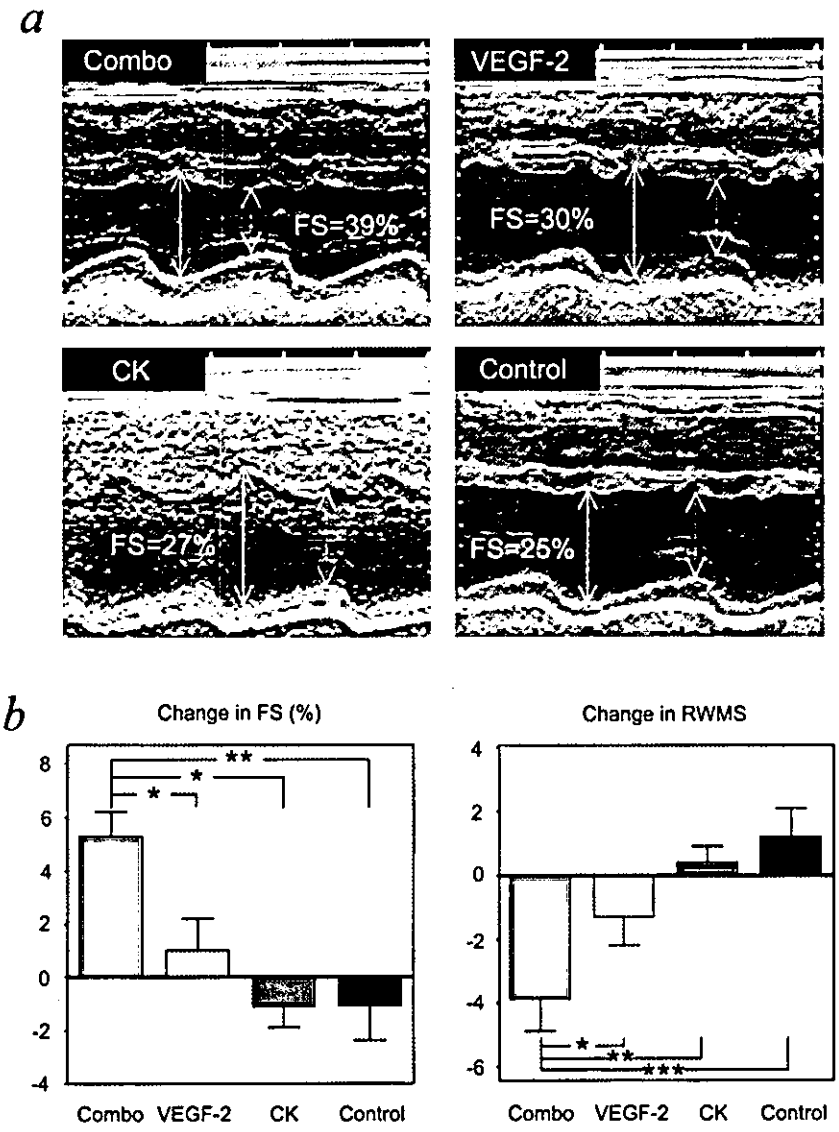

Figure 4. a, Representative findings of M-mode echocardiography 4 weeks after gene transfer in porcine chronic myocardial ischemia. b, Changes in FS and regional wall motion score during 4 weeks after the gene therapy in swine in Combo, VEGF-2, CK, and control groups. RWMS indicate regional wall motion score. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

method of coronary flow measurement, but no change in symptoms or physiologically induced ischemia was reported, and these initial findings have not yet been repeated or extended in further studies.

In our swine study, monotherapy with CKs failed to attenuate chronic myocardial ischemia, to increase vascularity in the ischemic myocardium, or to improve LV function. In contrast, as documented previously, monotherapy with VEGF-2 gene transfer significantly improved chronic myocardial ischemia as documented by NOGA mapping, improved capillary density, and resulted in a favorable trend in LV functional improvement. The results of VEGF-2 gene transfer were consistent with previous reports in preclinical and pilot clinical trials.[3,4] Most notably, however, the combination of VEGF-2 gene transfer plus CKs was superior to the monotherapies in terms of neovascularization and LV functional recovery. These favorable outcomes support the notion that progenitor cells play a key role in VEGF-induced local tissue revascularization and that the combination of BM mobilization and gene therapy can achieve superior therapeutic neovascularization.

To provide additional evidence for the enhanced contribution of BM-derived cells after combination therapy, BMT from eGFP mice into wild-type mice was performed. Histological examination revealed greater numbers of BM-derived cells in the myocardial neovasculature in mice receiving combination therapy than in those receiving monotherapy. These findings are consistent with prior observations. VEGF-1 has previously been shown to enhance mobilization of BM-derived EPCs into the circulation and to increase the incorporation of EPCs into sites of neovascularization.[23] Intramyocardial VEGF-2 gene transfer also increased circulating EPC counts.[11] These and other prior studies suggested that progenitor cells were an integral component of ischemia- and CK-induced neovascularization of ischemic tissues. The present findings provide additional evidence to support a fundamental role for EPCs in ischemia-induced neovascularization and suggest that therapies directed at enhancing the supply of these cells may be helpful in addressing the failure of native or CK-induced collateral vessel formation. Moreover, the failure of CK-induced EPC mobilization as a monotherapy in the setting of chronic ischemia indicates that

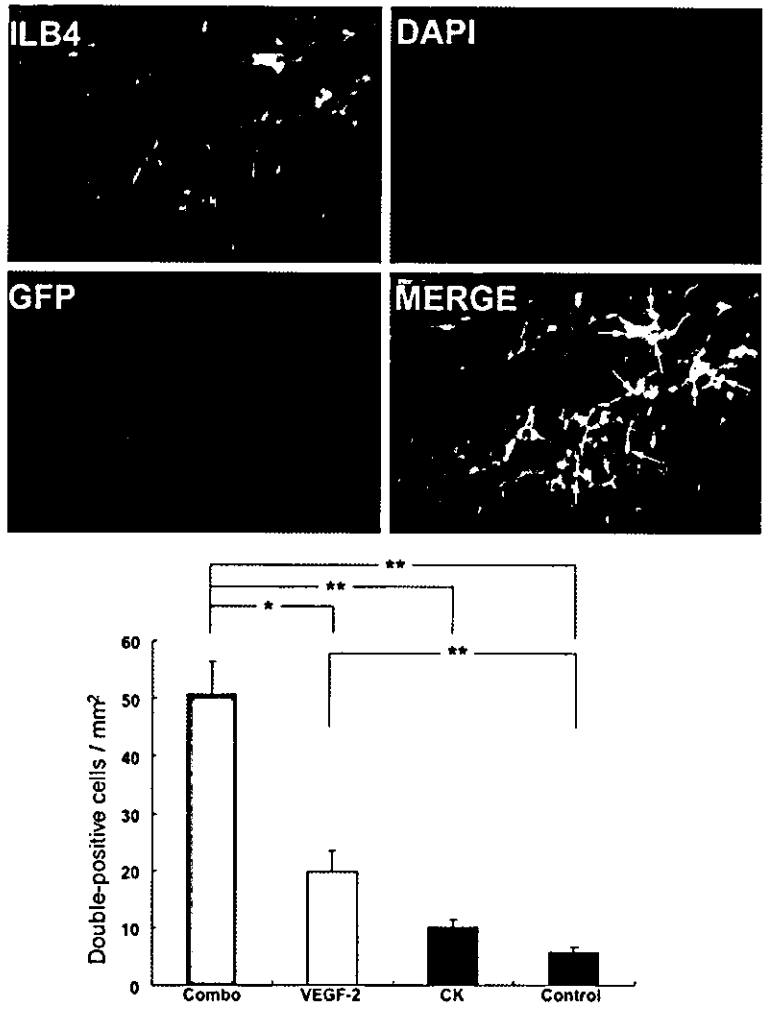

Figure 5. a, Representative double immunofluorescent microscopic findings from BM transplanted mice 1 week after MI. This example shows myocardial tissue from mouse in Combo group stained with antibody to isolectin B4 (ILB4) to identify endothelial cells, DAPI to identify nuclei, and antibody to GFP to identify BM-derived cells. In merge image, arrows identify yellow, resulting from overlap of red (ILB4 positive, endothelial cells) and green (GFP, BM-derived cells), thus identifying BM-derived cells that have relocated to myocardium and now express endothelial marker. b, Quantification of double-positive cells, revealing that number of double-positive cells in Combo group was greater than all other treatment groups and that VEGF group also had greater number of double-positive cells than controls. $^{*}P<0.01$; $^{**}P<0.001$.

a local signal, in this case provided by VEGF gene therapy, is required for recruitment and incorporation of circulating progenitors. The precise mechanisms governing the recruitment, retention, and incorporation of BM-derived progenitors into the myocardial tissue and the relative roles of each in the enhanced functional recovery documented remain to be elucidated.

Together, these findings underscore the likelihood that progenitor cells must be considered not only as a part of the native mechanisms that govern vascular biology but also as entities whose failure may play a fundamental role in the advent of vascular pathology.[38] Modulation of progenitor cell function therefore represents a reasonable therapeutic target for treatment of ischemic diseases.

Acknowledgments

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Magnetic resonance mapping of transplanted endothelial progenitor cells for therapeutic neovascularization in ischemic heart disease☆

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Abstract

Objective: Intramyocardial transplantation of endothelial progenitor cells (EPCs) has been previously correlated with significant augmentation of vascularity and improvement of left ventricular function following myocardial ischemia. However, precise intramyocardial localization of the transplanted cells and the extent of in situ cell migration are unknown. We present a novel technique using magnetic resonance imaging (MRI) to localize transplanted EPCs in ischemic hearts. **Methods**: CD34-positive cells were isolated from human peripheral blood by magnetic bead selection: CD34-positive cells adhere to CD34-negative antibody coated magnetic beads, while CD34-negative cells do not. All cells were labeled with fluorescent DiI-dye for histological localization. CD34-positive cells or CD34-negative cells (105, 1 × 106 and 2 × 106 cells) were transplanted into non-ischemic ($n = 6$) or ischemic myocardium ($n = 2$) of Sprague–Dawley rats. Rats were sacrificed 24 h after cell transplantation. The resected hearts were imaged ex vivo using 3 and 8.5 T magnets. Morphological correlation between the MRI findings and fluorescent microscopy for identification of retained CD34-positive cells was evaluated. **Results**: CD34-positive cells were identified as areas of low signal intensity on T2*-weighted images within the myocardium. These areas increased in size with the gradual increase in the echo time due to susceptibility effect. The extent of the low signal intensity at a given echo time was proportional to cell dosage. No areas of low signal were identified in the CD34-negative cell transplanted hearts. Histological localization of DiI-labeled CD34-positive cells documented a direct anatomic correlation with the localization of transplanted cells on the MRI images. **Conclusions**: Magnetically labeled EPCs transplanted for therapeutic neovascularization in myocardial ischemia can be visualized with ex vivo MRI at high-field strengths.

Keywords: Angiogenesis; Ischemic heart disease; Stem cell tracking; Magnetic resonance imaging

1. Introduction

Whereas most tissues in adult organs are composed of differentiated cells, stem or progenitor cells are maintained in a quiescent status locally or in the systemic circulation and are activated during physiological and pathological tissue regeneration. Haematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs) are derived from a common precursor, the hemangioblast (Fig. 1). HSCs and EPCs share certain antigenic determinants, including Flk-1, Tie 2, cKit, Sca-1, CD133 and CD34. These markers are subsequently lost as HSCs differentiate [1]. EPCs were first isolated, by Asahara et al., as CD34-positive mononuclear cells (MNCs) from adult peripheral blood by means of magnetic beads coated with antibody to CD34, as EPCs-enriched fraction [2].

Circulating EPCs have been shown to home to sites of neovascularization where they differentiate into endothelial cells (ECs) in situ, consistent with 'vasculogenesis' [3–5]. Intravenous transplantation of cultured human EPCs applied

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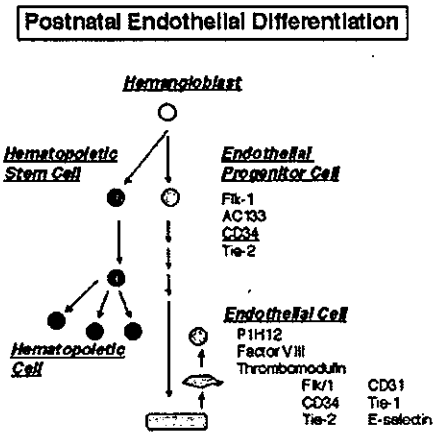

Fig. 1. Differentiation profile of endothelial lineage cells. Embryonic endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs) share among others CD34, lost by the second as they differentiate.

in a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs can incorporate into rat myocardial neovascularization, preserving left ventricular (LV) function, and inhibiting myocardial fibrosis [6]. Recently, Kocher et al. [7] attempted intravenous infusion of freshly isolated human CD34-positive MNCs (EPC-enriched fraction) into nude rats with myocardial ischemia. These experimental findings using immunodeficient animals suggest that both cultured and freshly isolated human EPCs have therapeutic potential in peripheral and coronary artery diseases [1]. In the above-mentioned studies, EPCs were always isolated by means of magnetic beads coated with antibody to the CD34 antigen [8]. The magnetic beads were not detached from the selected cells before transplantation. Previous data from our lab documents preserved viability and functionality of transplanted CD34-positive cells isolated by means of magnetic beads.

The development of progenitor and stem cell therapy in humans would be greatly enhanced by a technique to monitor their fate non-invasively thereby permitting serial assessment of the cellular biodistribution and migratory capacity [9]. To monitor the fate of transplanted cells, including their migration in vivo, cells are currently labeled ex vivo using a vital dye (e.g. a fluorochrome), a thymidine analog (e.g. BrdU), or a transfected gene (e.g. LacZ or green fluorescent protein, GFP), for later visualization using (immuno)histochemical procedures following tissue removal at a single time point. Considering the different delivery options for implementing myocardial cell transplantation (epicardial via intraoperative intramyocardial injection; endocardial, via catheter-based intramyocardial injection; intracoronary; retroperfusion, via cannulation of the coronary sinus; or intrapericardial, after transthoracic access to the pericardium) [10], a technique that could monitor the engraftment and localization of the transplanted cells is crucial to assess the safety and success of these procedures.

Most MR scanners used in clinical practice have magnetic fields equivalent to 1.5 T. Recent advances in the development of MR systems have allowed for scanners that operate at high magnetic fields. High-field MR scanners, including whole body clinical 3 T magnets for human use, provide excellent signal that allow for near microscopic resolution. The magnetic tagging of cells that facilitates separation also creates an opportunity to visualize these cells with magnetic resonance imaging (MRI).

MR signal results from the behaviour of protons in a magnetic environment. One behaviour is referred as the T2 of a tissue, an innate feature based upon its constituent molecules. The T2 characteristics of tissue are related to local interactions amongst protons. These so called 'spin–spin' interactions cause dephasing of the protons, which leads to the decay of MR signal over time. Disturbances in the local magnetic field affect the spin–spin interactions so that dephasing occurs more rapidly and therefore, the MR signal decay is also faster. This faster decay, or T2* decay, yields a lower signal intensity on the image. Thus, local magnetic field distortions caused by the tagged cells can be captured with MRI as a dark signal intensity particularly when T2* weighted sequences are used. An increase in the time between the excitation of the protons and the collection of the MR signal, or echo time (TE), allows for more dephasing of the protons. Hence, the longer the TE the greater decay in MR signal. By selecting gradually increasing echo times, the T2* effect resulting from the magnetic beads can be appreciated as an increase in the area of dark signal intensity or 'blooming effect' within the MR images. Gradient echo sequences (GRE) are a class of MRI strategies that are particularly sensitive to T2* effects. Finally, higher field strengths are more sensitive to T2* effects as compared with lower field strengths.

Conventional magnetic cell labeling techniques rely on surface attachment of magnetic beads ranging in size from several hundred nanometers to micrometers [11]. There are several prior reports describing magnetic resonance tracking of progenitor cells, in neural tissue, tumor or inflammatory tissue [9,11–14].

It has been established that bone marrow-derived EPCs present in the systemic circulation home to and incorporate into sites of neovascularization, and may be useful in therapeutic strategies of 'supply-side angiogenesis', for example, after myocardial ischaemia (MI) [2,10,15–20]. A technique that could monitor the engraftment and migration of intramyocardial injected EPCs, serially and non-invasive, could guide further advances for clinical application. We hypothesized that magnetic cell labeling would allow for intramyocardial visualization and localization of EPCs on high-field MRI.

The data presented in this study represent just a preliminary proof of principle where we try to define whether or not intramyocardial EPCs visualization and localization is possible with MRI.

2. Materials and methods

2.1. Animal models

All procedures were performed in accordance with St. Elizabeth's Institutional Animal Care Committee. We utilized male Sprague–Dawley rats (200–250 g weight) as our animal model.

2.2. Fresh isolation and intramyocardial transplantation of human EPCs

Human total peripheral blood MNCs were isolated from healthy volunteers by density-gradient centrifugation. CD34-positive mononuclear blood cells were isolated from total MNCs by means of colloidal super-paramagnetic beads (MACS-Microbeads) conjugated to monoclonal mouse anti-human CD34 antibody (isotype: mouse IgG1, clone: QBEND/10) (Miltenyi Biotec) as EPC-enriched fraction. Mononuclear cell isolation by density-gradient centrifugation is necessary for the initial step, because mononuclear cells in the systemic circulation contain a fraction capable of differentiation to endothelial lineage cells. After mononuclear cell isolation, total mononuclear cells are incubated with anti-CD34 antibodies coated with magnetic microbeads for fresh isolation. A magnetic column is used to collect only the cells binding to the antibodies with microbeads. After the isolation, CD34-negative MNCs were also collected. Both populations of CD34-positive and CD34-negative MNCs were labeled with fluorescent DiI-dye. Cells were counted using a haemocytometer and resuspended in 100 μl PBS. The magnetic beads were never detached from the isolated CD34-positive cells before transplantation. Non-ischemic (A) and ischemic (B) rat hearts were treated. (A) Non-ischemic: Sprague–Dawley rats ($n = 4$) were anaesthetized with ketamine i.p. (0.6 ml/100 g) and intubated. Left parasternal longitudinal thoracotomy was performed. After pericardectomy, DiI-labeled CD34-positive MNCs in 100 μl of PBS were injected intramyocardially in the anterior and/or lateral wall of the LV using a 27G needle. These four rats were treated with intramyocardial injection of 10^5, 2×10^5, 1×10^6 or 2×10^6 CD34-positive cells, respectively. Two additional rats received 1×10^6 or 2×10^6 DiI-labeled CD34-negative MNCs in 100 μl of PBS as negative controls. (B) Ischemic: Two rats underwent ligation of the left anterior descending coronary artery (LAD). Ten minutes after the operation these rats were injected with 10^5 DiI-labeled CD34-positive MNCs in 100 μl of PBS. Cells were injected in two sites within the ischemic vascular territory of the LAD using a 27G needle. The ischemic zone was macroscopically identified by the pale color of the anterior and lateral walls after LAD ligation.

All rats were sacrificed 24 h after intramyocardial transplantation of CD34-positive EPCs or CD34-negative MNCs. From our experience, it takes about 24 h for the rat heart to absorb the injected saline and that is why 24 h was chosen as time point for sacrifice. The hearts were resected and fixed with 4% paraformaldehyde.

2.3. MR imaging and histopathological correlation

To obtain a completely dark background we embedded the specimens in a perfluoropolyether (Fomblin, Fluortek AB, Sweden) devoid of proton signals. This polymer was found to be inert, effectively sealing the specimens from dehydration, with no observed effects on tissue morphology. Three-dimensional spin echo MR images were obtained by using a 9 cm bore 8.5 T Bruker magnet (Bruker Biospin, Billerica, MA).

Specimens were imaged with a 20 mm birdcage coil. Scan parameters were: TR/TE = 1700/25 ms, $256 \times 128 \times 128$ matrix with an FOV = $30 \times 15 \times 15$ mm, affording a 117 μ^3 resolution. Additionally, the hearts were scanned on a clinical 3 T magnet (General Electric Medical Systems, Milwaukee) using a 3-inch surface coil. Two-dimensional gradient echo MR images were obtained with the following parameters: TE = 10 and 20 ms, TR = 325, flip angle = 30°, slice thickness of 1 mm, matrix 512×256, FOV = 6 mm, NEX = 1. The GRE sequence was performed with two echoes to facilitate an evaluation of the susceptibility effect of the magnetic beads, the longer echo time having greater sensitivity to this effect.

Images were obtained in the long and short axes of the resected hearts. The area of T2/T2* effect (marked hypointensity) on each short axis slice-image was measured for each heart, dividing each heart in 1 mm thick slice-images; this analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The marked hypointensity is presented as percentage of the left ventricular volume, excluding the intra-ventricular cavity, after measuring all short axis slices. Imaging analyses were always done by a blind observer (MR technician). Correlation analysis by Pearson was performed. After MR imaging, the (fixed) heart specimens were embedded in OCT compound (Miles Scientific) and snap-frozen in liquid nitrogen and stored at −80 °C until it was analyzed in cryosections under fluorescence microscopy.

3. Results

Super-paramagnetic particles induce a T2/T2* effect that is detected by MR as regions with low signal intensity and appearing black relative to adjacent myocardium. Regions with CD34-positive cells were identified as intramyocardial areas of low signal intensity on T2*-weighted images at 8.5

and 3 T. Areas of susceptibility effect were localized within the anterior and lateral walls of the LV.

Susceptibility effect related to the presence of magnetic beads was confirmed by imaging the hearts with different echo times on the 3 T magnet. Intramyocardial areas of low signal intensity demonstrated increased magnetic susceptibility (blooming effect) with longer echo time. An increase in the echo time from 10 to 20 ms, resulted in a doubling of the area of low signal intensity, independently of the cell dosage injected. Computational planimetric analysis (NIH imaging software) demonstrated a proportional increase of the intramyocardial areas of low signal intensity on the MR images with the increase in the number of EPCs transplanted (cell dosage). Areas of low signal intensity related to transplanted EPCs increased proportionally to the cell dosage. MR images of each heart acquired with similar echo time demonstrated a linear relation between the number of cells injected and the area of low signal intensity ($P < 0.0001$).

For each heart, transverse cryosections corresponding to the injection sites were analysed under a fluorescence microscope. MR images were visually correlated with the results from histopathological analysis by fluorescence microscopy. There was an excellent correlation between the location of the areas of low signal intensity on MR images and the identification of labeled cells in the injection sites by fluorescent microscopy (Fig. 2).

There was no difference in depiction of the labeled cells between ischemic and non-ischemic myocardium, since it was not possible to enhance the MR signal of ischemic myocardium within 24 h postoperative ex vivo (data not shown).

Areas engrafted with fluorescent DiI CD34-positive cells matched closely with the areas of low signal intensity seen on the MR images. The areas of low signal intensity on the MR images were slightly larger than the areas with CD34-positive cells on fluorescence microscopy. This was likely caused by the blooming effect secondary to an extended-range susceptibility effect on the magnetic particles (Fig. 3).

Two hearts transplanted with CD34-negative cells demonstrated no areas of low signal intensity within the myocardium. Fluorescence microscopy confirmed the presence of the fluorescence DiI CD34-negative cells in areas of myocardium that showed no susceptibility effect on MR (Fig. 4). No areas of susceptibility effect were noted at the sites of the intramyocardial injection. The lack of susceptibility effect in these two hearts confirmed that the areas of low signal intensity were related to the magnetic beads attached to CD34-positive cells.

4. Discussion

Several clinical trials are being conducted that utilize exogenous stem or progenitor cells transplanted into damaged myocardium to augment myocardial performance and/or neovascularization after infarction or heart failure. Recently, Stamm et al. (EACTS 2002) reported results from ongoing clinical studies for intramyocardial transplantation of bone marrow progenitor cells isolated using antibodies coated with magnetic beads. These studies point to a need

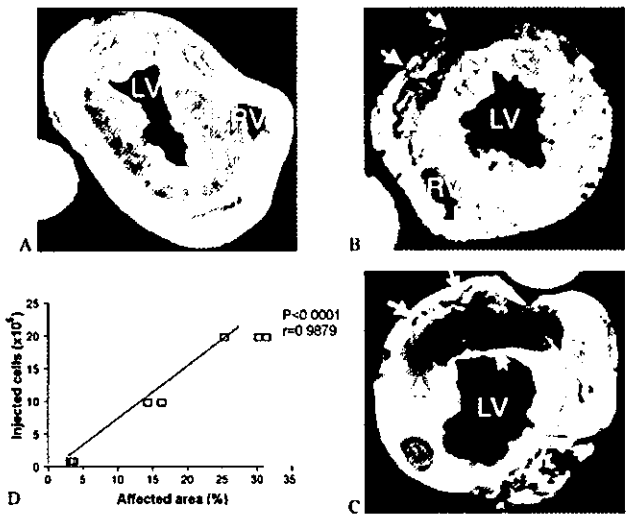

Fig. 2. T2-weighted images on 8.5 T Bruker magnet; representative transversal heart sections only. CD34-positive cells were identified as anterolateral intramyocardial areas of low signal intensity. (A) (Arrows) 105 CD34-positive cells. (B) (Arrows) 1 × 106 CD34-positive cells. (C) (Arrows) 2 × 106 CD34-positive cells. (D) The area of low signal intensity is proportional to the cell dosage. (LV) Left ventricle cavity. (RV) Right ventricle cavity. (Stars) Some areas of low signal intensity, far away from the injection sites, are usually seen in ex vivo preparations and are well known as haemosiderine precipitations after blood coagulation.

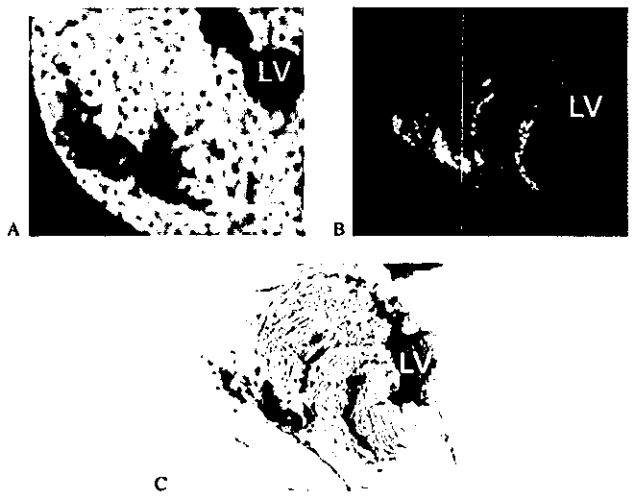

Fig. 3. T2-weighted image on 8.5 T Bruker magnet and fluorescent microscopy analysis. Excellent agreement between the areas of MR low signal intensity (black) (A) and histopathological fluorescence DiI staining for CD34-positive transplanted cells (red) (B) and (negative; black) (C). The cell location area in the MR image shows a blooming effect compared to the corresponding histological section caused by an extended-range susceptibility effect of the magnetic particles. (LV) Left ventricular cavity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

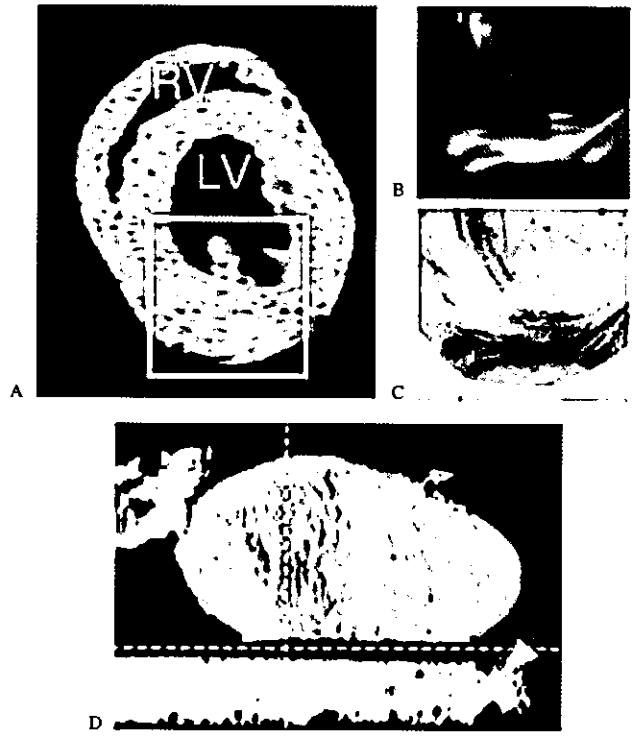

Fig. 4. No low signal intensity areas in hearts transplanted with 2 × 106 CD34-negative cells. (A) Image with 3 T magnet. (B and C) Histopathological fluorescence DiI staining for 2 × 106 CD34-negative cells. (D) Image of sagittal section, 3D T2-weighted, on 8.5 T magnet. (Arrow) Tube with PBS solution including CD34-positive cells as positive control.

for techniques that permit an evaluation of the efficiency of transplantation and an evaluation of the potential post-operative migration of the transplanted cells. Such a technique would provide an important tool for assessing patients following treatment. The recent occurrence of postoperative arrhythmic complications in patients post-cell-transplant underscores the requirement for careful evaluation of cell location and fate.

The technique we investigate in this study, could be one such method for tracking the location of these cells in treated myocardium. It has the advantage of using magnetic beads both to isolate EPCs from the mononuclear cell fraction of peripheral blood (a standard technique) and to serve as the magnetic label for MRI.

Further studies will be needed to determine whether cell surface magnetic labeling, previously used only for cell selection, is stable enough to allow mid- and long-term in vivo imaging studies [11]. Magnetically selected cells can be cultured with attached beads and the cells grow and adhere normally. In previous studies from our lab and others [1,5,6], EPCs were also always isolated by means of magnetic beads coated with antibody to the CD34 antigen [8].

In these studies the magnetic beads were never de-attached from the selected cells before transplantation. Therefore, based on our previous data we have established that there is a preserved cell viability and functionality of CD34-positive cells isolated by means of magnetic beads.

However, some authors [21,22] claim that after approximately three passages in culture the beads are diluted out. Others suggest that after in vivo administration there is a more rapid reticuloendothelial recognition and clearance of cells thus surface-labeled [11]. Conversely, there is some evidence that the magnetic coated antibodies might be internalised to the cytoplasm and remain intracellular.

There are several works reporting the possibility of intracellular labeling with superparamagnetic ironoxide nanoparticles or magnetodendrimers [9,11,23] using fluid-phase or receptor-mediated endocytosis. These methods allow in vivo cell tracking and the magnetic beads are stably retained intracellularly over time (up to 6 months has been reported). Unfortunately, labeling efficiency is generally low and cells need to be exposed to culture media for long incubation periods. This methodology would not be compatible with the transplantation of autologous freshly isolated cells, a consideration that avoids any contact with potential immunogenic agents.

In the present study we limited our resources and goals to realize whether it is possible or not to identify the magnetic labeled cells by means of MRI just to establish a proof of principle. The selected cell dosages were established aiming previous functional studies in a range from low to relatively high dosage. We first used a small bore 8.5 T magnet, which is currently used for experimental purposes only, still far from clinical use, but allowed us to draw on the full potential of MRI. In a next step we were able to corroborate these initial results with the 3 T magnets currently in clinical use. This is especially encouraging taking into account that we were measuring small rat hearts with a machine that is used for human adults.

Limitations of this study are the lack of experiments with big animals and, of course, the lack of measurements in living animals. These are surely the next steps to follow, being in vivo MRI of the heart a challenging issue because of the need for cardiac and respiratory gating in order to trigger the physiologic heart beats and lung movements.

In vivo imaging of animals would allow the investigator to follow the migration of transplanted cells in the heart and would provide considerable data related to the safety of cell transplantation procedures. Knowledge of the migration pattern of haematopoietic progenitor cells in vivo after homing to ischemic areas would be of considerable importance to understand their physiological impact.

In the present study, we demonstrate that magnetically labeled EPCs intramyocardially transplanted for therapeutic neovascularization can be accurately visualized with ex vivo MRI at high-field strengths. This can be achieved with an experimental, small bore 8.5 T magnet, as well as with the 3 T magnets currently in clinical use. We observed an excellent agreement between the areas demonstrating MR susceptibility effect and histopathological fluorescence DiI staining for CD34-positive transplanted cells. This study introduces MR tracking as a technique to monitor,

non-invasively, the localization of magnetically labeled cells after intramyocardial transplantation.

Hence, this ex vivo experiment constitutes a proof of principle of the utility of cellular magnetic tags for tracking cellular transplants currently being evaluated for use in cardiovascular medicine.

Acknowledgements

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Appendix A. Conference discussion

***Dr Hoerstrup** (Zurich, Switzerland)*: My question is concerning the clinical applicability of this concept. You mentioned that you have sacrificed the animals after 24 hours, and you said that for future clinical application you might use intracellular labeling with magnetic particles. How long will it be possible of tracing down these cells in an in vivo model?

Dr Weber: Well, there are several studies, mainly at Johns Hopkins, already tracing these cells in with intracellular labeling; this group even started clinical studies recently. The imaging of these cells appears to be much better than the surface tracing, and the in vivo tracking seems to be possible for long term.

Dr Hoerstrup: What would be a time frame seen from these other studies?

Dr Weber: The last studies point out that the intracellular magnetic labeling is still available 6 months after implantation. However, these are neural stem cells which don't divide as fast as EPCs, so we don't know, but in our case the tracking follow up might be a little shorter.

***Dr C. Stamm** (Rostock, Germany)*: Am I right to say that you do see the iron particle, not the cell? And if I am right, can you think of a way to image a cell to say something about the viability of the cell instead of simply saying that the iron particle that you injected is there?

Dr Weber: In this case, this experiment constitutes proof of principle of the utility of cellular magnetic tags for tracking cells in their localization

after intramyocardial injection. The next step will be to evaluate the follow-up and see, in the case of migration, if the correlation between cells and magnetic beads persists. However, in vitro experiments suggested that the magnetic-coated antibodies might be internalized to the cytoplasm and remain intracellular, mimicking the ex vivo intracellular labeling techniques. These methods, as I said before, demonstrated that the magnetic beads are stably retained intracellularly over time (up to 6 months, as reported by Johns Hopkins).

Dr Stamm: And once you have injected the cells, what you do see is the iron particle in the myocardium, right, you can't say this is a living cell inside the myocardium that you can image?

Dr Weber: In all our previous functional studies (see papers by Drs Isner and Asahara) the magnetic beads were never detached from the selected cells before transplantation. Therefore, based on our previous data we have established that there is preserved in vivo cell viability and functionality of CD34-positive cells isolated by means of magnetic beads.

Invited Review

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Endothelial progenitor cells for postnatal vasculogenesis

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Asahara, Takayuki, and Atsuhiko Kawamoto. Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* 287: C572–C579, 2004; 10.1152/ajpcell.00330.2003.—In the past decade, researchers have defined committed stem or progenitor cells from various tissues, including bone marrow, peripheral blood, brain, liver, and reproductive organs, in both adult animals and humans. Whereas most cells in adult organs are composed of differentiated cells, which express a variety of specific phenotypic genes adapted to each organ's environment, quiescent stem or progenitor cells are maintained locally or in the systemic circulation and are activated by environmental stimuli for physiological and pathological tissue regeneration. Recently, endothelial progenitor cells (EPCs) were isolated from peripheral blood CD34, Flk-1, or AC133 antigen-positive cells, which are considered to include a hematopoietic stem cell population, and were shown to be incorporated into foci of neovascularization. This finding, that circulating EPCs may home to sites of neovascularization and differentiate into endothelial cells in situ, is consistent with "vasculogenesis," a critical paradigm for embryonic neovascularization, and suggests that vasculogenesis and angiogenesis may constitute complementary mechanisms for postnatal neovascularization. Previous reports demonstrating therapeutic potential of EPC transplantation in animal models of hindlimb and myocardial ischemia opened the way to the clinical application of cell therapy: the replacement of diseased or degenerating cell populations, tissues, and organs. In this review, we summarize biological features of EPCs and speculate on the utility of EPCs for vascular and general medicine.

cell transplantation; ischemia; neovascularization; stem cell

IDENTIFICATION OF THE ENDOTHELIAL PROGENITOR CELL (EPC) was an introductory emergence of stem cell biology in the field of vascular biology. Evidence accumulated since our first publication on an isolation of EPCs has elucidated the significance of a postnatal vasculogenesis mechanism for neovascularization and vascular remodeling. This unique cell fraction among peripheral blood mononuclear cells (MNCs) derived from bone marrow has a similar profile to that of an embryonic angioblast, which proliferates and/or migrates in response to angiogenic growth factors and differentiates into mature endothelial cells (ECs) in situ for blood vessel formation. Considering the importance of blood vessel development on organogenesis, vasculogenesis by EPCs may be an essential cascade for tissue and organ regeneration following pathological damage in various critical diseases.

STEM CELL BIOLOGY FOR REGENERATION

Tissue regeneration for organ recovery in adults has two physiological mechanisms. One is the replacement of differentiated cells by newly generated populations derived from residual cycling stem cells. Hematopoietic cell regeneration is a typical example of this kind of mechanism. Whole hematopoietic lineage cells are derived from a few self-renewal stem cells by regulated differentiation under the influence of appropriate cytokines and/or growth factors. The second mechanism is the self-repair of differentiated functioning cells, preserving their proliferative activity. Hepatocytes, ECs, smooth muscle cells, keratinocytes, and fibroblasts are considered to possess this ability. After physiological stimulation or injury, factors secreted from surrounding tissues stimulate cell replication and replacement. However, regenerative activity of these fully differentiated cells is still limited because of finite proliferation by senescence and because of their inability to incorporate into remote target sites.

Whereas most cells in adult organs are composed of differentiated cells, which express a variety of specific phenotypic genes adapted to each organ's environment, quiescent stem or progenitor cells are maintained locally or in the systemic circulation and are activated by environmental stimuli for physiological and pathological tissue regeneration. In the past decade researchers have defined the stem or progenitor cells from various tissues, including bone marrow, peripheral blood, brain, liver, and reproductive organs, in both adult animals and humans (Fig. 1).

Among these stem/progenitor cells, the EPC has been identified recently and investigated to elucidate its biology for therapeutic applications. Because recent reports demonstrate that endothelial lineage cells play a critical role in the early stage of liver or pancreatic differentiation before formation of functioning blood vessels (38, 44), the significance of vascular

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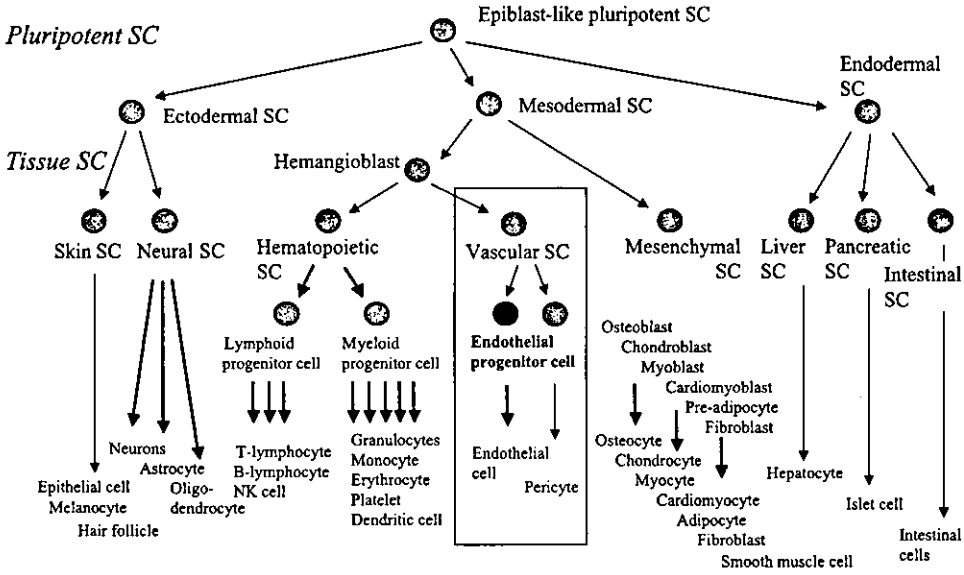

Fig. 1. Postnatal stem and progenitor cells. SC, stem cell.

development in organogenesis has become a crucial issue in regenerative medicine.

EMBRYONIC EPCs

Embryonic stem cell researchers in this decade have opened a novel door for vascular biology, as for any medical field, to elucidate the history of vascular development. Embryonic EPCs, or angioblasts, for blood vessel development arise from migrating mesodermal cells. EPCs have the capacity to proliferate, migrate, and differentiate into endothelial lineage cells but have not yet acquired characteristic mature endothelial markers. Available evidence suggests that hematopoietic stem cells (HSCs) and EPCs (46, 54) are derived from a common precursor (hemangioblast) (16, 27, 68). Growth and fusion of multiple blood islands in the yolk sac of the embryo ultimately give rise to the yolk sac capillary network (52); after the onset of blood circulation, this network differentiates into an arteriovenous vascular system (51). The integral relationship between the elements that circulate in the vascular system (blood cells) and the cells that are principally responsible for the vessels themselves (ECs) is implied by the composition of the embryonic blood islands. The cells destined to generate hematopoietic cells are situated in the center of the blood island and are termed HSCs. EPCs are located at the periphery of the blood islands.

The key molecular players determining the fate of the hemangioblast are not fully elucidated. However, several factors have been identified that may play a role in this early event. Studies in quail/chick chimeras showed that fibroblast growth factor-2 (FGF-2) mediates the induction of EPCs from the mesoderm (48). These embryonic EPCs express Flk-1, the type 2 receptor for vascular endothelial growth factor (VEGFR-2), and respond to a pleiotropic angiogenic factor, VEGF, for proliferation and migration. Deletion of the Flk-1 gene in mice results in embryonic lethality, lacking both hematopoietic and endothelial lineage development, supporting the critical importance of Flk-1 at that developmental stage, although the steps regulating differentiation into endothelial vs. hematopoietic cells had not yet been defined at the time of those studies. The Flk-1-expressing mesodermal cell has also been defined as an embryonic common vascular progenitor that differentiates into endothelial and smooth muscle cells (69). The vascular progenitors differentiated to ECs in response to VEGF, whereas they developed into smooth muscle cells in response to platelet-derived growth factor (PDGF)-BB. It remains to be determined whether embryonic EPCs or vascular progenitor cells persist with an equivalent capability during adult life and whether these cells contribute to postnatal vessel growth.

POSTNATAL EPCs

The identification of putative HSCs in peripheral blood and bone marrow and the demonstration of sustained hematopoietic reconstitution with these HSC transplants have constituted inferential evidence for HSCs in adult tissues (5, 35, 58). Recently, the related descendants, EPCs, have been isolated along with HSCs in hematopoietic organs. Flk-1 and CD34 antigens were used to detect putative EPCs from the mononuclear cell fraction of peripheral blood (2). This methodology was supported by former findings that embryonic HSCs and EPCs share certain antigenic determinants, including Flk-1, Tie-2, c-Kit, Sca-1, CD133, and CD34. These progenitor cells have consequently been considered to be derived from a common precursor, putatively termed a hemangioblast (16, 27, 68) (Fig. 2).

In vitro, these cells differentiated into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs were shown to incorporate into sites of active neovascularization. This finding was followed by diverse identifications of EPCs by several groups (20, 23, 39, 47, 59) using equivalent or different methodologies. EPCs were subsequently shown to express VE-cadherin, a junctional molecule, and AC133, an orphan receptor that is specifically expressed on EPCs but whose expression is lost once the EPCs differentiate into more mature ECs (47). Their high proliferation rate distinguishes circulating marrow-derived EPCs in the adult from mature ECs shed from the vessel wall (17). Thus far, a bipotential common vascular progenitor, giving rise to both endothelial and smooth muscle cells, has not been documented in the adult.

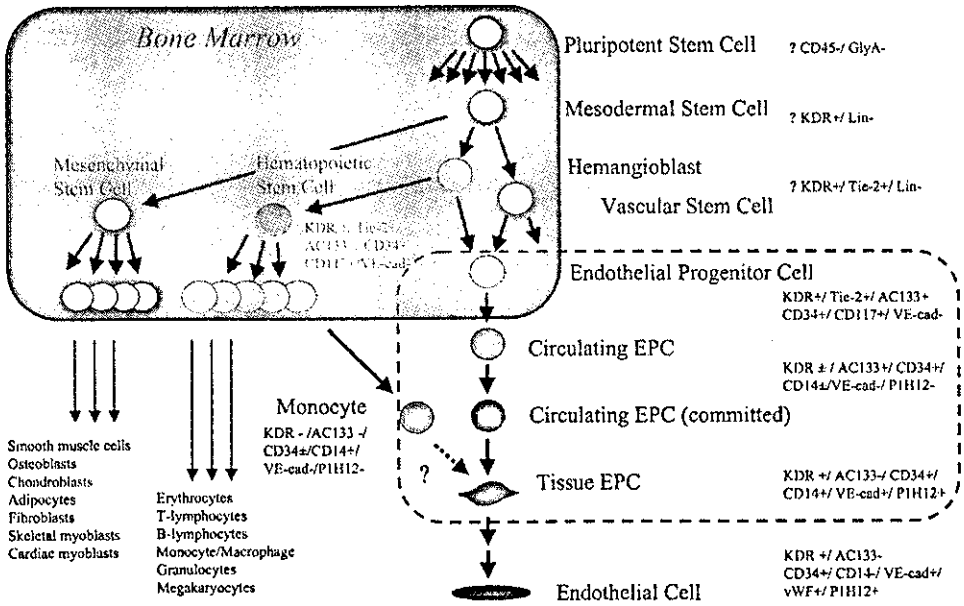

Fig. 2. Putative cascade and expressional profiles of human bone marrow-derived endothelial progenitor cell differentiation. EPC, endothelial progenitor cell.

These findings have raised important questions regarding fundamental concepts of blood vessel growth and development in adult subjects. Does the differentiation of EPCs in situ (vasculogenesis) play an important role in adult neovascularization, and would impairments in this process lead to clinical diseases? There is now a strong body of evidence suggesting that vasculogenesis does, in fact, make a significant contribution to postnatal neovascularization. Recent studies with animal bone marrow transplantation (BMT) models in which bone marrow (donor)-derived EPCs could be distinguished have shown that the contribution of EPCs to neovessel formation may range from 5 to 25% in response to granulation tissue formation (10) or growth factor-induced neovascularization (45).

IDENTIFICATION OF EPCs AND THEIR PRECURSORS

Since the initial report of EPCs (3), a number of groups have set out to better define this cell population. Because EPCs and HSCs share many surface markers, and because no simple definition of EPCs exists, various methods of EPC isolation have been reported (2, 3, 10, 20, 23, 39, 45, 47–50, 52, 59, 61, 63, 68, 69). The term "EPC" may therefore encompass a group of cells existing in a variety of stages ranging from hemangioblasts to fully differentiated ECs. Although the true differentiation lineage of EPCs and their putative precursors remain to be determined, there is overwhelming evidence in vivo that a population of EPCs exists in humans.

Lin et al. (39) cultivated peripheral MNCs from patients receiving gender-mismatched BMT and studied their growth in vitro. In that study, they identified a population of bone marrow (donor)-derived ECs with high proliferative potential (late outgrowth); these bone marrow cells likely represent EPCs. Gunsilius et al. (23) investigated a chronic myelogenous leukemia model and disclosed that bone marrow-derived EPCs contribute to postnatal neovascularization in humans (23). Reyes et al. (50) recently isolated multipotent adult progenitor cells (MAPCs) from bone marrow MNCs, which differentiated into EPCs. These findings strongly proposed MAPCs as the origin of EPCs (49). These studies therefore provide evidence to support the presence of bone marrow-derived EPCs that take part in neovascularization (Fig. 2).

EPC KINETICS FOR REGENERATION

Given the result of common antigenicity, bone marrow has been considered the origin of EPCs as HSCs in adults. The BMT experiments have demonstrated the incorporation of bone marrow-derived EPCs into foci of physiological and pathological neovascularization (2). Wild-type mice were lethally irradiated and transplanted with bone marrow harvested from transgenic mice in which constitutive LacZ expression is regulated by an EC-specific promoter: Flk-1 or Tie-2. Histological examination of the tissues in growing tumors, healing wounds, ischemic skeletal and cardiac muscles, and cornea micropocket surgery after BMT has shown localization of Flk-1- or Tie-2-expressing endothelial lineage cells derived from bone marrow in blood vessels and stroma around vasculatures. The similar incorporation was observed in physiological neovascularization in uterus endometrial formation after induced ovulation as well as estrogen administration (2). A source of EPCs other than bone marrow has never been identified because of the lack of appropriate animal models.

Previous investigators have shown that wound trauma causes mobilization of hematopoietic cells, including pluripotent stem or progenitor cells in spleen, bone marrow, and peripheral blood. Consistent with EPC/HSC common ancestry, recent data from our laboratory have shown that mobilization of bone marrow-derived EPCs constitute a natural response to tissue ischemia (26). The former murine BMT model presented the direct evidence of enhanced bone marrow-derived EPC incorporation into foci of corneal neovascularization after the development of hindlimb ischemia. Light microscopic examination of corneas excised 6 days after micropocket injury and concurrent surgery to establish hindlimb ischemia demonstrated a statistically significant increase in cells expressing β-galactosidase in the corneas of mice with, versus those without, an ischemic limb (63). This finding indicates that

circulating EPCs are mobilized endogenously in response to tissue ischemia, after which they may be incorporated into neovascular foci to promote tissue repair. This was confirmed by clinical findings of EPC mobilization in patients with coronary artery bypass grafting, burns (22), and acute myocardial infarction (60).

Having demonstrated the potential for endogenous mobilization of bone marrow-derived EPCs, we considered that iatrogenic expansion and mobilization of this putative EC precursor population might represent an effective means to augment the resident population of ECs that is competent to respond to administered angiogenic cytokines. Such a program might thereby address the issue of endothelial dysfunction or depletion that may compromise strategies of therapeutic neovascularization in older, diabetic, and/or hypercholesterolemic animals and patients. Granulocyte macrophage colony-stimulating factor (GM-CSF), which stimulates hematopoietic progenitor cells and myeloid lineage cells as well as nonhematopoietic cells including bone marrow stromal cells and ECs, has been shown to exert a potent stimulatory effect on EPC kinetics: mobilization from bone marrow, incorporation into sites of neovascularization, and proliferation and differentiation in culture (63). Such cytokine-induced EPC mobilization could enhance neovascularization of severely ischemic tissues as well as de novo corneal vascularization (63).

The mechanisms whereby these EPCs are mobilized to the peripheral circulation are in the early stage of definition. Among other growth factors, VEGF is the most critical factor for vasculogenesis and angiogenesis (6, 15, 57). Recently collected data indicate that VEGF is an important factor for the mobilization of EPCs from bone marrow, as well. Our studies performed first in mice (2) and subsequently in patients undergoing VEGF gene transfer for critical limb ischemia (32) and myocardial ischemia (32) established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is via mobilization of bone marrow-derived EPCs. The similar EPC kinetics modulation has been observed in response to other hematopoietic stimulators, such as granulocyte colony-stimulating factor (G-CSF) (20), angiopoietin-1 (24), and stroma-derived factor-1 (SDF-1) (47).

This therapeutic strategy of EPC mobilization has recently been implicated not only by natural hematopoietic or angiogenic stimulants but also by recombinant pharmaceuticals. The statins inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis. The statins rapidly activate Akt signaling in ECs, thereby stimulating EC bioactivity in vitro and enhancing angiogenesis in vivo (37). Recently, we (41) and Dimmeler and colleagues (12, 67) demonstrated a novel function for HMG-CoA reductase inhibitors that contributes to postnatal neovascularization by augmented mobilization of bone marrow-derived EPCs through stimulation of the Akt signaling pathway. With regard to its pharmacological safety and effectiveness on hypercholesterolemia, one of the risk factors for atherogenesis, the statin might be a potent medication against atherosclerotic vascular diseases.

THERAPEUTIC POTENTIALS OF EPC TRANSPLANTATION

The regenerative potential of stem cells is presently under intense investigation. In vitro, stem and progenitor cells possess the capability of self-renewal and differentiation into organ-specific cell types. In vivo, transplantation of these cells may reconstitute organ systems, as shown in animal models of diseases (1, 3, 14, 17, 32, 40). In contrast, differentiated cells do not exhibit such characteristics. Human EPCs have been isolated from the peripheral blood of adult individuals, expanded in vitro, and committed into an endothelial lineage in culture (2). The transplantation of these human EPCs has been shown to facilitate successful salvage of ischemic hindlimb and to improve blood perfusion in ischemic limbs of nude mice, whereas differentiated ECs (human microvascular ECs) failed to accomplish limb-saving neovascularization (32) (Fig. 3).

These experimental findings call into question certain fundamental concepts regarding blood vessel growth and development in adult organisms. Postnatal neovascularization was

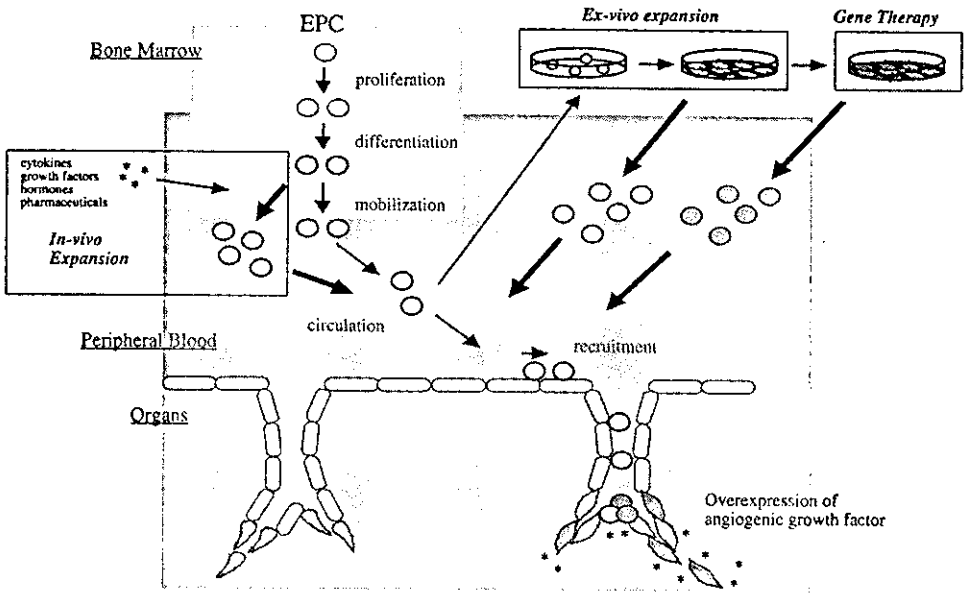

Fig. 3. Therapeutic application of EPCs for neovascularization.

previously considered synonymous with proliferation and migration of preexisting, fully differentiated ECs resident within parent vessels, i.e., angiogenesis (18). The finding that circulating EPCs may home to sites of neovascularization and differentiate into ECs in situ is consistent with vasculogenesis (51), a critical paradigm for establishment of the primordial vascular network in the embryo. While the proportional contributions of angiogenesis and vasculogenesis to postnatal neovascularization remain to be clarified, our findings together with recent reports from other investigators (25, 59) suggest that growth and development of new blood vessels in the adult are not restricted to angiogenesis but encompass both embryonic mechanisms. As a corollary, augmented or retarded neovascularization, whether endogenous or iatrogenic, likely includes enhancement or impairment of vasculogenesis.

We therefore considered a novel strategy of EPC transplantation to provide a source of robust ECs that might supplement fully differentiated ECs thought to migrate and proliferate from preexisting blood vessels according to the classic paradigm of angiogenesis developed by Folkman (19). Our studies indicated that ex vivo cell therapy, consisting of culture-expanded EPC transplantation, successfully promotes 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 (ECs as EPCs) rather than the ligand comprises the therapeutic agent was first demonstrated in the hindlimb ischemia model of immunodeficient mouse, using donor cells from human volunteers (32). These findings provided novel evidence that exogenously administered EPCs augment naturally impaired neovascularization in an animal model of experimentally induced critical limb ischemia. Not only did heterologous cell transplantation improve neovascularization and blood flow recovery, but important biological consequences, notably limb necrosis and autoamputation, were reduced by 50% compared with mice receiving differentiated ECs or control mice receiving media in which harvested cells were expanded ex vivo before transplantation. A similar strategy applied in a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs incorporated into rat myocardial neovascularization, differentiated into mature ECs in ischemic myocardium, enhanced neovascularization, preserved left ventricular (LV) function, and inhibited myocardial fibrosis (34).

Recently, Shatteman et al. (56) 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. Kocher et al. (28) attempted intravenous infusion of freshly isolated (not cultured) human CD34+ MNCs (EPC-enriched fraction) into nude rats with myocardial ischemia. This strategy resulted in preservation of LV function associated with inhibition of cardiomyocyte apoptosis. These experimental findings obtained using immunodeficient animals suggest that both cultured and freshly isolated human EPCs have therapeutic potential in peripheral and coronary artery diseases.

Induction of angiogenic diseases, such as diabetic retinopathy and malignant tumors, is a possible deleterious effect of EPC transplantation. Such harmful events should be carefully monitored in future clinical trials, although no adverse effects have ever been reported in previous basic and preclinical studies.

IMPACT OF CLINICAL PHENOTYPE ON EPCs

Preliminary clinical findings in patients with critical limb ischemia indicated that the response to phVEGF gene transfer was most robust and expeditious in young patients with premature atherosclerosis involving the lower extremities, so-called Buerger's disease (28). This clinical observation was supported by experiments performed in live animal models, specifically young (6–8 mo) vs. old (4–5 yr) rabbits and young (8 wk) vs. old (2 yr) mice. In both cases, native neovascularization of the ischemic hindlimb was markedly retarded in old vs. young animals. Retardation of neovascularization in old animals appeared in part to result from reduced expression of VEGF in tissue sections harvested from the ischemic limb (54).

Endogenous cytokine expression, however, is not the only factor contributing to impaired neovascularization. Older, diabetic, and hypercholesterolemic animals, like human subjects (7, 8, 13, 21, 30, 42, 62, 65), also exhibit evidence of age-related endothelial dysfunction. Although endothelial dysfunction does not necessarily preclude a favorable response to cytokine replacement therapy, indexes of limb perfusion fail to reach ultimate levels recorded in wild-type animals, reflecting limitations imposed by a less responsive EC substrate (9, 54, 55, 66).

It is then conceivable that unfavorable clinical situations (such as aging) might be associated with dysfunctional EPCs, defective vasculogenesis, and, thus, impaired neovascularization. Indeed, preliminary results from our laboratory indicated that replacement of native bone marrow (including its compartment of progenitor cells) of young mice with bone marrow transplanted from old animals leads to a marked reduction in neovascularization following corneal micropocket injury, compared with young mice transplanted with young bone marrow (53). These studies thus established evidence of an age-dependent impairment in vasculogenesis (as well as angiogenesis) and the origin of progenitor cells as a critical parameter influencing neovascularization. Moreover, analysis of clinical data in older patients at our institution disclosed a significant reduction in the number of circulating EPC both at baseline and after VEGF165 gene transfer (31); specifically, the number of circulating EPCs of younger patients with critical limb ischemia was five times more than the number in older individuals. Impaired EPC mobilization and/or activity in response to VEGF may thus contribute to the age-dependent defect in postnatal neovascularization. Recently, Tepper et al. (64) reported that proliferation and tube formation of EPCs was impaired in patients with type 2 diabetes compared with normal subjects.

GENE THERAPY OF EPCs

Given these findings, together with the limited quantity of EPCs available even under healthy, physiological conditions, one must consider a strategy that addresses this shortfall and mitigates the possibility of dysfunctional EPCs for therapeutic vasculogenesis in ischemic disorders complicated by aging, diabetes, hypercholesterolemia, and/or hyperhomocysteinemia. Genetic modification of EPCs to overexpress angiogenic growth factors, enhance signaling activity of the angiogenic

response, rejuvenate the bioactivity, and/or extend the life span of EPCs constitutes one potential strategy that might address these limitations of EPC transplantation and thereby optimize therapeutic neovascularization (Fig. 3).

Our recent findings provide the first evidence that exogenously administered, gene-modified EPCs augment naturally impaired neovascularization in an animal model of experimentally induced limb ischemia (29). Transplantation of heterologous EPCs transduced with adenovirus encoding VEGF not only improved neovascularization and blood flow recovery but also had meaningful biological consequences: limb necrosis and autoamputation were reduced by 63.7% compared with controls. The dose of EPCs used in the current in vivo experiments was subtherapeutic; i.e., this dose of EPCs was 30 times less than that required in previous experiments to improve the rate of limb salvage above that seen in untreated controls. Adenoviral VEGF gene transfer of EPC, however, accomplished a therapeutic effect, as evidenced by a functional outcome, despite a subtherapeutic dose of EPCs. Thus VEGF gene transfer of EPC constitutes one option to address the limited number of EPCs that can be isolated from peripheral blood prior to ex vivo expansion and subsequent autologous readministration.

EPCs IN OTHER FIELDS

EPCs have recently been applied to the field of tissue engineering as a means of improving biocompatibility of vascular grafts. Artificial grafts first seeded with autologous CD34+ cells from canine bone marrow and then implanted into the aorta were found to have increased surface endothelialization and vascularization compared with controls (4). Similarly, when cultured autologous ovine EPCs were seeded onto carotid interposition grafts, the EPC-seeded grafts achieved physiological motility and remained patent for 130 days vs. 15 days in nonseeded grafts (33).

EPCs also have been investigated in the cerebrovascular field. Embolization of the middle cerebral artery in Tie-2/LacZ/BMT mice disclosed that the formation of new blood vessels in the adult brain after stroke involves vasculogenesis/EPCs (70). Similar data were reported by investigators using gender-mismatched wild-type mice transplanted with bone marrow from green fluorescent protein-transgenic mice (26). However, whether autologous EPC transplantation would augment cerebral revascularization has yet to be examined.

To date, the role of EPCs in tumor angiogenesis has been demonstrated by several groups. Davidoff et al. (11) showed that bone marrow-derived EPCs contribute to tumor neovasculature and that bone marrow cells transduced with an anti-angiogenic gene can restrict tumor growth in mice. Lyden et al. (43) recently demonstrated the critical role of bone marrow-derived EPCs in tumor neovascularization. Id-mutant ($Id1^{+/-}Id3^{-/-}$) mice are angiogenic defective and tumor-resistant double-mutant mice in which implanted tumors rapidly regress in association with poor development of tumor neovessels. BMT from wild-type mice, not from Id-mutant mice, restored the tumor neovascularization and growth in Id-mutant mice (43). These data demonstrate that EPCs are not only important but also critical to tumor neovascularization. Although it is not known whether local administration of exogenous EPCs may augment tumor neovascularization, this issue should be carefully considered for clinical application of EPC cell therapy to treat cardiovascular diseases.

In conclusion, EPCs isolated from adult species, which have characteristics similar to those of embryonic angioblasts, have the capacity to proliferate, migrate, and differentiate into endothelial lineage cells but have not yet acquired mature endothelial markers. EPCs are mobilized from bone marrow into the circulation and then home to the site of neovascularization in response to physiological and pathological stimuli, thereby contributing to postnatal neovascularization. Since animal experiments on EPC transplantation proved the therapeutic potential of the cell-based strategy, the application of EPCs for regenerative medicine has been watched with keen interest. The clinical impact of EPC regenerative properties will be evaluated in a phase I-II trial being started at our institution.

However, a number of issues remain to be addressed in this research field. Some of the future perspectives are as follows: *1*) identification of a specific marker for EPC with which other lineage cells do not share; *2*) evaluation of EPC transdifferentiation in vitro and in physiological, pathological, and iatrogenic regeneration of tissues and organs; *3*) methodological optimization of EPC purification, expansion, gene transfer, and administration to improve the efficacy of EPC transplantation; and *4*) comparison of the therapeutic impact between purified EPCs and total bone marrow MNCs.

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Mechanisms of Angiogenesis
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Bone marrow-derived endothelial progenitor cells for neovascular formation

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Introduction

As the fertilized ovum undergoes repeated cell divisions to increase the cell population for tissue or organ formation exchange of material by simple diffusion becomes unable to nourish all cells. Before facing such a situation, the developing embryo begins to harbor the blood island, which consists of hematopoietic stem cells and angioblasts. Hematopoietic stem cells and angioblasts differentiate into blood cells and vascular endothelial cells in the blood islands, respectively. Then, the blood islands begin fusing with each other and form vasculature with blood cells in its lumen [1, 2]. With the cardiovascular development, the exchange of material by the circulatory system becomes effective and capable of nourishing the growing tissue or organ. In the adult, hematopoietic stem cells in the bone marrow continue asymmetric cell divisions and supply blood cells throughout the individual's life span. However, it was unknown whether hemangioblasts or other stem cells that can differentiate into vascular endothelial cells exist or not. Folkman [3] documented that the extent of tumor growth was dependent upon neovascularization and suggested that this relationship might be based on the angiogenic growth factors that were released by neoplasms. Since his report, neovascular formation derived from pre-existing neighbor vessels has been called angiogenesis. Subsequent investigations revealed the potential of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral blood flow in the ischemic tissue. This novel strategy to treat ischemic disease was termed therapeutic angiogenesis [4]. In the developing embryo, vessel formation is initiated by the fusion of blood islands. Then, vascular endothelial cells are supplied by cell division and differentiation of angioblasts in the foci of neo-vascular formation, which is called vasculogenesis [1]. After vessel formation by vasculogenesis, vascular elongation occurs. The vascular endothelial cells in this stage are supplied from endothelial cells of neighboring vessels, which is called angiogenesis [2]. As stated above, vascular formation in the embryo consists of vasculogenesis and angiogenesis, but only angiogenesis has been discussed in relation to adult neovascular formation [1].

What is the endothelial progenitor cell?

In 1997, Asahara et al. discovered that CD34-positive mononuclear cells in the human peripheral blood incorporated into the foci of vascular injury and differentiated into vascular endothelial cells [5]. When cultured on fibronectin-coated dishes with growth factors, such as vascular endothelial growth factor (VEGF) and/or basic fibroblast growth factor, the CD34-positive cells express endothelial nitric oxide synthase (eNOS), kinase insert domain receptor (KDR) and CD31. They then become spindle-shaped cells and take up acetylated low density lipoprotein (LDL), which are features of the endothelial lineage. Human CD34-positive mononuclear cells were isolated from peripheral blood and marked with red fluorescent dye. Then, they were administered to nude mice in the ischemic hindlimb model. After six weeks, some of the vascular endothelial cells in the hindlimb muscle demonstrated red fluorescence, which indicated that administered human CD34-positive mononuclear cells were incorporated into the ischemic muscle tissue and differentiated into endothelial cells [5]. A bone-marrow transplantation model was used in order to exclude the possibility that the cells stained with red fluorescent were not the administered cells but the mouse endothelial cells that had taken up the dye. Bone marrow mononuclear cells from *Flk-1/lac Z* or *Tie-2/lac Z* transgenic mice were transplanted to the background of immuno-deficient mice that were lethally irradiated before the bone marrow mononuclear cell transplantation. The donor *Flk-1/lac Z* or *Tie-2/lac Z* transgenic mice were genetically modified to express bacterial β-galactosidase under the control of an endothelial cell-specific promoter. In other words, only endothelial cells express bacterial β-galactosidase in these mice. It is possible to identify the cells expressing bacterial β-galactosidase by X-gal staining or immunohistochemistry. After bone marrow reconstruction by the transplanted cells, both physiological and pathological neovascularization models were examined in the recipient mice. We can observe the physiological neovascularization in the adult when the corkscrew-like arteries are formed in the endometrium of the proliferative phase [6]. The uteri of recipient female mice whose bone marrow was reconstituted with cells from *Tie-2/lac Z* transgenic mice were examined. In the uteri of mice in the late proliferative phase, frequent incorporation of cells into the vascular structures was observed by X-gal staining. This result indicates that a certain population of bone marrow-derived cells were incorporated in the spiral arteries and differentiated into endothelial cells. We created ischemic models by removing femoral arteries of recipient mice [6]. After two weeks, the hindlimb muscles were examined. Sections stained with X-gal demonstrated that the neovasculature of the ischemic lesions frequently comprised *Tie-2*-expressing cells in the vascular structures. In the next experiments, mouse syngeneic colon cancer cells were implanted subcutaneously into the recipient mice and tumor samples were excised after two weeks. X-gal staining revealed that bone marrow-derived endothelial cells were incorporated even in the developing tumor