated whether bFGF or BMCs have benefits to skeletal myoblast (SM) transplantation using a rat MI model.

MI was created by ligating the left coronary artery in syngenic Lewis rats. Four weeks after ligation, they were randomized into 3 groups: Neonatal SMs (5×10^6) were subepicardially transplanted in the MI area, and then a gelatin sheet containing 100μg bFGF was placed onto the LV surface to

cover the MI and peri-MI area (FGF/SM group); SM transplantation and a gelatin sheet without bFGF were applied in the same manner (SM group); culture medium alone was injected with a gelatin sheet without bFGF (control group). Four weeks later, in the FGF/SM group, LVEDD and the MI size was smallest. End-systolic elastance was highest and tau was lowest in the FGF/SM group. The graft volume in the FGF/SM was much larger than that in the SM group. Vascular density in the MI and peri-MI area was highest in the FGF/SM group. Within the graft, many arterioles with a diameter of >50μm were found in the FGF/SM group, while there were no such large vessels seen in the SM group.

In BMC study, we performed co-transplantation of SMs and BMCs using Lewis rats. MI was induced by ligating the left coronary artery. Four weeks later, the rats were randomized into three groups: SM/BMC group had co-transplantation of SMs (5×10^6) and BMCs (1×10^6); SM group had SM transplantation (5×10^6 cells); control group received culture medium alone. LVEDD had gradually increased in the control group, while those in the SM and SM/BMC groups were unchanged at 4 weeks. Fractional area change was unchanged in the control and SM groups, while that in the SM/BMC group was increased at 4 weeks. Histology showed that the survival extent of the transplanted SM was similar between the SM and SM/BMC groups. In addition, the number of vessels found in the peri-MI area was largest in the SM/BMC group, followed by the SM group.

In conclusion, it may be reasonable to attribute the arteriogenesis induced by a sustained release of bFGF to the increased SM-derived graft volume in SM transplantation with bFGF administration. Such effect was not observed in co-transplantation of BMCs, which mainly induce angiogenesis rather than arteriogenesis (Tambara K, et al. 2003).

Future Prospects of Controlled Release of bFGF

As demonstrated in this review, the sustained release of bFGF may be a safe and effective strategy of regenerative medicine for extensive cardiovascular therapy. However, the optimal regimen for sustained release of bFGF with gelatin hydrogel is still unclear (for instance, dosage, timing, and duration).

Although gene therapy or cell transplantation has been mainly employed in clinical trials of regenerative medicine, our strategy has several advantages as follows: biodegradable gelatin hydrogel do not cause immune or inflammatory responses like genetic materials; collection of autologous cells is unnecessary; ease of controllability of dosage and release profile of growth factors. In order to achieve safer and more effective strategies, we

must investigate optimal regimens for sustained release of bFGF, which are suitable for various clinical situations.

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Gene Therapy for Angiogenesis under a Ventricular Assist System

Yoshiaki Takewa[1], Yukitoshi Shirakawa[1], Yoshiyuki Taenaka[1], Eisuke Tatsumi[1], Yoshiki Sawa[2], Hikaru Matsuda[2], Soichiro Kitamura[1], Hisateru Takano[1]

[1]Department of Artificial Organs, National Cardiovascular Center Research Institute, 5-7-1, Fujishirodai, Suita, Osaka 565-8565 Japan
[2] Division of Cardiovascular Surgery, Department of Surgery, E1, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871 Japan

Summary. Cardiac regeneration therapy was recently attempted for recovering heart failure. However it is not clear whether the therapy is effective for severe failing hearts that need mechanical circulatory assistance. We examined a gene therapy for angiogenesis after acute myocardial infarction in goats under ventricular assist system (VAS).

Six adult goats (56-65kg) were created with impaired hearts by ligating the coronary artery and installing pulsatile bi-ventricular assist devices (VADs). Hepatocyte Growth Factor (HGF) was selected as a gene for an angiogenesis factor, which also has cardio-protective activities. The HGF group (n=3) were administered 2.0mg human HGF-cDNA plasmid in myocardium. The control group (n=3) were similarly administered beta-galactosidase plasmid. Four weeks after gene transfection, we tried to wean all goats from VADs.

The myocardia transfected with the hHGF-cDNA contained hHGF protein at levels as high as 1.0+/-0.3ng/g tissue 3 days after transfection. After weaning from VADs, the HGF group showed good hemodynamics, while the control group showed deterioration. The percent fractional shortening was significantly higher in the HGF group than the control group (HGF vs. control, 37.9+/-1.7% vs. 26.4+/-0.3%, $p<0.01$). LV dilatation associated with myocytes hypertrophy and fibrotic changes were detected in the con-

trol group, but not in the HGF group. Vascular density was markedly increased in the HGF group.

These results suggest that gene therapy using hHGF may enhance the chance of "bridge to recovery" in the impaired heart under VAS.

Key words. Gene therapy, Hepatocyte Growth Factor, Ventricular assist system

Introduction

Recently, cardiac regeneration therapy was attempted for recovering heart failure. However it is not clear whether the therapy is effective for severe failing hearts which need mechanical circulatory assist. Patients who are supported with left ventricular assist system (LVAS) have cardiac transplantation as a choice of destination therapy. However, the overall applicability of cardiac transplantation is limited by a severe shortage of donors. Many patients are now spending several months and even years on these devices. Although improvements in LVAS have resulted in clinically meaningful survival benefits and an improved quality of life for patients with severe CHF, further improvements are needed. There have been several recent reports of selected patients with end-stage CHF whose recovery of cardiac function by LVAD was sufficient that the device could be explanted successfully (Frazier OH, et al. 1999). However, such patients constitute only a small percentage of patients using LVAD. And the long-term outcome of recovery, the mechanism of recovery, and which patients are capable of recovery remain unclear. Because the number of patients with severe CHF continues to increase, there have been several efforts to seek alternatives, such as regeneration therapy.

Hepatocyte Growth Factor (HGF) is a potent angiogenic agent possessing mitogenic, motogenic, and morphogenic effects through its own specific receptor, c-Met, in various types of cells, including myocytes (Aoki M, et al. 2000). We have previously demonstrated that HGF exerts antifibrotic and antiapoptotic effects in the myocardium (Taniyama, Y et al. 2000). Considering the pathogenic characteristics of severe heart failure, such as progression of fibrosis, progression of endothelial dysfunction, loss of

functional capillaries, and apoptosis-related loss of contractile mass (Treasure CB, et al. 1993), HGF might have a beneficial effect in the impaired heart by attenuating these remodeling processes (Ono K, et al. 1997).

Therefore, gene transfection with the HGF gene may enable a "bridge to recovery" in the impaired heart under the support of LVAD. To investigate this possibility, we examined gene therapy with HGF in impaired goat hearts implanted with LVAD (Shirakawa Y, et al. 2004).

Materials and methods

Preparation of plasmid encoded human HGF cDNA

A human HGF (hHGF) was inserted into the Not I site of the pUC-SR α expression vector plasmid as described elsewhere (Seki K, et al. 1991). In this plasmid, expression of the hHGF cDNA is regulated under the control of the SR α promoter which is composed of simian virus 40 polyadenylation sequence. The purified plasmid containing 2000 μ g of hHGF-cDNA was reconstituted in sterile saline 2.0ml and was directly injected into the myocardium at ten points with a 2.5ml-syringe and 30 gauge-needle. The concentration of hHGF in the heart was determined by enzyme-linked immunosorbent assay (ELISA) with anti-human HGF antibody (Institute of Immunology, Tokyo, Japan). The antibody against hHGF reacts with only hHGF and not with goat HGF. The serum hHGF levels were also assessed with the same ELISA system at 1, 3, 5, 7, 14, 28 days after cDNA injection.

Animal model of heart impairment

Ten adult goats weighing 56 to 65kg were used for this study. All animals were treated humanely in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resource and published by the National Institute of Health (NIH Publication No.86-23, revised 1985).

Anesthesia was induced with ketamine (10mg/kg) and atropine (0.01 mg/kg) administered intramuscularly and was maintained with 1-2.5% isoflurane after endotracheal intubation. Muscle relaxation was achieved with suxamethonium chloride (1mg/kg). After a left thoracotomy at the 5th costal bed, Three fluid-filled polyethylene catheters were inserted into the LV through the LV free wall for measuring LV pressure (LVP), the aorta through the left carotid artery for measuring systemic blood pressure (BP) and the left jugular vein for intravenous infusion. Fiberoptic pulmonary artery catheters (Oximetrix; Abbott Critical Care systems, North Chicago, IL) were placed in the pulmonary artery to allow measurement of mixed venous oxygen consumption (SvO2) and pulmonary arterial pressure (PAP). Heart rate (HR) was continuously monitored by electrocardiography (ECG).

For measurement of aortic root flow (AoRF) and left bypass flow (LBF), electromagnetic flowmeters (MFV-2100; Nihon-Koden, Tokyo, Japan) were placed in the ascending aorta, LVAD outflow cannulae Hemodynamic parameters, including the ECG values, LVP, AoP, central venous pressure (CVP), AoRF, and LBF were recorded continuously using a polygraph (NEC San-ei, Tokyo, Japan) AoRF was used as an index of native cardiac output, and the VAD assist ratio was calculated as follows.

VAD assist ratio (%) = LBF (L/min.) / [AoRF (Lmin.) + LBF (L/min.)]

The impaired heart was created by ligation of the left anterior descending (LAD) coronary artery distal to its first diagonal branch (Fig.1**a**). After ligation, all goats underwent cardiogenic shock and developed severe arrhythmias, such as ventricular fibrillation and ventricular tachycardia. In order to maintain systemic circulation and unload the left ventricle, a biventricular assist system (BVAS) with two air-driven ventricular assist devices (VAD) (with stroke volumes of 70ml and maximum outputs of 7.0 L/min) (Toyobo, Osaka, Japan) was installed in a paracorporeal fashion (Fig.1**b**). Blood flowed from the right atrium and ventricle to the pulmonary artery (PA) for the right bypass, and from the left atrium to the descending aorta for the left bypass. The VADs were driven asynchronously with the native heart. The goats were divided into two groups randomly. In the HGF group, hHGF-cDNA plasmid, a total of 2.0mg hHGF-cDNA in 2.0ml of plasmid solution, was injected using 30G needles into the myo-

cardium at ten points of the ischemic area of the left ventricular wall (Fig.1**a**).

There were no changes in the hemodynamic conditions associated with the injection of hHGF-cDNA plasmid, and no obvious adverse effects, such as anaphylactic reaction, in the goats throughout this experiment. In the control group, an equivalent volume of beta-galactosidase plasmid was injected in the same procedure. The chest was then closed and goats allowed to recover from anesthesia. In all goats, systemic circulation was subsequently maintained under BVAD for 4 weeks.

Assessment of cardiac function

We estimated the changes of cardiac function by means of three-dimensional digital sonomicrometry (Sonometrics Corp., Ontario, Canada). Two ultrasonic crystals were implanted in the endocardium parallel to the short axis at the level of the papillary muscle at the time of operation (Fig.1). These crystals were placed in the anterior wall and its opposite site to assess myocardial contractility in the resion of the LAD coronary artery. The LV dimension at end-diastole (LVDd) and end-systole (LVDs) was determined by simultaneously measured LVP.

The LV percent fractional shortening (%FS) was calculated as follows:

$$\%FS\ (\%) = (LVDd - LVDs) / LVDd \times 100$$

Before and after ligation of the LAD coronary artery, and at 1, 2, 3, and 4 weeks after gene transfection, we measured %FS and cardiac output during conditions when the BVAD was turned off for short periods. These dimension signals and the LV pressure signals were simultaneously sampled at 7-msec intervals and stored by a data acquisition analysis system (Sonometrics Corp., Ontario, Canada), and also recorded continuously using the polygraph.

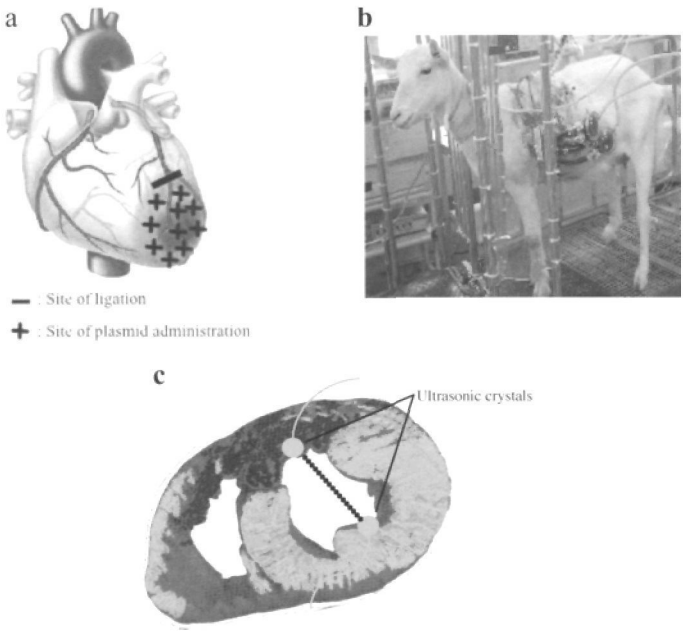

Fig. 1.a, b, c. Animal model of the impaired heart and experimental design. **a** Creation of myocardial infarction in adult goat hearts by ligating the left anterior descending coronary artery (LAD) also provided direct administration (crosses) of plasmid encoding hHGF cDNA or beta-galactosidase into the myocardium. **b** Bi-ventricular assist devices (BVAD) installed in all goats with impaired hearts. **c** Site of ultrasonic crystals. Two ultrasonic crystals were implanted in the endocardium parallel to the short axis of the left ventricle at the level of papillary muscle as shown in the figures at the time of operation

VAD off test

Four weeks after gene transfection, an attempt was made to wean all goats from BVAS. After systemic heparinization (300u/kg, intravenous injection), the BVAD was turned off. At 5, 15, and 30 minutes after turning off the BVAD, we measured HR, BP, SvO2, PAP, cardiac output, and LVDd.

Histological analysis

Four weeks after plasmid administration and after the VAD off test, all goats were euthanized with an overdose of sodium pentobarbital and the hearts were excised. The hearts were cut at the short axis into 5 pieces, and LV myocardium specimens were fixed with 10% buffered formalin and embedded in paraffin. A few serial sections from each specimen were cut into 5-μ m-thick slices and stained with hematoxylin and eosin for histological examination and measurement of cardiomyocyte cell diameter or with AZAN-trichrome stain to assess the collagen content. The proportion of the fibrosis occupying area at the border area neighboring the infarct area was measured on 10 randomly selected fields and the result was expressed as the percent fibrosis.

To label vascular endothelial cells so that the blood vessels could be counted in the border area neighboring the infarct area, immunohistochemical staining of Von Willebrand Factor-related antigen was performed according to a modified protocol. We used EPOS-conjugated antibody against Von Willebrand Factor-related antigen coupled with HRP (DAKO EPOS Anti-Human Von Willebrand Factor/HRP, DAKO) as primary antibodies. The stained vascular endothelial cells were counted as vascular density under a light microscopic at x200 magnification, using at least ten randomly selected fields per section. The result was expressed as the number of blood vessels/mm^2.

Computer analyses of pathology (cell diameter, percent fibrosis and vascular density) were performed by a Macintosh computer using a public domain image program developed at the US National Institute of Health.

Statistical analysis

All data are expressed as group mean ± standard deviation. Intergroup comparisons were made using ANOVA and the unpaired Student's t-test. All analyses were performed using the program StatView (version 5.0; Abacus Concepts, Inc., Berkeley, CA). Values of $p<0.05$ were considered to indicate statistical significance.

Results

Animal condition and systemic hemodynamic data

Just after infarction, all goats developed severe low output syndrome and cardiogenic shock. Native cardiac outputs decreased about 20 or 30 ml/kg/min. Under the support of BVAD, all animals were maintained in good condition, and the HR and BP did not differ between the two groups throughout this experiment. The VAD assist ratio was maintained at about 70% throughout this experiments, and this ratio did not differ markedly between the two groups (Fig.2**a**). However, at 4 weeks after the gene transfection, the cardiac output in the HGF group was significantly higher than that in the control group (85.0 +/- 1.4 ml/kg/min. in the HGF group vs. 64.6 +/- 6.0 ml/kg/min. in the control group, $p<0.01$;Fig.2**b**).

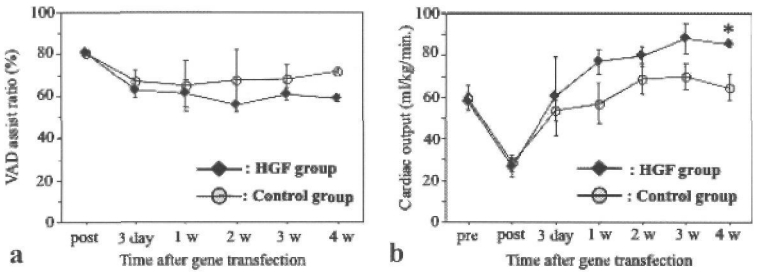

Fig. 2a, b. Comparison of VAD assist ratio **a** and native cardiac output **b** of the HGF group (squares) and the control group (circles) through this experiment. Data are presented as mean +/- SD. *P < 0.01 vs. the control group

Assessment of cardiac function

After infarction, the %FS was markedly decreased compared with the baseline values in both groups, and the degree of deterioration did not differ between two groups.

The %FS gradually recovered in the two groups after gene tranfection. However, the improvement of the %FS in the HGF group was significantly larger than the control group. The %FS in the HGF group 4 weeks after gene transfection recovered significantly more than the control group (37.9+/-1.7% in the HGF group vs. 26.4+/-0.3% in the control group, p<0.01;Fig.3).

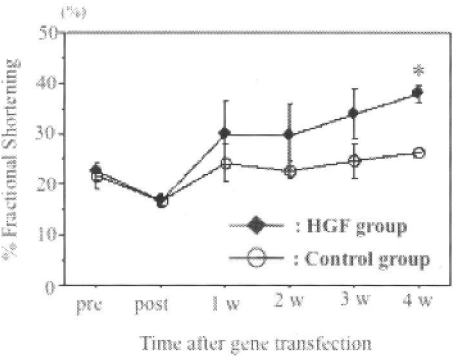

Fig. 3. Comparison of wall contractile function evaluated by percent fractional shortening (%FS) which was calculated by the sonomicrometry method. The HGF group was squares and the control group was circles. Data are presented as mean +/- SD. *P < 0.01 versus the control group.

VAD off test

Four weeks after gene transfection, we performed a VAD "off test" (Fig. 4). HR steadily increased and BP steadily decreased in the control group after the BVAD was turned off, but in the HGF group, these parameters remained stable and did not deteriorate. The SvO2 and the PAP of the control group also deteriorated relative to those of the HGF group. Cardiac output was significantly increased in the HGF group compared with the control group 30 minutes after weaning from VAD (80.1+/-6.2 ml/kg/min. in the HGF group vs. 61.2+/-4.3 ml/kg/min. in the control group, p<0.05;Fig.4). And in the control group, LVDd was significantly increased

relative to that in the HGF group 30 minutes after weaning from VAD (35.8+/-1.6 mm in the HGF group vs. 46.9+/-0.1 mm in the control group, p<0.05;Fig.4).

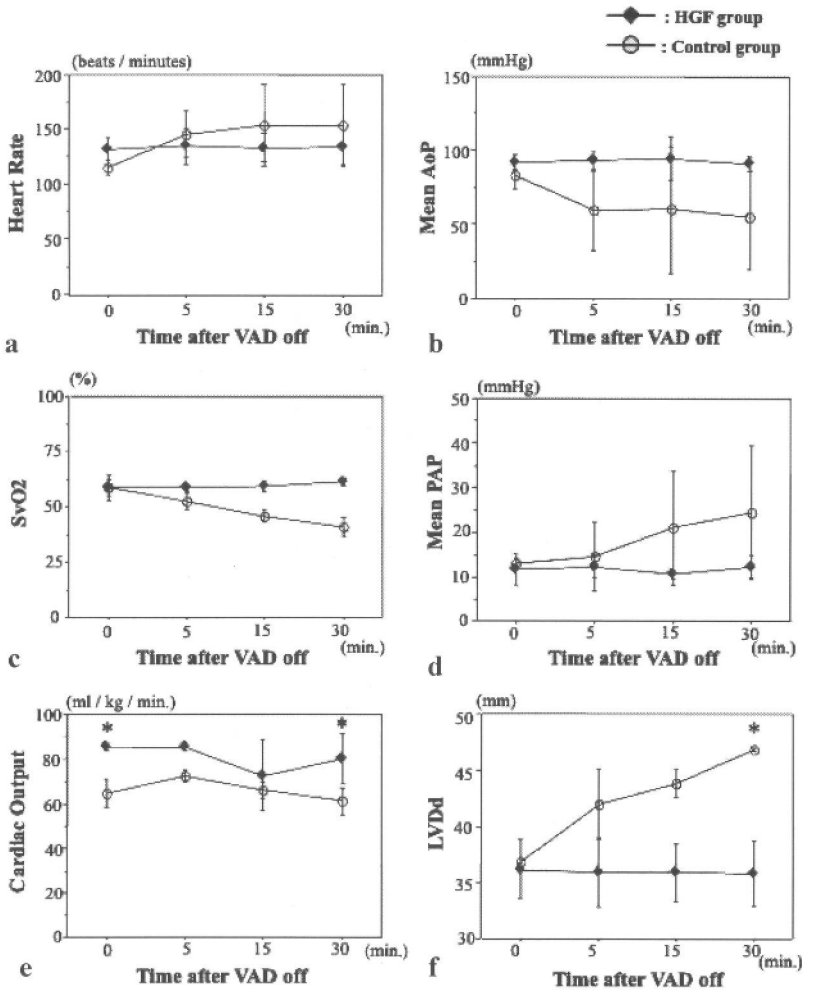

Fig. 4a,b,c,d,e,f. **a** Heart rate, **b** mean systemic blood pressure, **c** mixed venous oxygen saturation, **d** pulmonary arterial pressure, e native cardiac output and **f** left ventricular end-diastolic dimension after weaning from VAD. (in mean ±SD). *P < 0.05 versus the control group.

Histological assessment

Macroscopic findings of sacrificed hearts revealed that LV dilatation was markedly suppressed in the HGF group relative to that in the control group, although necrotic change and scar formation of the anterior wall were recognized in both groups (Fig.5**a**,**b**).

Azan staining of the myocardium in the neighborhood of the infarcted area revealed that fibrous change was also suppressed in the HGF group compared with that in the control group (Fig.5**c**,**d**).

The percent fibrosis was significantly reduced in the HGF group compared with the control group (13.9+/-1.7% in the HGF group vs. 22.3+/-1.3% in the control group, $p<0.01$;Fig.6**a**).

HE staining of the border zone revealed hypertrophic change of cardiomyocytes in the control group, but not in the HGF group (Fig.5**e**,**f**). The cell diameter was significantly smaller in the HGF group than in the control group (39.6+/-0.5 μ m in the HGF group vs. 54.4+/-0.6 μ m in the control group, $p<0.01$;Fig.6**b**).

Vascular density was examined in the border zone of the infarct area (Fig.5**g**,**h**). Vascular density was significantly higher in the HGF group than in the control group (35.2+/-2.1 vessels per field in the HGF group vs. 24.5+/-2.7 vessels per field in the control group, $p < 0.05$;Fig.6**c**).

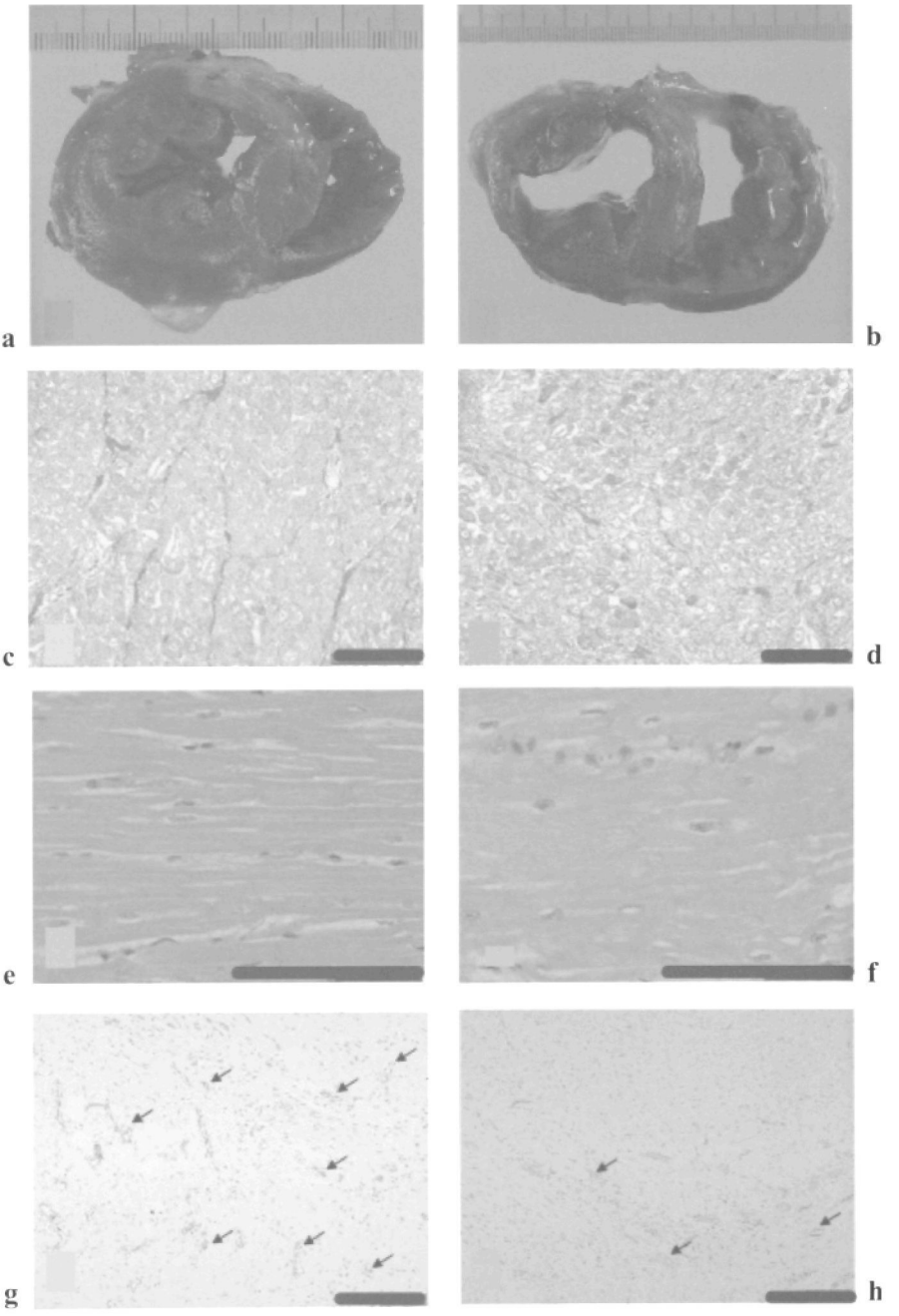
a
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Fig. 5a, b, c, d, e, f, g, h. Histological findings of the heart 4 weeks after gene transfection. **a,b** Macroscopic findings of short axis area of the left ventricle. **c,d** Azan-trichrome staining of the myocardium in the border zone of the infracted and normal area (bar = 100 μ m, original magnification x 100). **e,f** Hematoxylin and eosin staining of the myocardium of the border zone (bar = 100μm, original magnification x 200,). **g,h** Immunohistologic staining by von Willebrand antibody (bar = 100 μ m, original magnification x 200). Arrows indicate the example of von Willebrand antibody positive endothelial cells. **a,c,e** and **g** was the **HGF** group. **b,d,f** and **h** were the control group.

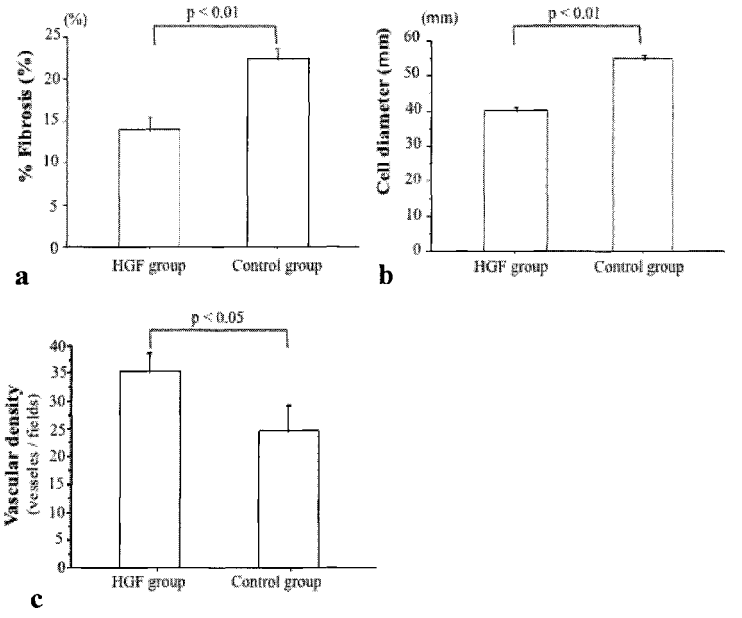

Fig. 6a, b, c. Evaluation of histopathological findings. **a** Percent fibrosis, **b** cell diameter of myocyte in the border zone. Data are represented mean +/- SD. **c** Vascular density. Data expressed as vessels per visual field (Magnifications; x 100).

Discussion

The major findings of this study were as follows: (1) left ventricular unloading by VAD alone could not achieve sufficient suppression of cardiac remodeling after myocardial infarction; and (2) gene transfection with HGF-cDNA plasmid attenuated cardiac remodeling in the impaired heart under mechanical unloading with VAD, and achieved markedly better improvement of cardiac function than that by VAD alone, suggesting its potential use as a "bridge to recovery".

Recently, several studies of regenerative therapy with gene therapy or cell transplantation have reported the effect on protection of cardiomyocytes and improvement of cardiac function in the impaired heart. But such therapies require time to take effect, and are not able to control heart failure immediately after treatment. VAD may not only support systemic circulation but provide an optimal environment for myocardial recovery along with ventricular unloading (Levin HR, et al. 1996). We, therefore, propose a combination therapy consisting of gene therapy and VAD as a new strategy for the treatment of severe heart failure. VAD provides sufficient time and suitable circumstances for myocardial regeneration, and regenerative therapy promotes myocardial recovery in the impaired heart resulting in the increase of a "bridge to recovery".

HGF is not only an angiogenic factor but also shows various physiological activities, including antifibrotic and cardiotrophic activities (Aoki M, et al. 2000). Therefore, we believe that HGF has an advantage for promoting myocardial regeneration. In the chronic phase of myocardial infarction, the progression of cardiac remodeling with reduced cardiac function is responsible for interstitial fibrosis as well as for the apoptosis of the cardiomyocytes. In particular, fibrosis remote from the infarcted area is considered to be the major cause of ventricular remodeling in ischemic cardiomyopathy. In this study, neoangiogenesis was induced and fibrosis was suppressed in the peri-infarcted area by HGF gene transfection. Some of the molecular contributors to fibrosis during cardiac remodeling have been identified (Goldstein S, et al. 1998). Transforming growth factor-β and angiotensin-II are believed to play an important role in the pathogenesis of fibrosis (Kapadia S, et al. 1998). These molecules are negative regu-

lators of local HGF production in various cell types (Taniyama Y, et al. 2000). In this study, increase of local HGF expression may prevent myocardial fibrosis, possibly by inhibiting the production of such molecules as previously reported (Aoki M, et al. 2000). Regarding delivery of HGF, we did not use any vector for gene therapy in this study. Because, we have already reported that direct administration of HGF-cDNA plasmid is enough for local and continuous intramural delivery of HGF to enhance angiogenesis and cardiac function in the infarct myocardium (Aoki M, et al. 2000). Moreover, HGF acts as a paracrine growth factor and its production by administration of HGF-cDNA plasmid in the myocardium continues about for 14 days so that its local synthesis without viral vectors might protect against the adverse effects without any detection in the serum HGF level during gene therapy. Thus, our results have promise for clinical applications.

To the best of our knowledge, this is the first report to demonstrate the effectiveness of regenerative therapy in the impaired heart under LVAD, and the protocol of this study could be used as one of the new therapeutic strategies for severe heart failure. However, several limitations of this study must be considered before developing a clinical application. First, due to limitations of the experimental protocol, we were not able to clarify the efficacy of this method with respect to scar thinning and expansion of the impaired myocardium in the chronic phase. When loss of contractile mass is markedly increased, such as in patients with dilated cardiomyopathy, regeneration of cardiomyocytes is insufficient to increase the contractile function even with the HGF gene transfection. Thus a method of supplementing the contractile mass, such as cellular cardiomyoplasty, may be needed to increase the treatment efficacy. Second, because of the lack of techniques for measuring goat HGF, the roles of endogenous HGF in this experiment are not clear. Third, the long-term effects of HGF is also unclear. To address these problems, further studies will be needed.

Thus, we demonstrated the therapeutic value of suppression of cardiac remodeling by hHGF transfection in the impaired heart under LVAD. Our results suggest that, in the setting of acute myocardial infarction causing cardiogenic shock, a combined therapy with HGF gene therapy and LVAD

can increase the chance of a "bridge to recovery" in the severely impaired heart under LVAD.

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The Role of Vascular Endothelial Growth Factor (VEGF) on Therapeutic Angiogenesis Using Bone Marrow Cells

Yoshikazu Maeda[1], Uichi Ikeda[2]

[1] Cardiovascular Division, Department of Medicine, Jichi Medical School
[2] Department of Organ Regeneration, Shinshu University Graduate School of Medicine

Summary. Therapeutic delivery of bone marrow derived mononuclear cells (BMMNCs) into ischemic tissues would be able to improve blood flow. Vascular endothelial growth factor (VEGF) is known as a potent angiogenic growth factor. We injected sFLT-1 expressing plasmid into mice anterotibial muscle. One week after gene transfer, gap unilateral femoral arterial segments were removed and murine BMMNCs ($1x10^4$ cells) were delivered into the thigh muscle to make BMMNC delivery model. SFLT-1 gene transfer inhibited BMMNCs delivery-induced blood flow recovery. This finding suggests that the effect of BMMNCs delivery is at least partially mediated via VEGF.

Key words. Bone marrow derived mononuclear cells, VEGF, sFlt-1, Angiogesis, Gene transfer

Introduction

Asahara T, *et al.* (1997) reported the presence of endothelial progenitor cells (EPC) in the human peripheral blood and indicated that they contribute to neovascular formation in the normal and pathological situations such as wound healing, and hindlimb ischemia. After that, not only angiogenesis, but also vasculogenesis is thought to be one of the mechanisms of neovascular formation.

Recently, many therapeutic bone marrow-derived mononuclear cells (BMMNCs) delivery experiments have been performed in animal hindlimb and myocardial ischemia models, and many of them demonstrated that BMMNCs delivery is efficient for the augmentation of blood flow in the ischemic area. Moreover, clinical trials revealed the benefit of therapeutic BMMNCs delivery in the human peripheral artery disease such as atherosclerosis obliterans and Burger's disease.

On the other hand, the precise mechanisms of this improvement of blood flow are still unclear. Majka M, *et al.* (2001) reported that numerous growth factors, cytokines, and chemokines are secreted by BMMNCs. They demonstrated that vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are mainly secreted by stromal cells. This finding suggests that therapeutic BMMNCs delivery into ischemic lesion could supply not only EPC but also growth factors.

VEGF is one of the important growth factors in angiogenesis, however, the role of VEGF in the therapeutic BMMNCs delivery has not been investigated in detail. A soluble form of the FLT-1(sFLT-1) VEGF receptor is known to be a selective and specific inhibitor of VEGF. This isoform is expressed endogenously by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant negative inhibitor against VEGF receptors. Previously, we have demonstrated that sFLT-1 gene transfer could efficiently inhibit VEGF-induced endothelial cellar proliferative activity *in vitro*. (Chen H, et al. 2000).

Here, we tried to block VEGF activity *in vivo* by transfecting plasmid DNA which encodes the murine sFLT-1 gene to examine the role of VEGF on the BMMNCs delivery models.

Methods

Isolation of mice BMMNCs

BMMNCs were obtained from the femoral bone of GFP transgenic mice and isolated by density-gradient centrifugation with Ficoll (NycoPrep, AXIS-SHIELD PoC AS).

Co-culture of BMMNCs and C2C12 and VEGF production

The C2C12 cells were cultured in 12-well plates with 1 ml DMEM containing 5% horse serum. Eight days after plating, differentiated C2C12 cells were starved with FCS-free DMEM for 24 hours, then isolated BMMNCs were added and further incubated for 24 hours. The concentration of mice VEGF in the cultured medium was estimated by ELISA (cytokine Mouse VEGF, American Research Products, Inc.).

Mouse hindlimb ischemia, sFLT-1 gene transfer, and BMMNCs delivery

After anesthesia with pentobarbital, male Bulb/c nude mice were injected with 1μg of sFLT-1 expressing plasmid or TE buffer into their right anterior tibial muscle. One week after gene transfer, mice underwent surgical ligation and resection of the right femoral artery to produce unilateral hindlimb ischemia. At the same time, isolated BMMNCs $1X10^4$ or RPMI buffer (50μL, respectively) were delivered into the right thigh muscle. The development of the collateral circulation was quantified by Laser Doppler perfusion imaging experiments (LDPI, Moor Instruments). Blood Flow Ratio (operated versus non-operated leg) was calculated for each animal.

Results

Isolated BMMNCs are cultured with 293 cells (human embryonic kidney cell lines) or differentiated C2C12 cells (mouse skeletal muscle cell lines) in a serum-free RPMI medium for 24 hours. Then, we measured VEGF concentration in the cultured medium by ELISA. Although C2C12

alone can secrete VEGF, VEGF production is augmented by co-cultured with C2C12 cells in a BMMNC number-dependent manner (10^3-10^5). On the other hand, this augmentation is not seen by co-cultured cells with 293 (Fig. 1).

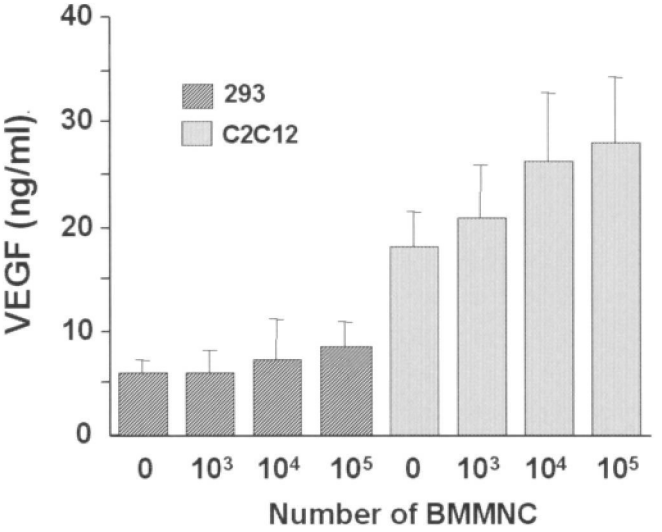

Fig. 1. VEGF concentration of cultured medium. Co-culture with C2C12 (murine skeletal muscle cell line) and BMMNCs produced VEGF in a BMMNCs number dependent (10^3-10^5) manner.

Next, we made hindlimb ischemia in Bulb/c nude mice and investigated the effects of BMMNCs delivery on the recovery of perfusion estimated by Laser Doppler Imaging. Representative Laser Doppler Images 2 weeks after femoral artery removal are shown in Fig. 2a. In these images, bright ares represent good perfusion, and dark areas indicate poor perfusion. As shown in these figures, right hindlimb with femoral artery removal shows poor perfusion compared with countralateral untreated left hindlimb (Control). On the other hand, 10^4 cells of BMMNCs delivery to ischemic hindlimb (BMMNC) results in a marked improvement of perfusion which almost has the same level of that in contralateral hindlimb, indicating that 10^4 cells of BMMNCs delivery is sufficient for our experiments.

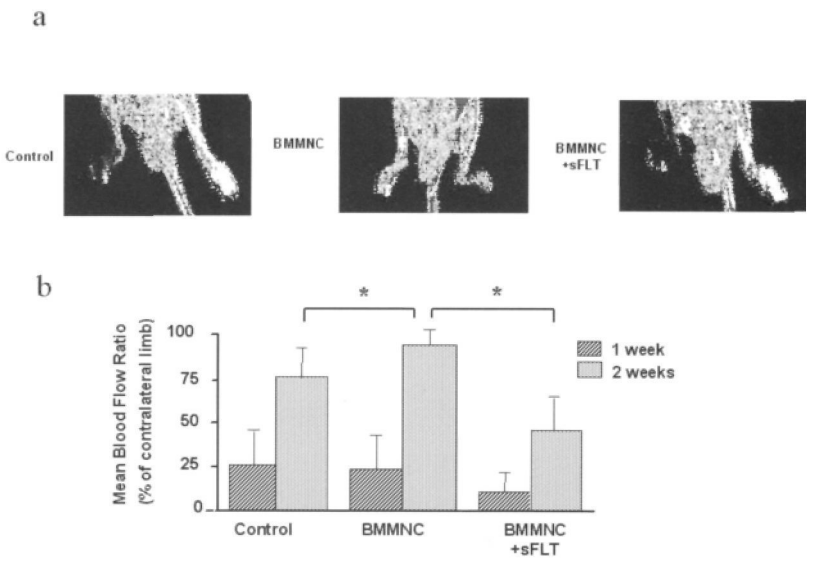

Fig. 2. **a.** Representative images of effects of BMMNCs delivery and sFLT-1 gene transfer on blood flow recovery in a murine model of hindlimb ischemia. **b.** Quantative analysis of blood flow ratio. **Control** : ischemia alone, **BMMNC** : BMMNCs delivered group, and **BMMNC+sFLT** : sFLT-1 gene transfer group.

To examine whether VEGF might contribute to blood flow recovery by BMMNCs delivery *in vivo*, we injected sFLT-1 plasmids into the anterior tibial muscles 1 week before arterial removal and BMMNCs delivery. Pretreatment with sFLT-1 gene transfer abolished the improvement of perfusion by BMMNCs delivery (BMMNC+sFLT).Fig. 2b demonstrates quantative analysis of this experiment. We measured Doppler signal intensity at the ankle joint (ROI), and calculated "blood flow ratio (BFR)" which is percent intensity in treated limb against untreated limb. In the control ischemic group, BFR was 25% at 1 week and 76% at 2 weeks. This increase suggests endogenous collateral formation in our model. In BMMNC delivered group, BFR at 1 week is almost similar to that in the control group, however, BFR at 2 weeks is significantly greater than that in the control group. This result indicates BMMNC delivery is successful

and that optimal results are obtained after at least 2-weeks. Pretreatment of sFLT-1 gene transfer into skeletal muscles markedly reduced the benefit of BMMNC delivery (BMMNC+sFLT).

Discussion

Since Asahara T, *et al.* (1997) indicated that EPC play a role in neovascular formation, many investigations and clinical applications have been performed in therapeutic angiogenesis strategy using bone marrow-derived cells.

One of the topics of therapeutic angiogenesis is that bone marrow cells can produce several kinds of angiogenic growth factors and chemokines.Kinnaird T, *et al.* (2004) investigated the hypoxia-induced gene expression profile on bone marrow-derived stromal cells by Affymetrix GeneChips representing 12000 genes. They found that a broad spectrum of angiogenic growth factors, such as basic fibroblast growth factor (bFGF)-2, 7, placental growth factor, interleukin-1, 6, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and VEGF are expressed in these cells. They also tried stromal cell delivery into mice ischemic hindlimb and confirmed its benefit on angiogenesis. They speculated that paracrine signaling is an important mediator of bone marrow cell therapy in tissue ischemia. Recently, Hiasa K, *et al.* (2004) demonstrated that gene transfer of stromal cell-delived factor 1α (SDF-1α) enhances ischemic vasculogenesis and angiogenesis. These reports suggest the importance of angiogenic growth factors and chemokines on neovascular formation.

On the other hand, the role of angiogenic growth factors and chemokines on therapeutic BMMNCs delivery is still unclear. In this study, we focused on the role of VEGF, one of the important angiogenic growth factors, in the therapeutic BMMNC delivery. In both animal and human therapeutic BMMNC delivery, BMMNCs are injected into skeletal muscles, we speculated that the interaction between BMMNCs and skeletal muscles could augment VEGF production. We investigated this hypothesis at first *in vitro*. Fig.1 indicates that unstimulated mice BMMNCs express little VEGF, however co-culture with BMMNCs and C2C12 produces

VEGF in a BMMNCs number dependent manner. Although we can not distinguish whether VEGF is secreted from BMMNCs or C2C12, this phenomenon might be one of many possible mechanisms of the therapeutic BMMNC delivery *in vivo*. Further investigation would be needed to explore the precise mechanism of this interaction on VEGF production.

As shown in Fig.2, BMMNCs delivery into ischemic hindlimb significantly augments blood flow recovery. Although we did not confirm the *in situ* VEGF production in this model, we speculated that the interaction between BMMNCs and skeletal muscle enhances VEGF production and plays a role in angiogenesis. Our preliminary study revealed that 1 μg of sFLT-1 plasmid injected into mice anterior tibial muscles 1 week prior to arterial removal could significantly inhibit endogenous blood flow recovery (data not shown). We applied this method to investigate the role of VEGF on BMMNCs delivery models. Pretreatment of sFLT-1 gene transfer completely inhibits blood flow recovery by BMMNCs delivery. This data suggests that VEGF, at least in part, plays an important role on therapeutic angiogenesis by BMMNCs delivery.

Recently, Hiasa K, *et al.* reported that the SDF-1α gene transfer mobilized EPCs into the peripheral blood and augmented the recovery of blood flow to the ischemic limb. These effects of SDF-1α are diminished by the blockade of VEGF using sFLT-1 gene transfer. This finding supports our hypothesis that VEGF might play a role in therapeutic angiogenesis.

In this study, we have demonstrated the importance of VEGF on BMMNCs delivery. VEGF may affect EPC mobilization, attachment, transdifferentiation, and incorporation of vascular structures.

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CHAPTER 5

CLINICAL RESULTS OF THERAPEUTIC ANGIOGENESIS AND VASCULOGENESIS

Clinical Survey of Cell Therapy in Japan

Yousuke Katsuda, Yoshiaki Takeshita, Ken Arima, Yutaka Saitoh, Tsutomu Imaizumi, Takayuki Asahara, Takeshi Nakatani, Teruo Okano, Akira Kishida, Hatsue Ishibashi-Ueda, Toshiharu Shin'oka, Ryozo Nagai, Yoshiki Sawa, Masashi Komeda, Yoshiaki Takewa, Hikaru Matsuda, and Hidezo Mori

Kurume University School of Medicine, and Collaborating Institutions of Cardiovascular Disease Grant from MHLW (13C-1)

Summary. Background: Therapeutic angiogenesis by using cells is being performed in Japan. However, it is unknown in how many centers and how these therapies are performed. The efficacy and side effects are also unknown. Thus, we conducted the survey by mailing questionnaire within Japan. Methods and results: Two surveys were performed in 2003. The first survey unveiled that cell therapy was performed in 32 facilities until October 2003. The second survey unveiled the followings. (1) The total number of performed cases was 221. 153 patients (69.2%) had arterio-sclerosis obliterance (ASO), 56 patients (25.3%) thromboangitis obliterans (TAO, Burger's disease), and 12 patients (5.4%) other conditions. (2) The sources of cells were bone marrow-mononuclear cells (61.5%), peripheral-mononuclear cells (9.5%), and peripheral $CD34^+$ cells (22.1%). A few patients (6.7%) were treated with a cytokine only (granulocyte-colony stimulating factor: G-CSF). (3) Inclusion criteria were the same for most facilities, such as patients with PAOD, especially with critical limb ischemia with rest pain, non-healing ischemic ulcers and non-candidates for non-surgical or surgical revascularization. All facilities excluded patients with histories of malignant disorder during the past 5 years, proliferative diabetic retinopathy, pregnancy, proliferative blood disease, uncontrolled ischemic heart disease, rheumatic arthritis, or psychiatric disease. (4) Subjective improvement was observed in 138 of 199 patients (69%). Objective improvements for ABI, TcO2 or angiographic

findings were observed in 98 of 182 patients (53.8%). (6) Three of 221 patients (1.4%) died after cell therapy. One died from cerebro-vascular attack (thrombo-embolizm), and two died from acute myocardial infarction (AMI). Conclusion: Our clinical survey has shown that cell therapy is being performed in many medical centers in Japan. It seems to be safe and effective for patients with PAOD with no surgical options

Key Words. Cell therapy, Clinical survey, Angiogenesis, PAOD

Introduction

Recently, it has been reported that treatment with angiogenic growth factors, such as vascular endothelial growth factor, for patients with peripheral artery obstructive disease (PAOD) could increase collateral blood flow in ischemic limbs (Asahara T and Isner M 1999). Therapeutic angiogenesis can be achieved by use of not only growth factors (Baumgartner I, et al. 1998) but also cells, such as bone marrow cells (Shintani S, et al. 2001), peripheral cells (Kalka C, et al. 2000) and colony stimulating factor (CSF)-induced stem cells (Seiler C, et al. 2001). Preclinical studies have shown that such cell therapy increased collateral flow of ischemic limbs, and also could relieve ischemic pain and ulcers (Tateishi-Yuyama E, et al. 2002). Because cell therapies use their own cells (autologous transplantation), they are associated with fewer problems, such as immunological complications and ethical issues. However, it is unknown in how many centers and how these therapies are performed. The efficacy and side effects are also unknown. Thus, we conducted the survey by mailing questionnaires in Japan as a project funded by Japanese Ministry of Health, Labor, and Welfare Grant-in-Aid for Scientific Research in Japan.

Methods

Two steps of survey of cell therapy were performed in 2003. The first survey was performed to examine whether cell therapy was performed for patients with PAOD. With the permission of the Japanese Circulation So-

ciety questionnaire were mailed to 190 facilities throughout Japan (125 Internal Medicine or Cardiology Department of universities, 65 national hospitals). The basic data such as names and addresses of facilities performing cell therapy were obtained.

Then, in order to examine the following issues, the questionnaire was mailed to the facilities in which cell therapy was performed (second survey): (1) Cause of chronic limb ischemia, (2) modes of cell therapy, (3) number of patients, (4) inclusion and exclusion criteria, (5) efficacy and (6) complications. The survey was co-performed with TACT (Therapeutic Angiogenesis by Cell Transplantation) group.

Table 1. Etiologies of PAOD performed cell therapy

Etiology of PAOD	No of patients
Total	221
ASO	153 (69.2%)
TAO	56 (25.3%)
Others	12 (5.4%)

ASO: arteriosclerosis obliterans, TAO: thromboangitis obliterans

Results

In the first survey, we obtained responses from 146 of 190 facilities (76%). The first survey unveiled that cell therapy was performed in 32 facilities until October 2003. In the second survey, we obtained response from 28 of 32 facilities (88%). The second survey unveiled the following.

Cause of chronic limb ischemia treated with cell therapy:

Etiologies of PAOD are shown in **Table 1**. The total number of cases performed was 221. 153 patients (69.2%) had arterio-sclerosis obliterance

(ASO), 56 patients (25.3%) thromboangitis obliterans (TAO, Burger's disease), and 12 patients (5.4%) had other conditions.

Modes of cell therapy performed:

The sources of cells were bone marrow-mononuclear cells (136 cases, 61.5%), peripheral-mononuclear cells (21 cases, 9.5%), and peripheral $CD34^+$ cells (49 cases, 22.1%). A few patients (6.7%) were treated with a cytokine only (granulocyte-colony stimulating factor: G-CSF). (Table 2).

Table 2. Modes of cell therapy

Source of cells using cell therapy	No. of patients
Total	221
Bone marrow- mononuclear cells	136 (61.5%)
Peripheral-mononuclear cells	21 (9.5%)
Peripheral stem cells ($CD34^+$)	49 (22.2%)
Administration of G-CSF only	15 (6.8%)

G-CSF: granulocyte-colony stimulating factor

Inclusion or exclusion criteria of cell therapy:

Inclusion criteria were similar for most facilities. They included patients with PAOD, especially with critical limb ischemia with rest pain, non-healing ischemic ulcers and non-candidates for non-surgical or surgical revascularization. Fontaine's classification was used in 31 of 32 facilities (96.9%). In most facilities (81.3%), Fontaine's classification more than III was indicated. All facilities excluded patients with a history of malignant disorders during the past 5 years, proliferative diabetic retinopathy, pregnancy, proliferative blood disease, uncontrolled ischemic heart disease, rheumatic arthritis, and psychiatric disease. To exclude malignant disorders, occult blood in stool, measurement of tumor maker, such as CEA or SCC, and brain CT were performed. Upper gastro-intestinal fiber, total colon fiber, computed tomography of chest or abdominal area, and

upper abdominal echo were also performed as necessarily. Written informed consent was mandatory in all facilities.

Assessments of efficacy:

To evaluate the subjective efficacy, the severity of pain was assessed by pain scale or visual analogue scale in all facilities. To assess the objective changes, the following parameters were monitored: the size of ischemic ulcer and ankle-brachial index (ABI) in all facilities, and laser doppler flow ratio or transcutaneous oxygen pressure (TcO2) in some facilities. Angiography, including digital subtraction angiography, was performed in all facilities.

Efficacy:

The data for subjective outcomes were not obtained in 22 of 221 patients (10.0%). Subjective improvement was observed in 138 of 199 patients (69%). The objective outcomes could not be evaluated in 32 of 221 patients(14.5%). Objective improvement of at least one parameter was observed in 98 of 182 patients (53.8%).

Adverse events or complications:

Three of 221 patients (1.4%) died after cell therapy. One died from cerebro-vascular attack (thrombo-embolizm), and two died from acute myocardial infarction (AMI) after bone marrow cell transplantation. In the patients with AMI, autopsy showed coronary restenosis of previous percutaneous coronary intervention (Tateishi-Yuyama E, et al. 2002). No relation of death to cell therapy was demonstrated in this survey. No embolic episode was reported for G-CSF treated patients. Although, various lineages of cells, such as fibroblasts, osteoblasts, and endothelial cells, may have been implanted with endothelial progenitor cells, angioma, bone formation or increased fibrosis was not reported. Minor edema or pain of the iliac crest was reported in some patients with bone marrow puncture. No other complications were reported in this survey.

Discussion

This clinical survey disclosed two important information of cell therapy performed in Japan. First, over 200 patients were treated by cell therapy in a few years. The most common etiology of chronic limb ischemia was ASO, and the next one was TAO. The types of cell used were bone marrow cells, peripheral cells and G-CSF stimulated cells. Bone marrow cells were most frequently used and then peripheral cells.

Second, this survey disclosed the efficacy and safety of cell therapy. Subjective improvement was observed in 69%, and objective improvement was observed in 53.8% of all cases. Most facilities extensively monitored adverse outcomes, and no major adverse effect was reported. In conclusion, cell therapy seems to be safe and effective for patients with PAOD with no surgical options.

Study limitations

First, the inclusion or exclusion criteria were not strictly uniform among facilities and various etiologies of PAOD might have been included. Second, subjective and objective outcomes were not assessed using the same criteria. Third, information on the detailed method of cell therapy was lacking. These limitations may have resulted in some differences in the assessment of the effects of cell therapies among facilities. In order to determine the efficacy and safety of cell therapies, a double-blind placebo controlled trial with the uniform protocol is desired.

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A Novel Micro-Angiography Detecting Angiogenesis, Application for Autologous Bone Marrow Mononuclear Cells Transplantation in the Patients with Critical Limb Ischemia

Kazuhiro Nishigami, Takeshi Nakatani, Masaaki Chiku, Hidezo Mori

National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

Summary. Conventional Anigiographic Findings in Autologous Bone Marrow Mononuclear Cells Transplantation for Critical Limb Ischemia: Bone marrow mononuclear cells have many of the characteristics of stem cells for mesenchymal tissues, and secrete many angiogenic cytokines. We performed autologous transplantation of bone marrow mononuclear cells in six patients with critical limb ischemia due to Buerger disease, who were not candidates for catheter or surgical revascularization. Leg pains at rest and skin ulcers improved after bone marrow transplantation in all patients, although significant collateral developments after the therapy by conventional angiography could not be observed. Autologous transplantation of bone marrow mononuclear cells including stem cells improved critical limb ischemia due to Buerger disease. Neovascularization after therapeutic angiogenesis might be quite small and could not be visualized by conventional angiography.

Novel Micro-angiograpy:We developed in-hospital micro-angiographic equipment which consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera). We visualized mid-zone collaterals after femoral arterial exfoliation with and without therapeutic angiogenesis in rabbit ischemic limbs and assessed the radio-absorptions in a clinical setting. The micro-angiography clearly demonstrated mid-zone collaterals after the treatment with a diameter of down to 50μm, but the conventional angiography did not. The sum of ra-

dio-absorptions for 10 seconds in clinical settings was 300 mSv. The newly developed in-house micro-angiography could illuminate micro-vessels with a diameter of down to 50μm in clinical settings safely and could be useful in the evaluation of therapeutic angiogenesis.

Keywords. Micro-angiography, Angiogenesis, Autologous bone marrow mononuclear cells transplantation, Critical limb ischemia, Buerger disease

Introduction

Endothelial progenitor cells (EPCs) possess the ability to mature into cells that line the lumen of blood vessels(Asahara T, et al. 1997). Therapeutic angiogenesis could be induced by the transplantation of bone marrow mononuclear cells including EPCs. Several studies demonstrated that therapeutic angiogenesis using autologous bone marrow mononuclear cells transplantation (BMT) was effective for ischemic vascular diseases although conventional angiography could not precisely detect developed collaterals after therapeutic angiogenesis(Iba O, et al. 2002, Inaba S, et al. 2002, Shintani S, et al. 2001,Tateishi-Yuyama E, et al. 2002). We developed an in-hospital micro-angiographic equipment which consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera).

The purpose of the present study was to evaluate the clinical effects and conventional angiographic findings on BMT for critical limb ischemia, and to validate the usefulness and safety of the novel micro-angiography technique for the evaluation of therapeutic angiogenesis.

Methods

Patients

Patients qualified for autologous BMT if they had chronic critical limb ischemia including rest pain and/or non-healing ischemic ulcers for a minimum of 4 weeks without evidence of improvement in response to

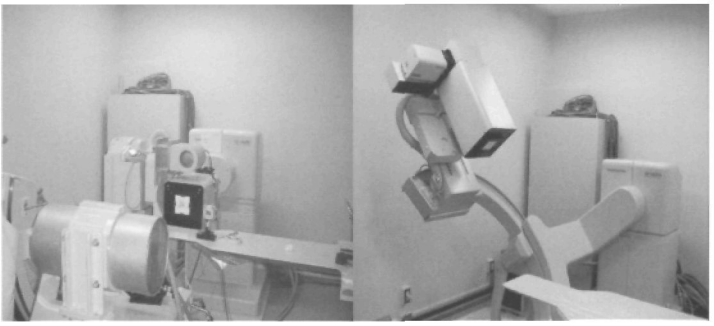
conventional therapies and were not optimal candidates for surgical or catheter revascularization. Buerger's disease was diagnosed by segmental occlusion of small- and medium-sized arteries, absence of atherosclerosis, and corkscrew collaterals circumventing the occlusion in angiogram and the exclusion of autoimmune diseases such as scleroderma or systemic lupus erythematosus, hypercoagulable states, diabetes, or acute arterial occlusion secondary to embolism. Patients with retinopathy and/or malignancy were excluded. Although 30 patients with atherosclerotic peripheral artery disease were candidates for BMT, they were excluded from the present study due to their systemic atherosclerotic complications. Six patients with Buerger's disease were recruited for the present study. All patients had legs pain at rest and five patients had foot ulcers. Written consent was obtained from all participants of this study. This clinical trial of autologous BMT for the treatment of patients with critical ischemia was approved by the Medical Ethics Committee of the National Cardiovascular Center.

Autologous BMT

Bone marrow fluid (700-800ml) was collected from the iliac bone under general anesthesia. The harvested bone marrow fluid was diluted with RPMI 1640 (Nikken Bio Medical Laboratory, Kyoto, Japan) containing heparin, then stored in a sterile pack from the Bone Marrow Collection Kit (Baxter, IL, USA). The mononuclear cell fraction was prepared with a Fresenius AS104 (AMCO, USA). The injection volume was 0.5ml and injections were spaced 2-3cm apart, using a 1ml syringe and a 27-gauge needle. Leg pains were measured by a visual analog pain scale and foot ulcers were evaluated by area and appearance.

Novel micro-angiography

The in-hospital micro-angiographic equipment consisted of a high power X-ray source for computed tomography and an avalanche type detector characterized by a high spatial resolution (20μm) and high sensitivity (100 times of CCD camera) (Fig.1).



a b

Fig. 1a, b. The micro-angiographic equipment that we developed. High-voltage power X-ray source **a** and a detecting system with a high spatial resolution(25μm) and high sensitivity (100 times of CCD camera) **b.**

Limb ischemia models in rabbits were made by ligating the femoral artery and treated by fibroblast growth factor 4 (FGF-4) genes incorporated to gelatin hydro gel (GHG). One month after the treatment, we evaluated collateral micro-vessels by using conventional and micro-angiographic systems. The approach was via the left femoral artery so that the catheter was located in the abdominal aorta. A 5ml bolus of Iodine contrast medium (300mg/ml) was injected at 3ml/sec using an auto-injection system. Imaging was recorded using a digital source in 1000 x 1000 pixels. The sum of radio-absorptions for 10 seconds in clinical settings was studied.

Results

Autologous BMT for Critical Limb Ischemia

The number of transplanted bone marrow mononuclear cells were one to five multiplied 10^9. Rest pains decreased or disappeared in one month after BMT (Fig.2) and Skin ulcers improved in one to three months after BMT in all patients (Fig.3).

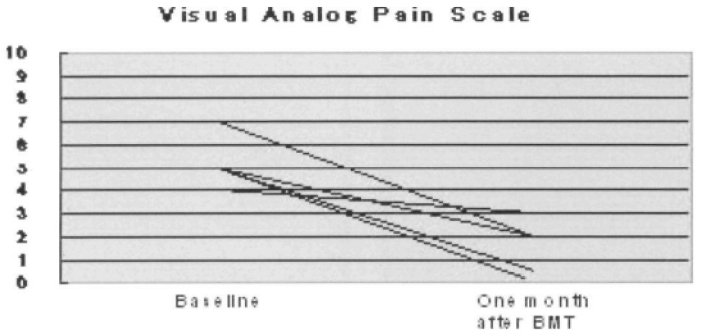


Fig. 2. The Visual analog pain scale in all patients.

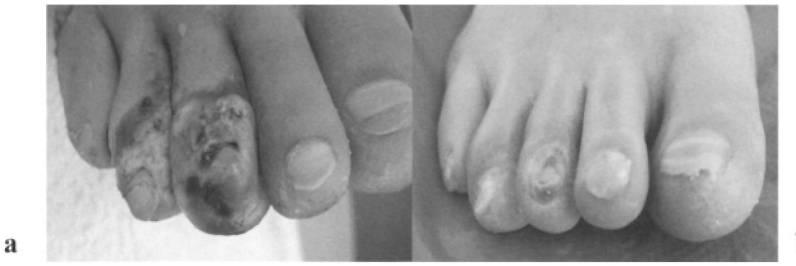


Fig. 3a, b. The skin ulcers in a patient before **a** and one month after autologous bone marrow transplantation **b**.

Conventional angiography was performed before and one month after BMT, but there was no significant changes in any of the patients (Fig.4).

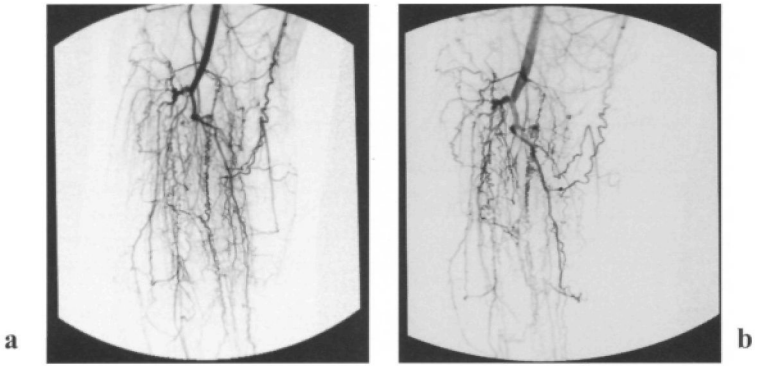


Fig. 4a, b. The conventional angiographic findings in the patient before **a** and one month after autologous bone marrow transplantation **b**.

Novel micro-angiography

The novel micro-angiography can detect to within a limit 50 of μm, although a detection limit of a conventional angiography is 250μm (Fig.5).

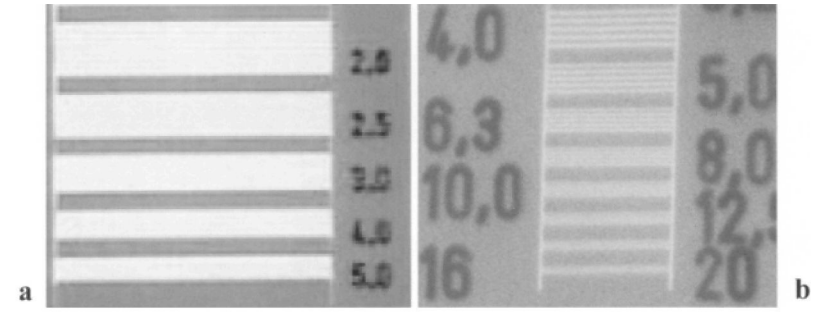


Fig. 5a, b. The detection limits on a conventional angiography **a** and the novel micro-angiography **b** using a line chart

Collateral micro-vessels, which were 100-500μm or less in diameter, were demonstrated more clearly in micro-angiography than conventional angiography (Fig.6).

The sum of radio-absorptions at the point of 1m distance from the X-ray source in clinical settings was 300 mSv. for 10 seconds.

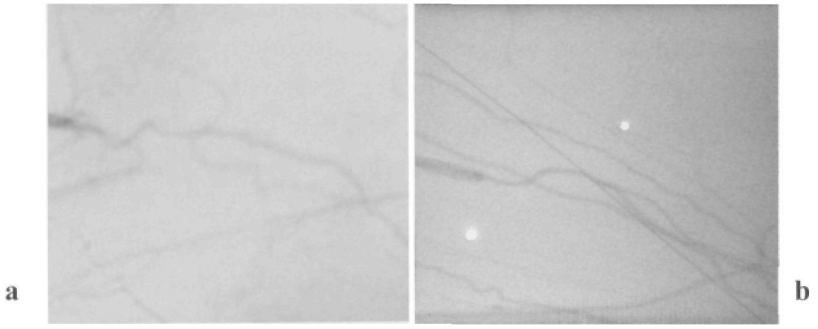


Fig. 6a, b. In 2.5x2.5cm view size, Collateral micro-vessels after therapeutic angiogenesis in the rabbit limb ischemia model. Vessel sizes in the range of 100-500μm or less, were demonstrated in the novel micro-angiography **b** more clearly than in a conventional angiography **a**. The diameter of the line in the micro-angiography is 130μm.

Discussion

Autologous BMT improved chronic severe limb ischemia due to Bue rger's disease. Conventional angiography could not disclose develope d collateral vessels after BMT. A novel micro-angiography technique could illuminate promoted collateral vessels after therapeutic angioge nesis in rabbit models although a conventional angiography did not. The sum of radio-absorptions in the novel angiography could be acc-epted in clinical settings.

Autologous BMT and Buerger's disease

Bone marrow harvests need an amount of more than 500ml bone marrow fluid and general anesthesia in therapeutic angiogenesis using BMT. Such factors have practical limitations to select candidates with peripheral artery disease complicated with systemic atherosclerosis and aging for BMT. Buerger's disease is a segmental vasculitis that affects the distal arteries of the upper and lower extremities. It typically occurs in young people. The majority of patients with Buerger's disease have pain at rest and digital

ulcerations and are hard to treat by revasculaizations, including catheter angioplasty and surgical bypass grafting, because of peripheral artery lesions. Patients with Buerger's disease, however, tend to have less systemic atherosclerotic lesions and normal cardiac function. These suggest that patients with Buerger's disease are the ideal candidates for therapeutic angiogenesis using autologous BMT.

Discrepancy between clinical improvements and conventional angiographic findings after BMT

BMT improved critical limb ischemia clinically. Promoted collateral vessels after the treatment were not, however, visualized well by conventional angiography. These vessels are quite small and the detection limit of small vessels by conventional angiography is about 200μm in diameter.

Novel micro-angiography

Recently, synchrotron radiation system characterized by high brightness, monochromatic and collimated nature bypass, revealed micro-vessels in situ. However the high cost of a synchrotron system strictly limits its clinical application (100 million dollars or more). We developed an in-house micro-angiographic system with a relatively low cost of approximately 1million dollars, which consisted of a high-voltage power X-ray source and a detecting system with a high spatial resolution (25μm) and high sensitivity (100 times of CCD camera). We evaluated collateral micro-vessels one month after therapeutic angiogenesis by using the conventional and micro-angiographic system. The in-house micro-vessel angiographic system could detect the micro-vessels more precisely than conventional angiographic system. We thought that the present micro-angiography should be useful for evaluating efficacy of therapeutic angiogenesis in clinical settings.

Conclusions

Conventional angiography failed to disclose the promoted collateral vessels after BMT although BMT improved the critical limb ischemia clini-

cally. The in-house micro-angiographic system could detect the micro-vessels more precisely than conventional angiographic system and the sum of the radio-absorption in the equipment could be acceptable in clinical settings. The novel in-house micro-angiographic system can be useful in the evaluation of therapeutic angiogenesis clinically.

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Angiogenesis Induced by Intramyocardial Implantation of Autologous Bone Marrow Mononuclear Cells for the Treatment of Ischemic Heart Disease

Tao-Sheng Li, Masunori Matsuzaki, and Kimikazu Hamano

Department of Medical Bioregulation, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan

Summary. Therapeutic angiogenesis has been successfully induced by various methods, and developed clinically as a new treatment for ischemic diseases. We have tried to induce therapeutic angiogenesis for the treatment of ischemic heart disease by the implantation of bone marrow mononuclear cells (BM-MNCs), because BM-MNCs consist of complex populations, including the endothelial progenitor and angiogenic cytokine-producing cells. Using the ischemic heart model in rats and dogs, we have demonstrated that the intramyocardial implantation of BM-MNCs is a feasible and safe method for inducing therapeutic angiogenesis and improving deteriorated cardiac function. Then, we performed the intramyocardial implantation of autologous BM-MNCs in eight patients with ischemic heart disease who underwent coronary arterial bypass graft (CABG) simultaneously. A specific increase in myocardial perfusion was seen in five patients, and two patients showed improved left ventricular wall motion, 1 and 12 months after treatment. No postoperative complications, new-onset malignant arrhythmias, mass formation or calcification, or any other abnormal signs related with this new therapy were detected in any of the patients by 3 years follow-up examination. Although further investigation on the safety and effect of cell-based therapy are required, our preliminary results indicated that the intramyocardial injection of BM-MNCs is a feasible and safe method of inducing therapeutic angiogenesis and improving cardiac function in patients with severe ischemic heart disease.

Key words. Bone marrow mononuclear cells, Ischemic heart disease, Angiogenesis

Cell-based therapeutic angiogenesis and BM-MNCs

Despite remarkable advances in medication, percutaneous catheter intervention, and coronary artery bypass grafting (CABG), some patients with severe ischemic heart disease are unsuitable candidates for these conventional treatments. Several new therapies, including the induction of therapeutic angiogensis, have been developed to treat these patients (Horvath KA, et al. 1997; Losordo DW, et al. 1998; Schumacher B, et al. 1998). Cell-based therapy has recently been the new method of focus for inducing angiogenesis because endothelial progenitors are found in peripheral blood and bone marrow, which could contributed to vasculagenesis (Asahara T, et al. 1997). Bone marrow mononuclear cells (BM-MNCs) are one of the most viable cell sources for clinical application because BM-MNCs consist of many endothelial progenitor (stem) cells and angiogenic cytokine-producing cells. Furthermore, the implantation of autologous BM-MNCs does not have the problems of immunological rejection and ethical objection for clinical application.

To investigate whether BM-MNCs are the suitable cell source for inducing angiogenesis, we examined the potency of angiogenic cytokines production and endothelial differentiation from BM-MNCs. Immunostaining analysis have shown that about 16% of nuclear bone marrow cells positively expressed vascular endothelial growth factor (VEGF) and 4% basic fibroblast growth factor (bFGF) (Hamano K, et al. 2000). Furthermore, VEGF and bFGF were produced from the BM-MNCs in vitro and the production was enhanced by hypoxia stimulation (Hamano K, et al. 2000). After 7 days of cultivation with the supplement of growth factors of VEGF, bFGF, and IGF-1, we have also found that on these BM-MNCs was positive expression of about 15% CD34, 5% of VE-cadherin, and 7% of Flk-1, indicating endothelial differentiation from BM-MNCs. Using a rat cornea micropocket model, we have also observed the angiogenic potency induced by BM-MNCs in vivo (Hamano K, et al. 2000). All of these have indicated clearly that BM-MNCs are one of the suitable cell sources for inducing angiogenesis. We speculate that the formation of new collateral vessels by intramyocardial implantation of BM-MNCs may result from both angiogenesis (induced by the angiogenic cytokine produced from implanted BM-MNCs) and vasculogenesis (raised by the in situ differentiation and incorporation of endothelial progenitors in BM-MNCs after implantation) (Figure 1).

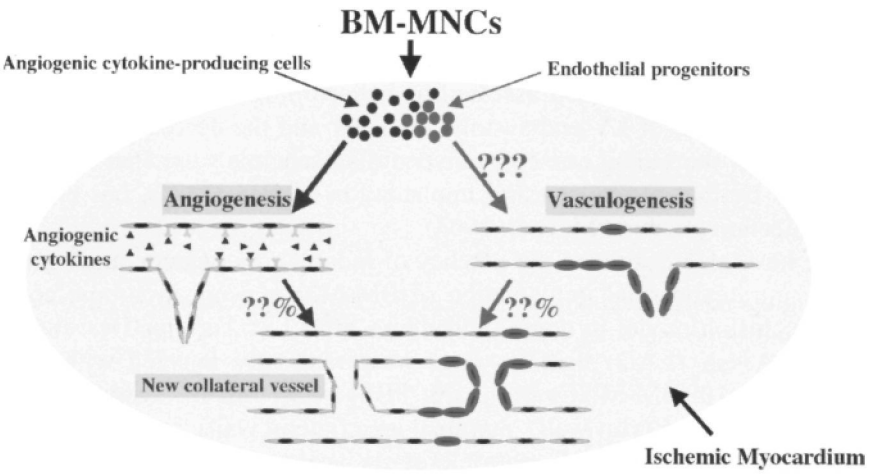

Fig. 1. The speculated mechanism of therapeutic angiogenesis induced by intramyocardial implantation of bone marrow mononuclear cells (BM-MNCs) for the treatment of ischemic heart disease. After the implantation of BM-MNCs into ischemic myocardium, both angiogenesis (angiogenic cytokines produced from BM-MNCs) and vasculogenesis (in situ differentiation and incorporation of endothelial progenitors in BM-MNCs) will contribute to the formation of new collateral vessels.

Preclinical study of intramyocardial implantation of BM-MNCs into ischemic myocardium in rats and dogs

Induction of angiogenesis and improvement of cardiac function by intramyocardial implantation of BM-MNCs

To investigate whether intramyocardial implantation of BM-MNCs will induce angiogenesis and improve deteriorated cardiac function, we have created a hypoperfusion heart model in rats by ligating the left anterior descending artery placed against a copper wire (275 μm in diameter), then pulling out the wire immediately. Then, the left ventricular (LV) anterior wall was injected directly at six points, each with 1 x 10^7 bone marrow cells in 10 μL of phosphate-buffered saline (PBS) or with PBS only, respectively. Western blotting analysis showed that bone marrow cell implantation treatment increased significantly the levels of angiopoietin-1

and WEGF in the LV anterior wall. Compared with PBS injection only, intramyocardial implantation of BM-MNCs increased significantly the microvessel density, blood flow, and thickness of the LV anterior wall after treatment. Cardiac function assessed by echocardiography also found that both the increase of LV end-systolic diameter and the decrease of percent of fractional shortening caused by myocardial ischemia were attenuated effectively by the intramyocardial implantation of BM-MNCs, but not the PBS injection (Nishida M, et al. 2003).

We have also examined the potency of inducing therapeutic angiogenesis by intramyocardial implantation of BM-MNCs using a chronic coronary occlusion model in dogs. Thirty days after Lef Anterior Descending Conary Artery (LAD) ligation, dogs were randomly injected with BM-MNCs (2 x 10^7 BM-MNCs in 0.1 mL PBS) or 0.1 mL PBS only into the infarction area (anterior wall), marginal area (lateral wall), and normal area (posterior wall) of the left ventricle at six points. Immunohistochemical staining showed that the microvessel density in the marginal area was significantly higher 30 days after the treatment with the implantation with BM-MNCs than the injection of PBS only (127.7 ± 20.1 versus 88.0 ± 10.2, $p = 0.007$). Furthermore, a significant increase of wall thickening in the marginal area was observed only by the implantation with BM-MNCs, but not by the injection of PBS (14.5 ± 2.28 versus 8.1 ± 3.00, $p = 0.002$) (Hamano K, et al. 2002)..

All of these findings indicate that intramyocardial implantation of autologous bone marrow cells can induce therapeutic angiogenesis and improve the perfusion of ischemic myocardium, thereby preventing LV remodeling and improving deteriorated cardiac function caused by myocardial hypoperfusion.

Feasibility and safety

Eight healthy dogs were used to investigate the systemic and local toxicity of intramyocardial implantation of autologous BM-MNCs. The dogs were randomly to receive the intramyocardial implantation of autologous BM-MNCs (2 x 10^7 BM-MNCs in 0.1 mL PBS, n = 7) or 0.1 mL PBS only (n = 7), at six points in the left ventricular wall. Before and after 1, 3, 7, 30, and 240 days of treatment, the electrocardiograph (ECG), echocardiograph, and systemic biochemistry indexes were recorded for monitoring the dysfunction of heart, liver, kidney, and bone marrow. The dogs were killed 240 days after the treatment and the local myocardium was collected for histologic examination. Compared with PBS injection only, neither systemic nor local toxicity were detected in the acute or chronic phases after the intramyocardial implantation of autologous BM-MNCs. However,

fibrotic changes are seen in the myocardium of both groups but it was localized only at the site of injection and was less than 1% in area. No other significant histologic changes such as calcification, hemetoma formation, or inflammatory cell migrations were observed in the hematoxilin-eosin stained specimens from the myocardium 240 days after intramyocardial implantation of autologous BM-MNCs (Hamano K, et al. 2002).

Clinical Trial of Intramyocardial implantation of BM-MNCs for the treatment of ischemic heart disease

Patients selection

After confirming the therapeutic potency and safety by preclinical study in animal models (Kobayashi T, et al. 1999, Hamano K, et al. 2002), we began a clinical trial of intramyocardial implantation of autologous BM-MNCs for the treatment of ischemic heart disease in October 1999 (Hamano K, et al. 2001). This clinical trial was approved by the Medical Ethics Committee of Yamaguchi University School of Medicine, and The Medical Ethics Committee stipulated that this therapy only be performed concomitantly with CABG.

The patients were selected as the only candidates when they, 1) are scheduled to undergo CABG, 2) with at least one ischemic area unsuitable for the treatments of percutaneous catheter intervention or surgical CABG to the stenotic coronary artery, 3) no wall thinning change in the targeted area if evidence of old myocardial infarction, 4) informed consent was obtained.

Collection, separation, and intramyocardial injection of BM-MNCs

Bone marrow cells are collected from the iliac bone under general anesthesis. The harvested bone marrow cells are diluted with RPMI 1640 (NIKKEN BIO MEDICAL LABORATORY, Kyoto, Japan) containing heparin. The bone marrow fluid (200-600 ml) is saved in sterile pack of the Bone Marrow Collection Kit (Baxter, IL, USA). CABG was begun in the usual way after collecting bone marrow fluid. The mononuclear cell fraction is prepared with a COBE Spectra Apheresis System (Gambro, Stockholm, Sweden). The separated BM-MNCs were adjusted for injection, at a cell density of $5\sim10\times10^{8}$ cells/ml in serum-free RPMI 1640 medium.

When bypass grafting is completed and the heart is still arrested under cardiopulmonary bypass, the autologous mononuclear cell-rich fraction of bone marrow cells is injected into the targeted area of ischemic myocardium that is without a graft (Figure 2). The injection volume is 0.1 ml and injections are spaced 1 cm apart, a 1ml syringe and a 26-guage needle are used. After the final injection, all other surgical procedures proceeded as usual. All procedures were finished without any intraoperative trouble.

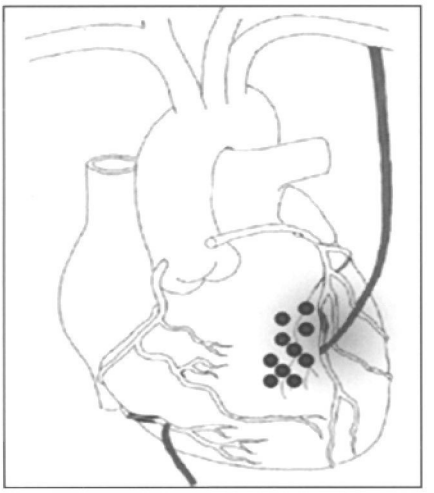

Fig. 2. Scheme of the intramyocardial injection of BM-MNCs into targeted zone.

Follow-up examinations and outcomes

Eight selected patients (six men and two women aged between 50 and 73 years old) were given intramyocardial implantation of BM-MNCs after CABG. The intramyocardial implantation of BM-MNCs was targeted into a small perfusion area in the initial four patients because we were unsure about the safety of this new therapy (two in the posterior descending artery perfusion area, and one in the first diagonal artery and the posterolateral branch perfusion area), and into the perfusion area of the left circumflex artery in the other four patients (Table 1).

Echocardiography was performed preoperatively, then 1, 12, and 24 months after treatment. LV angiography was also done preoperatively and 1 month after treatment. Improvement in cardiac function was evaluated by LV ejection fraction and LV wall motion. To detect specific improve-

ment in myocardial perfusion, ^{201}thallium-chloride SPECT myocardial perfusion scintigraphy was done preoperatively, then 1, 12, and 24 months after treatment.

As shown in Table 1, two of our eight patients had specific improvement of regional wall motion on echocardiography or LV angiography, and five patients had an obvious increase in myocardial perfusion in the targeted area on scintigraphy. Furthermore, the improvement of regional perfusion and wall motion in the targeted area continued through their follow-up period after treatment.

We also performed standardized quantitative analysis on bulls-eye views by calculating the mean percent uptake of the tracer in the respective perfusion area of the left circumflex artery according to the 25-segment model (Everaert H, et al 2000). Statistical analysis of mean percent uptake showed significant improvement of the regional perfusion in the targeted area that received an injection of BM-MNCs, from 72.8±11.1 at baseline to 87.1±3.3 at 1 month follow-up and 87.7±4.0 at 12 months follow-up($P<0.05$).

Table 1. Characteristics and profiles of the patients received intramyocardial implantation of autologous bone marrow mononuclear cells.

Case	Age(y) /Sex	Target of BMCI	Bypass graft to	Cell injection		Perfusion *(Scintigraphy)*			LV function *(Echo.)*			Outcomes
				Cells	Points	1 mo	12 mo	24 mo	1 mo	12 mo	24 mo	
1	67 /M	15PD	LAD, D1, 14PL	5×10^7	6	↑	↑	↑	---	---	---	Improvement
2	68 /F	14PL	LAD, OM1, OM2	5×10^7	11	---	---	---	---	---	---	Inestimable
3	59 /M	15PD	LAD, 14PL, 4PD	1×10^8	5	↑	↑	↑	---	---	---	Improvement
4	61 /M	D1	LAD, OM, 4PD, 4PL	1×10^8	10	---	---	---	---	---	---	Inestimable
5	73 /M	LCX	LAD, D1, 4PL, 4PD	1×10^8	22	↑	↑	↑	---	---	---	Improvement
6	70 /F	LCX	LAD, D1, D2	1×10^8	17	↑	↑	↑	↑	↑	↑	Improvement
7	72 /M	LCX	LAD, RCA	1×10^8	18	---	---	---	---	---	---	No change
8	50 /M	LCX	LAD, D1, 4PL	5×10^7	20	↑	↑	↑	↑	↑	↑	Improvement

(BMCI: bone marrow cell implantation, LAD: left anterior descending artery, RCA: right coronary artery, LCX: left circumflex artery, LV: left ventricular, mo: month, Echo.: Echocardiography, ↑ Improved; --- no change)

Feasibility and safety

No postoperative complications developed in any of the patients who received an intramyocardial injection of up to about 2×10^9 BM-MNCs. Within the first 30 days of follow up after treatment, the serum level of CPK, CPK-MB, CRP, BUN, creatinine, GPT, and GOT did not differ significantly between the patients that received intramyocardial injection of BM-MNCs and these patients received CABG only. No anemia, leukocytopenia, or any other sign of hemopoietic abnormality was seen in any of the patients that received intramyocardial injection of BM-MNCs in 3 years of follow up. Holter electrocardiography showed no new-onset malignant arrhythmias after intramyocardial BM-MNC injection. Furthermore, no sign of atopic tissue formation, including hematoma, teratogenic tumor, bone, or other tissue, was detected in the myocardium by 3 years follow-up examinantion. All these findings indicated that the intramyocardial injection of autologous BM-MNCs had had minimal, if any, local and systemic side effect.

Limitations

Although our experimental studies and clinical trial suggest that the implantation of BM-MNCs may be a feasible and safe new method for the treatment of ischemic heart disease, many questions regarding this new therapy need to be answered.

First, the effectiveness of the intramyocardial implantation of BM-MNCs for inducing angiogenesis need to be confirmed by double-blind randomized trial with a long-term follow up. Experimental investigations have demonstrated that the implantation of bone marrow-derived cells can improve cardiac function by the induction of angiogenesis and the enhancement of myocardial blood flow, or by the regeneration of new myocardium from mesenchymal stem cells in BM-MNCs (Kocher AA, et al. 2001; Kamihata H, et al. 2001; Orlic D, et al. 2001). Similar with our results, some clinical trials have also reported that the intracoronary infusion or intramyocardial injection of bone marrow-derived cells helps to improve myocardial perfusion and wall motion, and increase global LV ejection fraction (Assmus B, et al. 2002; Fuchs S, et al. 2003; Hamano K, et al. 2001; Stamm C, et al 2003; Strauer BE, et al. 2002; Tse HF, et al. 2003). However, these clinical trials were limited in an open study with very few patients, which is difficult to conclude that the intramyocardial implantation of BM-MNCs is an effective new therapy for ischemic heart disease.

Second, the safety of this new therapy is yet to be confirmed because BM-MNCs consist of heterogeneous cell fractions, including mature mononuclear cells, hematopoietic stem cells, mesenchymal stem cells, and other pluripotential stem cells (Lee GR, et al. 1998). So, severe inflammatory response may be evoked and atopic tissue may be formed in heart by intramyocardial implantation of BM-MNCs. Although we have not detected any side effects in eight patients by three years follow-up examination, further investigation is required to clarify the long-term safety of the intramyocardial implantation of autologous BM-MNCs by implanting a greater number of cells and following up more patients for a longer period.

Third, it is necessary to clarify the cellular and molecular mechanisms of this new therapy. Although the mechanisms of angiogenesis induced by the implantation of BM-MNCs was considered to be related with both angiogenesis and vasculogenesis (Figure 1), the complex cellular and molecular mechanisms of cell-based therapy are still poorly understood. We do not know how much of the angiogenic potency results from angiogenesis, and how much from vasculogenesis. It is open to dispute whether the vasculogenesis is, in fact, a rare event that contributes very little to the improvement of regional perfusion (Patterson C, 2003).

Otherwise, many basic questions on the methodology of cell-based therapy for inducing angiogenesis also still exist. We do not know how many cell numbers of which subpopulation of BM-MNCs, should be implanted and by what kind of delivery route, for the treatment of ischemic heart disease. By now, the cell number, cell population, and delivery routes have differed from one to another, in both experimental studies and clinical trials (Li TS, et al. 2003; Hayashi M, et al. in press).

Taken together, these findings from our experimental investigations and clinical trial provide substantial evidence about the safety and feasibility of the intramyocardial implantation of freshly collected autologous BM-MNCs for the treatment of ischemic heart disease. Although further study is required to confirm the safety and curative potency, cell-based therapeutic angiogenesis provides a viable treatment option for patients with severe ischemic heart disease.

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Effect of Bone Marrow Transplantation in Patients with Critical Limb Ischemia

Yousuke Katsuda, Yoshiaki Takeshita, Ken Arima, Yutaka Saitoh, Ken-ichiro Sasaki, Shinichi Shintani, Toyoaki Murohara, Tsutomu Imaizumi

Department of Medicine III, Kurume University School of Medicine, 67 Asahi-machi, Kurume, 830-0011, Japan

Summary. Background: Endothelial progenitor cells in the $CD34^+$ stem-cell fraction of adult human peripheral blood take a part in postnatal neovascularization after mobilization from bone marrow. We investigated whether transplantation of bone marrow-mononuclear cells (BM-MNCs), including endothelial progenitor cells (EPCs), into ischemic limbs rescues ischemia in patients with peripheral artery occulusive disease (PAOD). Methods and Results: 22 patients with severe PAOD (atherosclerosis obliterance and Thromboangiitis obliterance / Burger's disease) were enrolled. After collecting MNCs from the autologous bone marrow using the density gradient centrifugation method, we transplanted them into the ischemic limb. Four weeks after transplantation, pain quantified by the Visual Analog Scale was significantly improved. Skin ulcers healed in 6 of 10 patients and the size was reduced in the rest of them. Ankle brachial blood pressure ratio index (ABI) increased (0.58 ± 0.2 to 0.69 ± 0.26 at 4weeks). Furthermore, most of patients revealed angiographical improvements. No adverse event was observed.

Conclusion: Our findings indicate that therapeutic angiogenesis by transplantation of autologous BM-MNCs is effective and safe for patients with PAOD.

Key words. Angiogenesis, PAOD, Bone marrow-mononuclear Cells, EPCs

Introduction

Peripheral artery obstructive disease (PAOD) is the main cause of critical limb ischemia, which was estimated to develop in 500-1000 individuals per million per year (Second European Consensus Document on Chronic Critical Leg Ischemia 1991). Some patients are unsuitable for operative or percutaneous revascularization and there is no optimal medical treatment for critical limb ischemia. Preclinical studies have demonstrated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral ischemia, a concept of therapeutic angiogenesis (Isner M, et al. 1999). Recently, it was reported that endothelial progenitor cells in the $CD34^+$ stem-cell fraction of adult human peripheral blood take part in postnatal neovascularization after mobilization from bone marrow (Asahara T, et al. 1997). Therefore, it is anticipated that transplantation of bone marrow-mononuclear cells (BM-MNCs), including endothelial progenitor cells (EPCs), into ischemic limbs increases collateral vessel formation (Asahara T, et al. 1999). We recently demonstrated the possibility of therapeutic angiogenesis for ischemic tissues by transplantation of autologous BM-MNCs in animal models (Shintani S, et al. 2001). On the basis of these results, we evaluated whether this approach is safe and effective for patients with PAOD.

Patients

Patients with PAOD were enrolled. They had critical limb ischemia, including rest pain, non-healing ischemic ulcers, and were not suitable candidates for non-surgical or surgical revascularization. We excluded patients with a history of malignant disorders during the past 5 years, and with proliferative or diabetic retinopathy. We obtained written informed consent from all patients. The ethics committee of our university approved the protocol.

Methods

Transplantation of BM-MNCs: Transplantation of BM-MNCs was performed as described before (Tateishi-Yuyama E, et al. 2002). Bone marrow (500-800ml) was aspirated from the iliac crest under general anesthesia, and gathered into a plastic bag containing heparin. We collected mononuclear cells from the bone marrow using a CS3000-Plus blood-cell separator (Baxter, Deerfield, USA), and concentrated them to a final volume of about 50ml. We injected them with a 23-gauge needle (1.5cm deep) 3 hours after marrow aspiration into the ischemic skeletal muscle at 50 points (1.4×10^9 to 4.4×10^9 cells, average 2.7×10^9 cells/patient).

Patient Follow-up and Assessment

The purpose of this study was to clarify safety and feasibility of the treatment, such as improvements in visual analog scale, size of skin ulcer, laser doppler blood flow and digital subtraction angiography score, and critical cardiovascular events. Critical cardiovascular events were defined as death of cardiovascular origin, myocardial infarction, unstable angina, coronary angioplasty, coronary artery bypass, graft surgery, stroke and transient ischemic attack. We evaluated them 1 week before and 4 weeks after the treatment. "Visual analog scale" was score of pain at rest (from none 0 to maximum 10). "Size of skin ulcer" was measured by a caliper and represented as length$\times$width. "Laser doppler blood flow (LDBF)" was the blood flow estimated by laser doppler. "Digital subtraction angiography score" was the extent of capillary vessel formation estimated by digital subtraction angiography. Digital subtraction angiography was performed with the strictly fixed amount of contrast agent, force of contrast injection, and position of the catheter tip before and after treatment. New collateral vessel formation was assessed as –1 (decrease), +0 (no collateral development), +1 (slight increase), +2 (moderate increase), or +3 (great increase). Angiograms were assessed at the time at which contrast flow in the main conducting arteries was most clearly visible. Ischemic ulcers and necrotic lesions were managed in a standard fashion, and antibiotics were prescribed as necessary. Patients continued to take medications at constant doses throughout screening and follow-up.

Statistical Analysis

All data are presented as mean±SD. Statistical significance was accepted when p was <0.05. A paired t test was used for comparisons before and after the treatment.

Table 1. Patient characteristics

	Number of patients (n=22)
ASO / TAO	7 / 15 (31.8% / 68.2%)
Age (years)	54.6 ± 16.0
Sex (male/female)	19 / 3 (86.4% / 13.6%)
Fontaine classification (IIb / III / IV)	6 / 4 / 12 (27.3% / 18.2% / 54.5%)
Coronary risk factor	
Hypertension	10 (45.5%)
Hyperlipidemia	7 (31.8%)
Diabetes	7 (31.8%)

ASO: arteriosclerosis obliterans, TAO: thromboangiitis obliterans

Results

Demographical Characteristics

First recruitment started in Nov 2000 and thereafter 22 patients with PAOD were enrolled. The follow-up period was 24 weeks. Clinical features for 19 men and 3 women are shown in **Table 1.** Most of them had severe ischemia as shown by the Fontaine classification. Skin ulcers were present in 12 limbs of 10 patients before transplantation.

Clinical outcomes

The transplantation of BM-MNCs into the ischemic limbs induced minimal local discomfort for a few days after the injection. The serial creatinine phosphokinase measurements remained within the normal range. No systemic or local inflammatory reactions were noted.

No worsening of diabetic retinopathy was observed. Because of deterioration of a large skin ulcer, amputation of the treated limb was required in

one patient. Twelve of the 22 patients were able to walk at their own pace before transplantation of BM-MNCs, and the maximum walking distance was significantly improved from 133.0±73.9 at baseline to 264.0±142.7 after 4 weeks (Fig.1). Complete healing of skin ulcers was achieved in 7 of 15 ulcers (46.7%, Fig.2), and rest pain was alleviated (complete resolution: 36.8%, partial resolution: 95.0%). ABI in the legs implanted with BM-MNCs was improved (0.58±0.20 at baseline to 0.69±0.26 at 4 weeks, $p<0.01$). Angiograms showed significant increases in the number of visible collateral vessels or blood flow in 13 of 19 patients (DSA score, 0.0±0.0 at baseline to 0.84±0.69 at 4 weeks, $p<0.05$). The LDBF ratio was increased from 0.73±0.34 at baseline to 0.82±0.22 at 4 weeks ($p<0.05$). Representative angiograms are shown in **Fig.3.**

No patient died during the follow-up. No adverse events were observed.

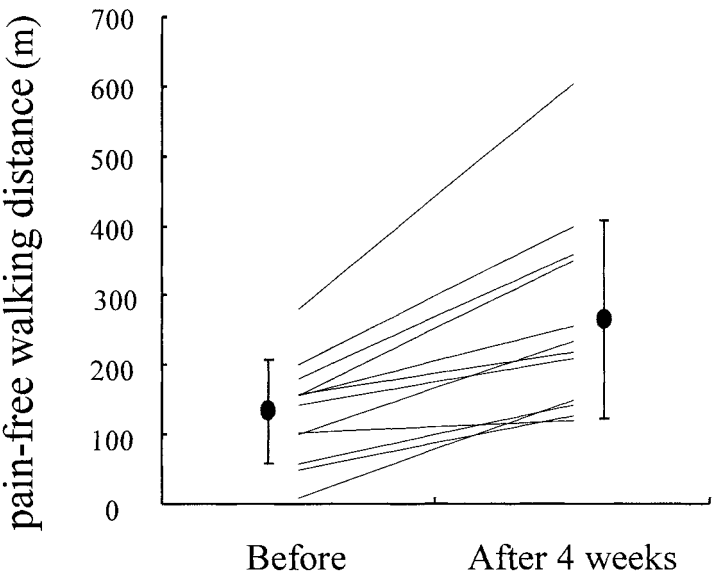

Fig. 1. Improvement of pain-free walking distance. Twelve of 19 patients were able to walk before transplantation. The maximum pain-free walking time was significantly ($p<0.01$) improved after transplantation.

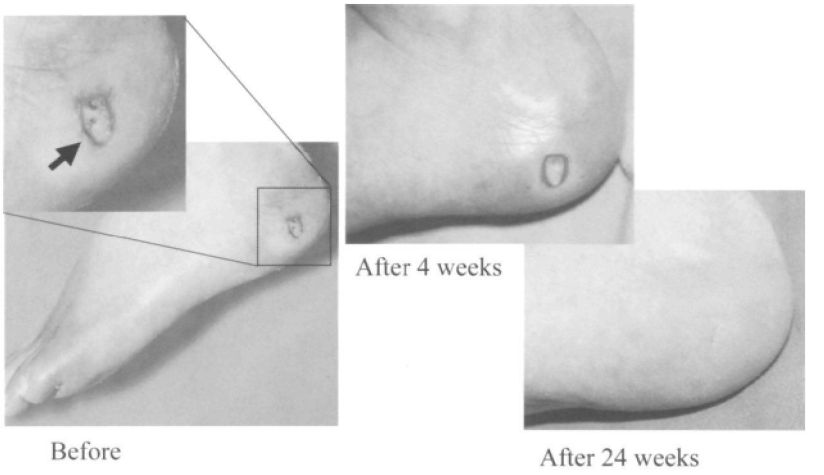

Fig. 2 Healing of an ischemic ulcer in a 44-year-old man. Left panel: This patient presented an ischemic ulcer at the left heel with severe pain. Middle panel: 4 weeks after transplantation, the ischemic ulcer was improving. Right panel: after 24 weeks, the ischemic ulcer was completely healed, and rest pain completely disappeared.

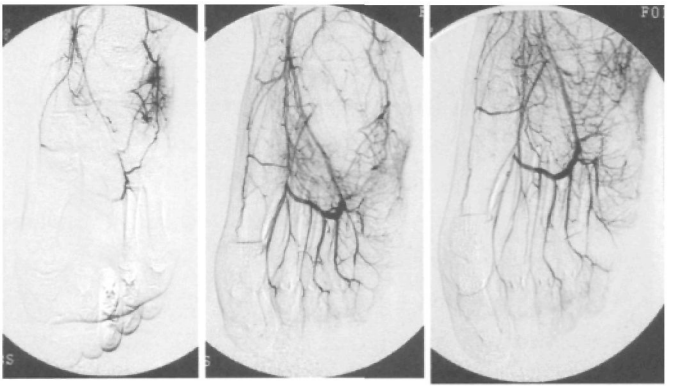

Fig. 3 Time course of angiographic images after transplantation in a 29-year-old man. Left panel: Angiography before transplantation demonstrated poor visualization of vessels. Mid panel: After 4 weeks of transplantation, angiography demonstrated improved blood flow and newly visible collateral vessels. Right panel: after 24 weeks, these improvements were maintained at 4 weeks.

Discussion

We have shown that transplantation of BM-MNCs significantly increased blood flow, as assessed by increases in ABI, DSA score, and LDBF ratio. The maximum pain free walking distance was extended. Furthermore, transplantation of BM-MNCs significantly alleviated rest pain and ischemic ulcers healed in most patients. Since marrow cells include cells of various lineages, such as fibroblasts, osteoblasts, myogenic cells (Prockop DJ 1997), such mixed populations could differentiate into various mesenchymal cells. However, in our study, neither bone formation nor increased interstitial fibrosis was detected. Furthermore, no adverse effects were observed. Thus, autologous transplantation of BM-MNCs could be a safe and effective strategy for achievement of therapeutic angiogenesis in patients with PAOD.

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Therapeutic Angiogenesis for a Patient with Arteriosclerosis Obliterans by Autologous Transplantation of Bone Marrow Mononuclear Cells

Kazuteru Fujimoto[1], Hiroo Miyagi[1], Yuji Miyao[1], Ichiro Kajiwara[1], Youko Oe[1], Fumio Kawano[2] and Michihiro Hidaka[2]

Division of [1]Cardiovascular Medicine and [2]Internal Medicine, National Hospital Organization Kumamoto Medical Center1-5 Ninomaru, Kumamoto, Kumamoto, Japan

Summary. A 60 year-old man with atherosclerosis obliterans had received a bypass operation twice, but came to notice resting pain in the left calf. As it was not possible to perform further bypass operations (resulys of arteriography), we conducted conservative treatments. We therefore performed therapeutic angiogenesis by autologous transplantation of bone marrow mononuclear cells since the symptoms were not improved at all. Resting pain was improved the next day after the operation and completely disappeared on the third day. The patient was only able to walk 180m in the preoperative state by crutch-assisted, but walked 180m without a stick on the second day and 900m on the fourth day. This treatment did not cause any inconvenience in everyday life and the patient showed no resting pain eight weeks later. In addition, the ankle-brachial index improved to 0.48 from the preoperation level of 0.36 after two months.

Therapeutic angiogenesis by autologous transplantation of bone marrow mononuclear cells was very useful.

Key words. Arteriosclerosis obliterans, Angiogenesis, Bone marrow mononuclear cells

Introduction

Neovascular formation in adults has been considered to result exclusively from the formation of new blood vessels by sprouting of preexisting mature endothelial cells, a process referred to as angiogenesis(Folkman J, 1995, Schper W, et al. 1971, Risau W, 1997). In contrast, vasculogenesis is referred to as the creation of primordial blood vessels from endothelial progenitor cells(Risau W, 1997, Risau W, 1995, Risau W, et al. 1988). Recently, circulating endothelial progenitor cells have been discovered in adult peripheral blood and human umbilical cord blood(Asahara T, et al. 1997, Murohara T, et al. 2000). Circulating endothelial progenitor cells have been shown to accumulate at active angiogenic sites and to participate in neovascularization(Takahashi T, et al. 1999). Endothelial progenitor cells in adults originate from bone marrow, and recently it has been demonstrated that implantation of autologous bone marrow mononuclear cells effectively augmented ischemia-induced neovascularization in animal studies (Shintani S, et al. 2001). as well as in human trials (Tateishi-Yuyama E, et al. 2002). Therapeutic angiogenesis is effective for limb salvage in patients with critical limb ischemia.

Case

A 60 year-old man with atherosclerosis obliterans had received a bypass operation twice, but came to notice resting pain in the left calf. Using arteriography, good collaterals were recognized in the left femoral region, but, less so in the knee region, therefore a bypass was impossible. Conservative treatments such as oral administration of aspirin and breaprost sodium with drip infusion of alprostadil were tried, but none of the symptoms were improved. So we induced therapeutic angiogenesis by autologous transplantation of bone marrow mononuclear cells.

Under general anesthesia, we gathered about 200ml of bone marrow from both iliac crests. Bone marrow mononuclear cells were isolated from the bone marrow by CS 3000 (Baxter company) and we obtained $1.0X10^9$ cells. We injected the bone marrow mononuclear cells into the left calf and the sole.

Resting pain was improved after the operation next day and completely disappeared on the third day (Fig. 1).

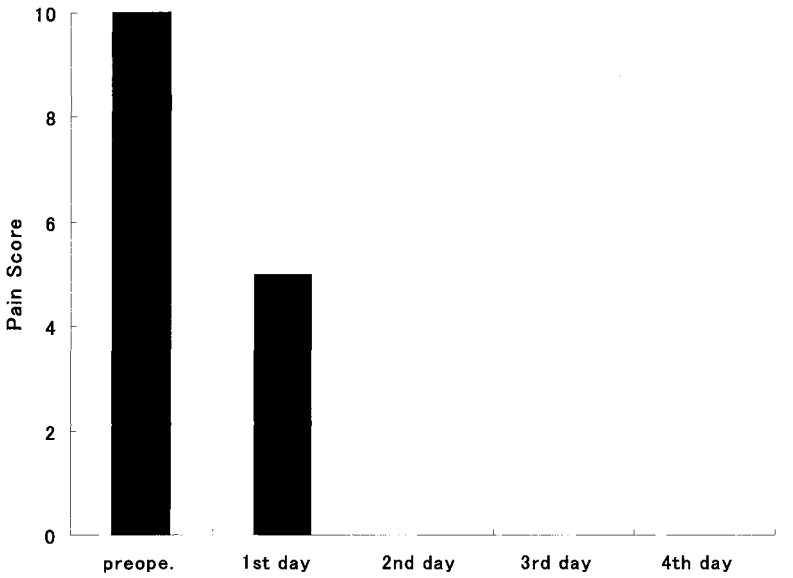

Fig. 1. shows the pain score. We showed a preoperative pain for ten. The pain score was five on the first day and zero on the second day.
The patient was only able to walk 180m by, but improved to 180m white walking without a stick on the second day and increased to 900m walking on the fourth day (Fig. 2).

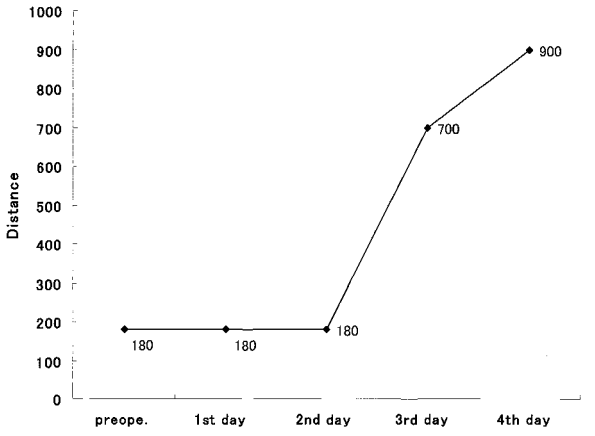

Fig. 2. shows the distance that he could walk. Only 180m were able to walk by preoperative crutch walking, but he came by 180m walk without a stick on the second day and came to cut it by 900m walk on the fourth day.

He did not have any incoveriences in everyday life and showed no resting pain eight weeks later. In addition, ankle-brachial index was improved to 0.43 one week later, 0.42 two weeks later, 0.42 three weeks later, 0.45 four weeks later and 0.48 eight weeks later from the preoperation 0.36 (Fig.3).

The symptoms were improved remarkably but, the arteriography suggested that collaterals did not clearly increase.

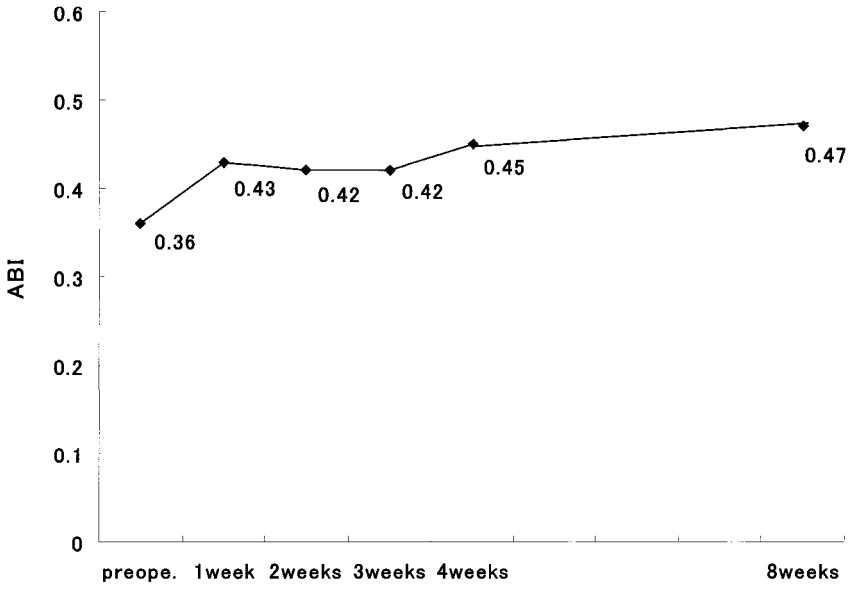

Fig. 3. Fig 3. shows ankle-brachial index. Ankle-brachial index was improved to 0.43 one week later, 0.42 two weeks later, 0.42 three weeks later, 0.45 four weeks later and 0.48 eight weeks later since preoperation 0.36.

Discussions

Surprisingly, symptoms began to improve from the next day and completely disappeared after 2nd. In addition, the distance that he could walk improved, too by eight weeks later these were no hindrance to everyday life. For visible sign evidence, ankle-brachial index was improved a little, and in arteriography, but we did not find a clear increase of collaterals. In addition, about 1 year 6 months later, he could walk freely without being

aggravated. Other researchers also have made similar reports. We are missing in detail now why such a thing occurs.

There are more questions, but therapeutic angiogenesis is an effective for limb salvage in patients with critical limb ischemia when there are no other therapies.

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Autologous Bone Marrow Implantation for Buerger's Disease

Masakatsu Ohtani[1], Toshihiro Soma[2], Akihiko Taguchi[2]

[1]Department of Cardiovascular Surgery, [2]Department of Medicine.

National Hospital Organization, Osaka Minami Medical Center, 2-1 Kido-higashi-cyou, Kawachinagano, Osaka 586-8521, Japan

Summary. Critical limb ischemia in Buerger's disease may lead to limb loss because reconstructive surgery is usually impossible. Recently, the autologous bone-marrow implantation into an ischemic limb has been demonstrated to enhance angiogenesis. We applied this new modality to two patients in Fontaine IV. The two patients were successfully treated without major amputations and we did not recognize any complications related to the bone-marrow implantation. Autologous bone-marrow implantation was effective for critical limb ischemia of Buerger's disease by promoting neoangiogenesis. However, the long-term efficacy of this treatment may be limited, probably because it does not control the vasculitis.

Key words. Buerger's disease, Autologous Bone-marrow implantation

Introduction

Buerger's disease is an aggressive form of inflammatory vasculitis affecting predominantly small and medium sized arteries and veins[1]. The obstructive deterioration of arteries may develop rest pain or ischemic ulcers, and the involved limbs are threatened with amputation in severe cases. Reconstructive surgery is usually impossible because of the diffuse lesion and extreme distal nature of the disease. Recently, the autologous bone-marrow implantation into the ischemic limb has been demonstrated to enhance angiogenesis and have promising therapeutic effects[2]. However, this modality is still a challenge in the treatment of severe limb ischemia. This report summarizes our experience in 2 patients with Buerger's disease.

Case Report

Patient 1

A 37-year-old man presented non-healing ulcers on the fingers of his right hand (Fig. 1) and a chronic abscess on his left thumb. The patient smoked until 7 years ago and had undergone finger amputation seven times, and sympathectomy. Any medication had not healed the ulcers or controlled the pain. Measurement of the skin perfusion pressure by laser Doppler (Getz Bros. Chicago, IL, U.S.A.) revealed poor perfusion to the fingers[3]. Arteriography revealed multiple palmar and digital occlusions with corkscrew-shaped vessels (Fig. 2). We made the diagnosis of Buerger's disease based on these arteriographic and clinical findings. Revascularization surgery was impossible because of diffuse involvement in the distal arteries. We concluded that autologous bone-marrow implantation was the only treatment of choice. The human research committee and a case committee at our hospital authorized our protocols for autologous bone marrow implantation with the informed consent of the patient.

Under the general anaesthesia, bone marrow cells (800ml) were obtained from the iliac crest and mononuclear cells were isolated using a Baxter CS3000 plus system according to the manufacturer's directions. By this method, 2×10^{10} mononuclear cells were obtained in a volume of 30 ml. Cell surface maker analysis using a fluorescence cell sorter revealed that 4.2% of these cells were CD34 positive. These mononuclear cells were injected into the forearm muscles (in 0.5-ml aliquots) as well as into

the palmar and interosseous muscles (in 0.2-ml aliquots) in a total of 50 sites on both hands.

The ulcers had been covered by thin skin and his pain had resolved by 2 weeks. Measurement of skin perfusion pressure by laser Doppler revealed a marked improvement in both hands. Three months after the implantation, the ulcers had been covered by normal skin and had completely healed. Angiography revealed new collateral vessels and dilatation of the pre-existing vessels (Fig. 3). We had not observed any complications related to the bone-marrow cell implantation. However, the patient developed the recurrence of thinning of skin and small ulcers on the finger-tips 3 years after the implantation. As the intensive medication and hand-care were not effective, the autologous bone-marrow cell implantation was performed again and, consequently, the ulcer had healed within 2 weeks.

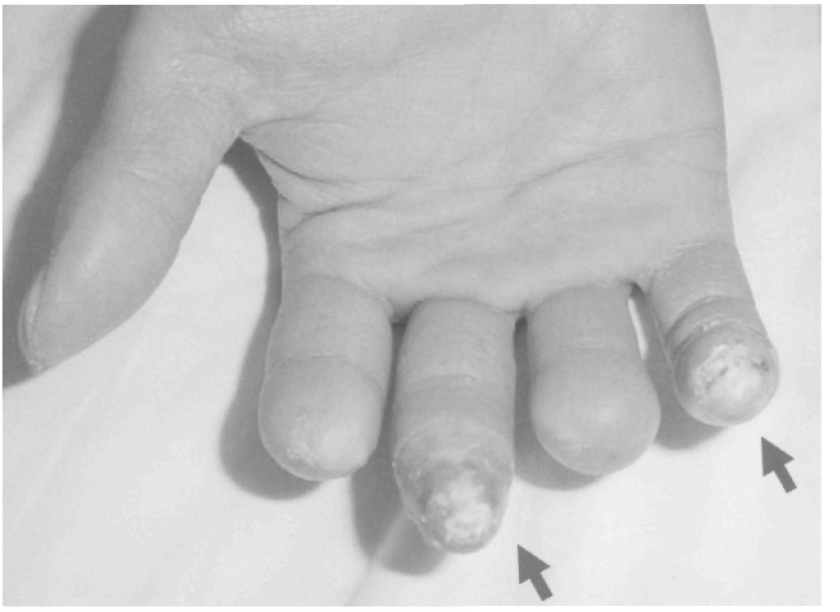

Fig. 1. Non-healing ulcers (arrows)

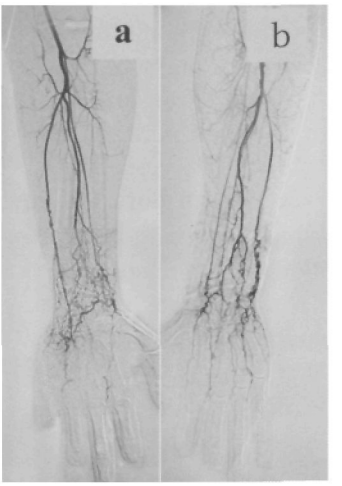

Fig. 2a, b. Arteriography of the right hand **a** and the left hand **b** before treatment

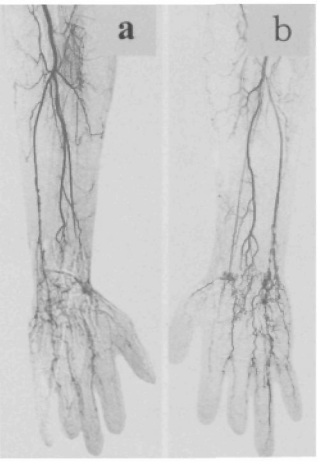

Fig. 3a, b. Arteriography of the right hand **a** and the left hand **b** performed 3 months after treatment

Patient 2

A 31-year-old man complained of a gangrenous lesion on the thumb of the left foot and a chronic abscess on 5th toe of the right foot (Fig. 4). He had been smoking for 10 years. Cessation of smoking and intensive foot care did not improve the foot lesions. Arteriography revealed that the leg arteries were abruptly occluded distal to the popliteal bifurcation with collateral vessels in both lower legs. Themography showed the decrease in the skin temperature of both feet, especially the toes. Based on the diagnosis of Buerger's disease, the autologous bone marrow implantation was performed in the same protocol and the gangrenous tissues were also removed to control infection. The obtained volume of bone-marrow cells was 480 ml and 5 x 10^9 mononuclear cells were observed in a condensed volume of 30ml. Subsequently, amputation of the necrotic left toe and primary skin closure were performed 2 weeks after the implantation. Foot lesions were completely healed by 1 month. We have not observed any complications related to the bone marrow implantation for 1 year.

Comment

Augmentation of neovascularization by autologous bone marrow implantation has been demonstrated in a model of limb ischemia[4]. Bone marrow mononuclear cells include cells of various lineages, such as endothelial progenitor cells (included in the CD34+ fraction), nonhematopoietic stromal cells, fibroblasts, osteoblasts, myogenic cells, and endothelial cells. Recently, it was reported that CD31 positive vessels are found in an increased frequency in human marrow-implanted legs, and that the CD34 negative fraction in bone marrow mononuclear cells synthesizes angiogenic factors including VEGF and bFGF. From these studies, we concluded that the supplement of endothelial progenitor cells and the multiple angiogenic factors released from CD34 negative cells promotes angiogenesis in ischemic organs.

We have demonstrated that autologous bone-marrow implantation is effective for critical limb ischemia of Buerger's disease by promoting neoangiogenesis. However, the long-term efficacy of this treatment may be limited as shown in the first case, probably because it does not control the vasculitis.

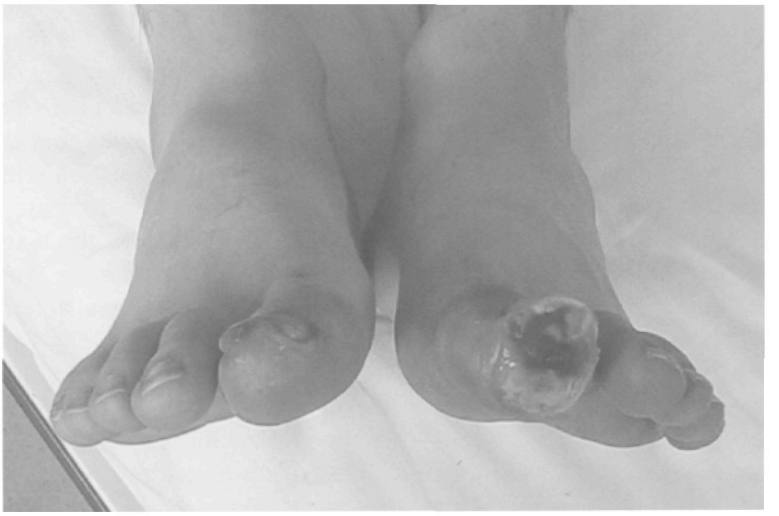

Fig. 4. A gangrenous lesion of the left thumb and an abscess on the right thumb

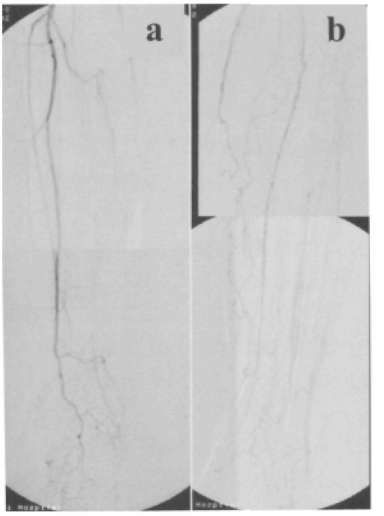

Fig. 5a, b. Arteriography of the right leg **a** and the left leg **b** before treatment

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Closing Remarks

Recent advances in molecular biology have been remarkably effective in elucidating the pathophysiology and mechanisms of various cardiovascular diseases. New techniques in genetic, cellular, and tissue engineering have had significant impact and sparked a revolution in therapy for severe diseases.

In the heart, cardiac myocytes have long been considered terminal differentiated cells without the potential to multiply and participate in tissue repair. This is in contrast to hepatocytes, which can regenerate when injured. However, developments in molecular cardiology and angiology have raised the possibility of neovascularization as well as the regeneration of myocardium.

Japanese scientists working in the field of cardiovascular disease have been at the forefront of research into the regeneration of impaired heart tissue using methods as diverse as angiogenesis, myogenesis, and tissue engineering. These studies, featuring both experimental and clinical assessment, are gaining momentum rapidly and drawing attention to translational research. As shown in this publication, angiogenic cytokines, cardiovascular stem cells, and tissue engineering tools are substantially contributing to this revolution. Another highlight has been the development in many leading centers and hospitals in Japan of therapeutic angiogenesis and vasculogenesis for the treatment of ischemic diseases of the myocardium and the limbs. We expect that the developments summarized in the book may have a substantial impact on the progress of regeneration medicine.

Finally, we express thanks for the following research grants: Cardiovascular Disease (13C-1 and 16C-6) and Health and Labor Sciences Research Grants (Saisei-003 and Cardiovascular Res-001). These grants have facilitated significant progress in the field of regeneration therapy in cardiovascular disease.

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Hikaru Matsuda, M.D., Ph.D.
Professor, Division of Cardiovascular Surgery, Department of Surgery
Osaka University Graduate School of Medicine

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