H. Mori, H. Matsuda (Eds.)

Cardiovascular Regeneration Therapies
Using Tissue Engineering Approaches
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.

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CHAPTER 1

VASCULAR PRECURSOR CELLS AND THEIR POTENTIATION
EPC and Their Potentiation by Adenovirus Gene Delivery

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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 \textit{in situ} ECs and BM-derived or \textit{in situ} EPCs, respectively.

**The potential of EPC transplantation**

The regenerative potential of stem/progenitor cells is currently under intense investigation. \textit{In vitro}, stem/progenitor cells possess the capability of self-renewal and differentiation into organ-specific cell types. When placed \textit{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 $5.0 \times 10^6$ cells per 100ml of blood on day 7. Our animal studies suggest that heterologous transplantation requires systemic
injection of $0.5 \sim 2.0 \times 10^4$ 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).
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 β-galactosidase expression (Fig. 3).

Human EPCs were transfected with 1,000 MOI of Ad/VEGF or Ad/βgal 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.

![Graph](image)

**Fig. 3.** Profile of transfection efficiencies for Ad/β-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/βgal for 3 hrs in 1% serum media (see note 2). *p<0.01, non-Tf vs Tf/β-EPCs.

**Proliferative activity assay**

At 24 hours post-gene transfer, reseed EPCs transduced with Ad/VEGF (Tf/V-EPCs), Ad/β-gal(Tf/β-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.](image-url)

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/β-EPCs were stained with fluorescent carbocyanine Dil. Dil-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, Dil, for cell tracking. Incubate Dil-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 Dil-labeled EPCs were not significantly different between Tf/V-EPCs and Tf/β-EPC transplanted animals (2.7±0.2 vs. 2.2±0.3, p=ns) (Fig. 5b). In activated HUVECs, however, adhesion of Dil-labeled Tf/V-EPCs exceeded that of Tf/β-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 Dil-labeled Tf/V-EPCs exceeded that of Tf/β-EPCs (7.0±0.5 vs. 3.5±0.5, p<0.01) (Fig. 5b). In activated HUVECs, incorporation of Dil-labeled Tf/V-EPCs also exceeded that of Tf/β-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/β-EPCs in HUVEC monolayer at 24 hrs after transduction with Ad/VEGF or Ad/βgal, respectively. Left panel is the Tf/β-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/β-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. Dil-acetylated-LDL incorporation: Purified low density lipoprotein was acetylated and then labeled with the fluorescent probe, Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) for labeling endothelial cells and macrophages. Aseptically dilute the Dil-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 Dil-Ac-LDL, wash twice by DPBS, and count cells for each experiment.
References


Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H Inai, Y, Silver M, and Isner, JM (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells EMBO J. 18, 3964-3972


Kawamoto A, Gwon HC, Iwaguro H et al. (2001) Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 103:634-637


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

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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