basis of the physicochemical interaction forces between the growth factor and gelatin. In this release system, the growth factor immobilized is not released from the hydrogel unless the hydrogel carrier is degraded to generate water-soluble gelatin fragments. The growth factor release can be controlled only by changing the hydrogel degradation.[24] In addition, we have experimentally confirmed that cationized gelatin hydrogels, prepared by introducing amine residues to the carboxyl groups of gelatin, achieved the controlled release of plasmid DNA based on the hydrogel degradation following intramuscular implantation.[28,29] The cationized gelatin hydrogel incorporating plasmid DNA enhanced the gene expression level to a significantly greater extent than the plasmid DNA injected in solution form, while it prolonged the duration period of gene expression which basically depends on the release period of plasmid DNA.[28-29]

This study was undertaken to prepare a micelle from this gelatin by PEG grafting. It is expected that the micelle of PEG and gelatin can retain in the blood circulation. Additionally, this micelle may release the drug immobilized to gelatin molecules in a controlled manner accompanied with gelatin degradation. The release mechanism driven by the degradation of the release carrier is quite different from that of drug or DNA diffusion from the release carrier, such as PLA and PLGA, which has been reported. Gelatin was grafted with PEG, and the micelle formation and body distribution of PEG-grafted gelatin (PEG-gelatin) were evaluated in terms of the molecular weight of PEG grafted and the PEGylation degree.

Materials and Methods

Materials. The gelatin sample with an isoelectric point of 5.0 (M_w = 100 000), prepared by an alkaline process of bovine bone, was kindly supplied from Nitta Gelatin Inc., Osaka, Japan. Succinimidyl succinate-methoxy PEG (M_w = 2000, 5000, and 12 000) was kindly provided by NOF Corp., Tokyo, Japan. *N*-Phenyl-1-naphthylamine (PNA) was obtained from Wako Pure Chemical, Ltd., Osaka, Japan.

PEG Grafting of Gelatin. Gelatin (1.0×10^{-5} mol) was dissolved in anhydrous dimethyl sulfoxide (DMSO, 10 g) at room temperature. Various amounts of succinimidyl succinate-methoxy PEG with molecular weights of 2000, 5000, and 12 000 (0.15, 0.3, 0.6, 1.5, 3.0, and 6.0×10^{-4} mol) were dissolved in 10 g of DMSO, and the solution was slowly added to the gelatin solution, followed by 3 h of stirring at room temperature for PEG grafting. The reaction mixture was dialyzed in a cellulose tube (the cutoff molecular weight = 12 000−14 000, Viskase Companies, Inc) against double-distilled water (DDW) for 48 h at room temperature and freeze-dried to obtain a PEG-gelatin. The percentage of PEG introduced to the amino groups of gelatin (PEGylation degree) was determined by the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS) method.[30]

Light Scattering Measurements. To investigate the hydrodynamic radius of PEG-gelatin, the dynamic light scattering (DLS) measurement was carried out on a DLS 700 (Otsuka Electronics, Japan) equipped with a He−Ne laser at a detection angle of 30°, 90°, and 120° at room temperature. The gelatin and PEG-gelatin were dissolved in phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C to prepare the respective solutions (10 mg/mL) in the presence or absence of 6 M guanidine hydrochloride as a hydrophobic interaction breaker. The solution was filtered by a disposable syringe filter (pore size: 0.8 μm; Millipore Co.) for DLS measurements. The PEG-gelatin solution (10 mg/mL, 5 mL) was added to the cell at room temperature, and the hydrodynamic diameter of gelatin and PEG-gelatin was analyzed based on the cumulants method and automatically calculated by the computer software equipped to express as the apparent molecular size. Electrophoretic light scattering (ELS) measurements were carried on an ELS-7000 (Otsuka Electronic Co. Ltd., Osaka, Japan) at room temperature and an electric field strength of 100 V/cm. Gelatin and PEG-gelatin solution were prepared by the same procedure as the DLS measurement. The zeta potential was automatically calculated using the Smoluchouski equation. Each experiment was done 10−20 times independently unless otherwise mentioned.

Affinity Assay of Gelatin and PEG-Gelatin. An affinity assay with HiTrap Blue HP column (Amersham Pharmacia Biotech AB), which has an affinity for protein, was performed to structurally characterize the PEG-gelatin. Gelatin and PEG-gelatin with various PEG molecular weights and PEGylation degrees were dissolved in PBS (5 mg/mL) at 37 °C. After the solution (0.5 mL) was applied to the HiTrap column, the column was washed with 5 mL of PBS and 400 μL of each elution fraction was collected by microcentrifuge tubes. Then, the column was washed with 5 mL of 2 M sodium chloride aqueous solution, followed by the fraction collection similarly. After desalting of the fractions by a PD-10 column (Amersham Pharmacia Biotech AB), the gelatin concentration of each fraction was determined by the protein assay Lowry kit (Nacalai tesque, Kyoto, Japan).

Critical Micelle Concentration (CMC) Measurements. The critical micelle concentration (CMC) of PEG-gelatin was measured according to the conventional method of PNA incorporation.[31] Gelatin and the PEG-gelatin solution were prepared by the same procedure as the DLS measurement. The concentration of gelatin and PEG-gelatin ranged from 0.04 to 10 mg/mL. A small aliquot (10 μL) of PNA methanol solution (5 mM) was added dropwise to 1 mL of gelatin or PEG-gelatin solution at room temperature and 37 °C. After 2 min of stirring, the absorbance of the mixed solution was measured at a wavelength of 500 nm (Ultrospec 2000; Amersham Pharmacia Biotech AB). The concentration of PEG-gelatin at which the solution absorbance changed drastically was measured and defined as the CMC.

Body Distribution Studies of PEG-Gelatin and the Pharmacokinetis Analysis. The body distribution of gelatin and PEG-gelatin was evaluated in female ddY mice (16−18 g weight) (Japan SLC, Inc., Hamamatsu, Japan) by the radiotracing procedure. Gelatin and PEG-gelatin were radioiodinated according to the chloramine T method.[32] The ^{125}I-labeled gelatin and PEG-gelatin (2 mg/mouse) were injected into the jugular vein of mice in a PBS volume of 100 μL. At predetermined time intervals, the mice were sacrificed by ether inhalation, and their tissues were excised,

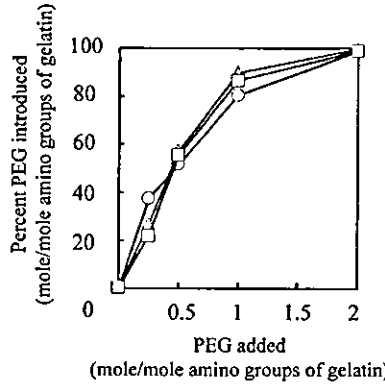

Figure 1. Percentage of PEG introduced to the amino groups of gelatin as a function of the amount of PEG added for grafting reaction. The molecular weight of PEG used was 2000 (○), 5000 (△), and 12 000 (□).

washed quickly with cold saline to remove surface blood, and weighed, and the radioactivity of whole organ was counted on a gamma counter (ARC-301B, Aloka, Tokyo, Japan). Blood samples (100 μL) were obtained in duplicate by cardiac puncture in preweighed tubes. The radioactivity was divided by that of injected initially and the tissue weight to calculate the percentage of injected dose per g of tissue, while the results were normalized to a 20 g mouse to exclude the error by weight variation according to the formula of Sato et al.[33] The percent remaining = [Radioactivity in the tissue/total injected dose/tissue weight (g)] × [body weight (g)/20] × 100. All of the animal experiments were carried out according to the Institutional Guidance of Kyoto University on Animal Experimentation. The mean area under the percent remaining-time curve (AUC) of gelatin and PEG-gelatin in the blood circulation was calculated by the trapezoidal method during the experimental period ($AUC_{[0-24]}$) to compare the stability in the blood circulation between the samples.

Statistical Analysis. All of the data were expressed as the mean ± the standard derivation of the mean. Statistical analysis was performed based on the unpaired Student's *t* test (two-sided) and significance was accepted at $p < 0.05$.

Results

Characterization of PEG-Gelatin Prepared. PEG was successfully grafted onto gelatin, and nonreacted PEG was completely removed by the dialysis because no peak of PEG was observed by gel permeation chromatography (data not shown). Figure 1 shows the percentage of PEG introduced to the amino groups of gelatin. The number of amino groups of native gelatin was 30 (mol/mol of gelatin). The introduction percentage increased with an increase in the amount of PEG added initially. The PEGylation degree of gelatin could be controlled by changing the addition molar ratio. No effect of the PEG molecular weight on the percentage of PEG introduced was observed.

Table 1 summarizes the apparent molecular size of PEG-gelatin prepared from PEG with molecular weights of 2000, 5000, and 12 000 with various PEGylation degree. The apparent molecular size did not depend on the measurement angle. However, when the guanidine hydrochloride was added, the PEG-gelatin did not show a specific hydrodynamic diameter. Neither gelatin alone nor PEG-gelatin with low degrees of PEGylation exhibited any hydrodynamic diameter. The zeta potential of PEG-gelatin with PEG-introduced percentages of 85 and 100 tended to increased to reach almost 0 mV, in marked contrast to the PEG-gelatin with the PEG molecular weight of 2000 (data not shown). However, there was no significant difference in the zeta potential between the PEG-gelatin samples for any molecular weight of PEG grafted and PEGylation degree.

Figure 2 shows the elution profiles of gelatin and PEG-gelatin prepared from PEG with a molecular weight of 12 000 by HiTrap Blue HP column. The percentage of respective fraction eluted was summarized in Table 1. Because this column has a specific affinity for gelatin, the affinity of gelatin will be reduced by the PEGylation. Gelatin alone and the PEG-gelatin with low PEGylation degrees were adsorbed on the affinity column, whereas the PEG-gelatin with higher PEGylation degrees was not adsorbed.

Figure 3 shows the change in the solution absorbance of PNA incorporated in gelatin grafted with PEG of molecular weights 12 000 as a function of the PEG-gelatin concentra-

Table 1. Physicochemical Properties of PEG-Gelatin

molecular weight of PEG grafted	number of PEG chains grafted (mol/1mol of gelatin)	PEGylation degree[a] (mol/mol%)	percent of eluted[b] PBS (pH 7.4)[c]	percent of eluted[b] 2 M NaCl aq.[d]	hydrodynamic diameter (nm)	CMC[e] (mg/mL)
2000	10	30	0	100	ND[f]	ND[f]
	17	55	34	66	ND	ND
	25	85	58	42	60 ± 10	ND
	30	100	93	7	65 ± 13	0.2
5000	10	30	21	79	ND	ND
	17	55	45	55	ND	ND
	25	85	94	6	78 ± 15	0.3
	30	100	95	5	80 ± 16	0.3
12 000	10	30	13	87	ND	ND
	17	55	73	27	110 ± 17	ND
	25	85	97	3	112 ± 16	1.0
	30	100	95	5	120 ± 27	1.0
gelatin	0	0	0	100	ND	ND

[a] The percentage of PEG introduced to the amino groups of gelatin. [b] Eluted from HiTrapTM Blue HP column which has an affinity for gelatin. [c] The column was washed with PBS and each elution fraction was collected. [d] The column was washed with 2 M sodium chloride aqueous solution, followed by the fraction collection similarly. [e] Critical micelle concentration (CMC) was measured by the *N*-phenyl-1-naphthylamine (PNA) uptake in PEG-gelatin micelle. [f] Not detected.

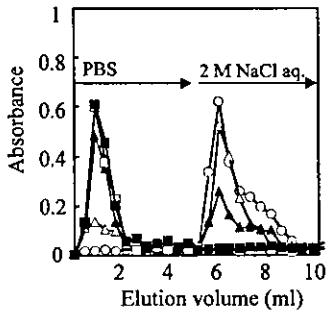

Figure 2. Elution profiles of gelatin and gelatin grafted with PEG of molecular weights 12 000 by HiTrap Blue HP column. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%.

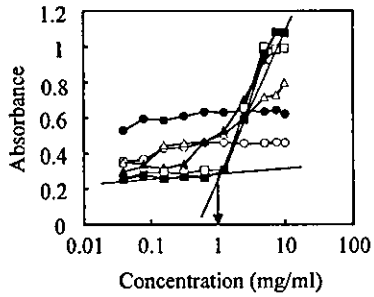

Figure 3. Change in the solution absorbance of PNA incorporated in gelatin grafted with PEG of molecular weights 12 000 as a function of the PEG-gelatin concentration. The PEGylation degree was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%.

tion. The results of the other PEGs are summarized in Table 1. The solution absorption of PEG-gelatin was drastically increased at concentrations of 0.2, 0.3, and 1.0 mg/mL for the PEG-gelatin with PEG-introduced percentages of 85 and 100. The similar CMC value was observed at room temperature and 37 °C for each PEG molecular weight. The bending point corresponds to the CMC where the intermolecular aggregation of PEG-gelatin takes place. On the other hand, for gelatin and PEG-gelatin with PEG-introduced percentages of 0, 30, and 55, such a drastic increase was not observed.

Body Distribution of PEG-Gelatin Intravenous Administration. Figure 4 shows the percent of remaining of gelatin and PEG-gelatin in the blood circulation following intravenous administration of ^{125}I-labeled PEG-gelatin. The radioactivity of ^{125}I-labeled PEG-gelatin with PEG-introduced percentages of 85 and 100 was retained in the blood circulation compared with that of ^{125}I-labeled gelatin and the PEG-gelatin with PEG-introduced percentages of 0, 30, and 55. At the same PEGylation degree, the blood concentration was significantly higher for the PEG-gelatin prepared from the PEG molecular weight of 12 000 than that of other molecular weights (2000 and 5000).

Figure 5 shows the $AUC_{[0-24]}$ value of gelatin and PEG-gelatin prepared from different molecular weights of PEG at various PEGylation degrees. The $AUC_{[0-24]}$ for the PEG-gelatin with PEG-introduced percentages of 85 and 100 increased with the increasing molecular weight of PEG grafted. On the other hand, the $AUC_{[0-24]}$ for the PEG-gelatin with PEG-introduced percentages of 0, 30, and 55 hardly increased, irrespective of the molecular weight of PEG.

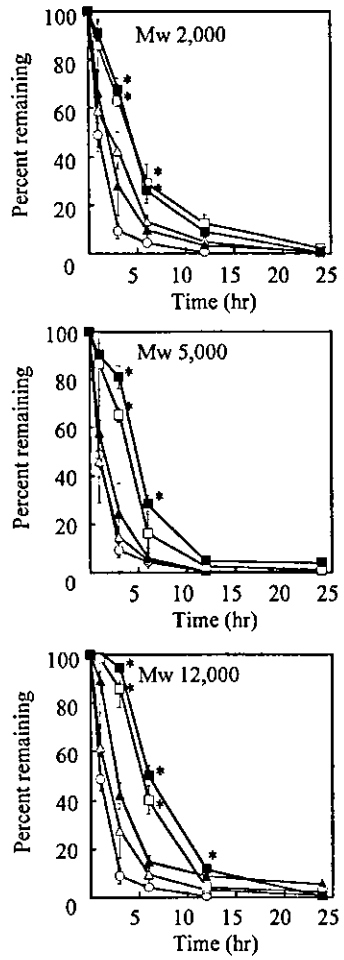

Figure 4. Decrement patterns of gelatin and gelatin grafted with PEG of molecular weights 2000, 5000, and 12 000 in the blood circulation after intravenous administration. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%. *, $p < 0.05$: significant against the percent remaining of native gelatin-injected, control mice.

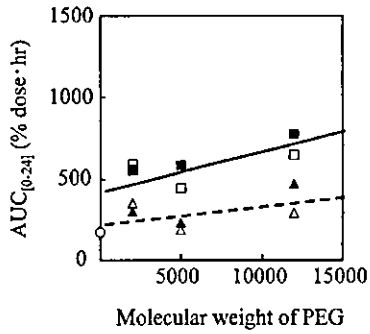

Figure 5. Effect of the molecular weight of PEG grafted on the AUC of PEG-gelatin. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%. The solid and dotted lines indicate the PEG-gelatin of micelle and unimer structures, respectively.

Figure 6 shows the mean percent remaining radioactivity in the liver, spleen, and kidney after intravenous administration to mice of gelatin and PEG-gelatin. The PEG-gelatin with PEG-introduced percentages of 85 and 100 tended to accumulate in the liver compared with that with PEG-

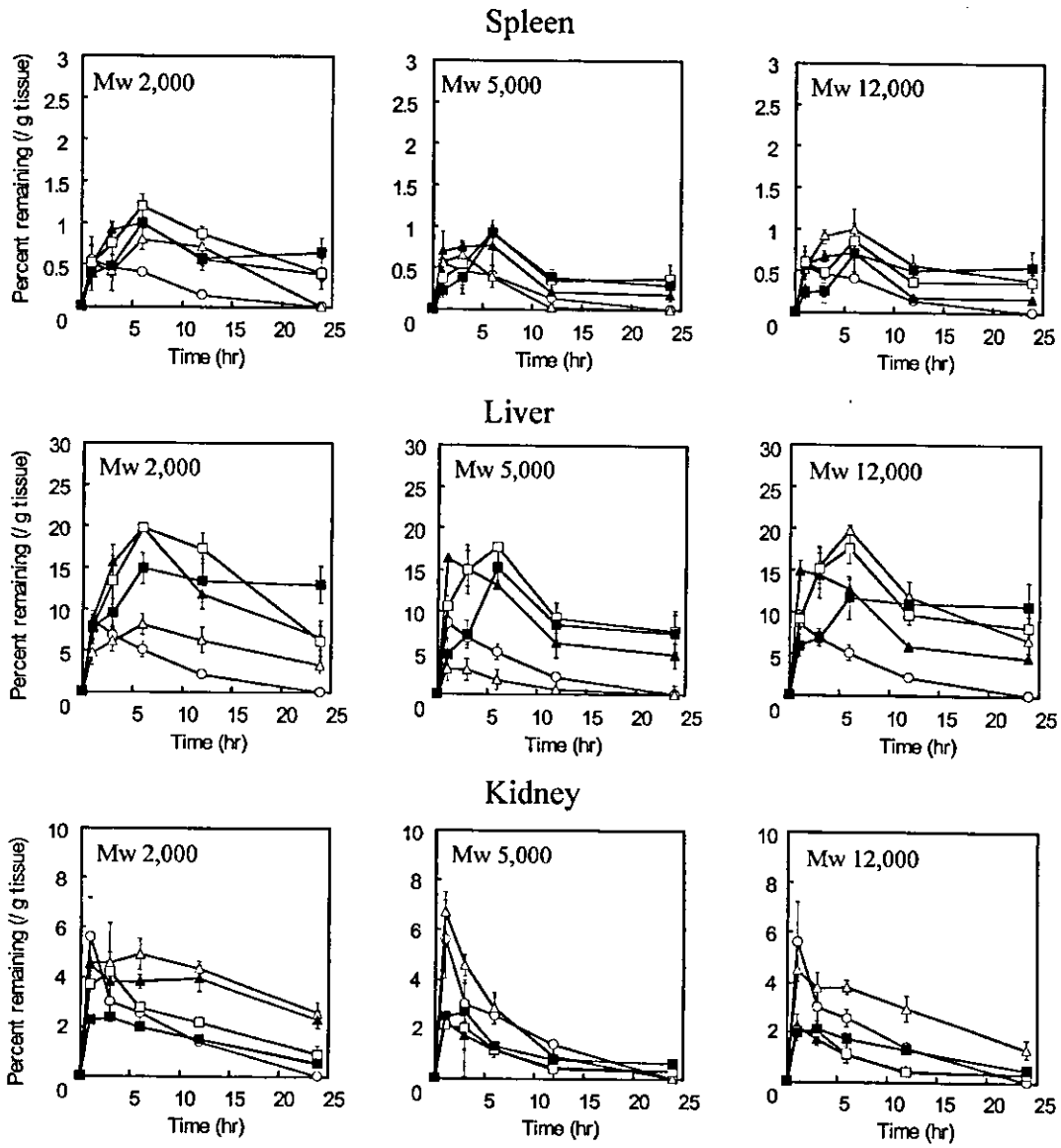

Figure 6. Effect of the molecular weight of PEG grafted and the PEGylation degree of PEG-gelatin on the time profile of PEG-gelatin accumulation in the spleen, liver, and kidney. The PEGylation degree of PEG-gelatin used was 0 (○) (native gelatin), 30 (△), 55 (▲), 85 (□), and 100 (■) mol/mol%.

introduced percentages of 0, 30, and 55. However, there was no significant difference between the PEGylation degree and molecular weight of PEG in the accumulation in the liver, spleen, and kidney.

Discussion

The present study clearly demonstrates that when gelatin was grafted with PEG at the PEG-introduced percentages of 85 and 100 percent the PEG-gelatin showed a micellar structure and tended to circulate in the blood stream for a long time period. No adsorption of the PEG-gelatin to the gelatin affinity column (Table 1 and Figure 2) indicates that the molecular surface of the PEG-gelatin micelle was covered with PEG molecules grafted on gelatin. The PEG-gelatin micelle showed an apparent molecular size in the nanometer range, but the nanomicelle disappeared by the addition of guanidine hydrochloride. Taken together, the PEG-gelatin with PEG-introduced percentages of 85 and 100 built a nanometer-size micelle structure of which the surface is covered with PEG molecules in PBS based on the hydrophobic interaction of gelatin moiety. The nature of PEG-gelatin micelles depended on the molecular weight of PEG grafted. Gelatin grafted with PEG of molecular weight 2000 showed a slightly smaller CMC value than that of PEG with molecular weight 12 000 (Table 1), indicating that the intermolecular interaction force to form the former micelle was stronger than that of the latter micelle. Because the hydrophilicity of PEG was increased with increasing PEG molecular weight, the CMC value was increased with increasing molecular weight of PEG grafted. Moreover, the former micelle showed a smaller slope than the latter one. This means that PEG-gelatin prepared from PEG of high molecular weight has a larger capacity to entrap the

hydrophobic PNA molecules. Some experiments on the molecular structure of PEG-gelatin like the number of molecular aggregation are presently in progress.

The body distribution study indicates that gelatin and PEG-gelatin were readily cleared from the plasma and consequently accumulated in the MPS organs. However, the $AUC_{[0-24]}$ value was increased as the molecular weight of PEG grafted increased at the same PEGylation degree (Figure 6). The diameter of the PEG-gelatin micelle prepared is smaller than 200 nm. The micelle will be promising to prepare a long-circulating drug carrier smaller than 200 nm in diameter being considered optimal for the stealth system to prevent a filtering mechanism in the MPS organs and an increased uptake by macrophage.[16] In fact, it is shown that long-circulating microparticulate drug carriers with sizes of 100−200 nm were effectively accumulated in various tumors tissues based on the EPR effect or "impaired filtration" mechanism.[6,7] This is extremely important from the clinical viewpoint. It is interesting to compare the in vivo behavior of these PEG-gelatin micelles with nanospheres previously reported from PEG-PLGA copolymers which are conventionally prepared as a long circulating carrier.[13] In both the cases, the blood concentration increases as the chain length increases for the same PEG density. It is important to consider that the diameter of PEG-gelatin micelles (65−120 nm) is comparable to that of PEG-PLGA nanospheres (80−150 nm) and that the PEG-gelatin micelles have a higher PEG content: 40−80% of PEG w/w in PEG-gelatin micelles compared to 10% w/w in PEG-PLA nanospheres. Irrespective of PEG molecular weight, the remaining time period of PEG-gelatin micelle in the blood circulation was longer compared with that of PEG-PLGA nanospheres.[34] In addition, the percent remaining of accumulation in the liver or spleen by the MPS was lower than that of the PEG-PLGA nanospheres. Moreover, longer PEG chains can sterically prevent enzymatic attack to gelatin molecules.[34] It is possible that the difference in the blood concentration for PEG-gelatin micelles prepared from PEG of different molecular weights is caused by the difference in their enzymatic degradation rate rather than their susceptibility to removal from the circulation by phagocytic cells.

Another question is about the biological fate of PEG. It has been demonstrated that the urinary clearance of PEG has a pronounced change at a molecular weight around 30 000, suggesting that this value represents the glomerular filtration cutoff for such nonionic polymer.[35] Thus, considering their better efficiency in reducing clearance of micelle in vivo, it is suitable to use PEG with the molecular weights of 12 000 in the micelle composition because the blood concentration was significantly higher for the PEG-gelatin prepared from the PEG molecular weight of 12 000 than that of other molecular weights (2000 and 5000). Nevertheless, the PEG density at the micelle surface could be further optimized by using other amphiphilic biodegradable polymers with more than one PEG chain per gelatin chain to obtain PEG distances as small as used in preparation of stealth liposomes[12] and nanospheres.[17] Furthermore, it would be advantageous to prepare PEG-gelatin micelles smaller than 200 nm, this being considered optimal for stealth systems to prevent a filtering mechanism in the spleen and an increased uptake by macrophages,[17] although this would reduce the payload per particle. Even if gelatin is grafted with PEG, finally the gelatin intravenously administered will be degraded enzymatically in the blood circulation. The PEG-gelatin degraded is removed by the MPS or excreted from the kidney. This is confirmed by the biodistribution result that the radioactivity of PEG-gelatin was accumulated with time in the liver rather than excreted from the kidney (Figure 6). It is well-known that the Kupffer cells play an important role in the MPS uptake in the liver. It is possible that the PEG-gelatin degraded may be taken up by the Kupffer cells.

This study indicates the micelle formation of gelatin by the PEGylation to show a higher residence behavior than gelatin alone. Interaction of this micelle with growth factor, plasmid DNA, and anticancer drugs is underway at present to create the controlled release system of drugs governed by carrier degradation together with the in vivo high residence nature based on the micelle formation. In conclusion, the pharmacokinetics and body distribution profile made this system a new candidate for intravenous administration of drugs which need to remain in the blood compartment and accumulate in the tumor or inflammation site by EPR effect.

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Novel PVA–DNA nanoparticles prepared by ultra high pressure technology for gene delivery

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Abstract

Polyvinyl alcohol (PVA)–DNA nanoparticles have been developed by ultra high pressure (UHP) technology. Mixture solutions of DNA and PVA having various molecular weights (Mw) and degree of saponifications (DS) were treated under 10,000 atmospheres (981 MPa) condition at 40 °C for 10 min. Agarose gel electrophoresis and scanning electron microscope observation revealed that the PVA–DNA nanoparticles with average diameter of about 200 nm were formed. Using PVA of higher Mw and degree of saponifications, the amount of nanoparticles formed increased. The driving force of nanoparticle formation was the hydrogen bonding between DNA and PVA. In order to apply the PVA–DNA nanoparticles for gene delivery, the cytotoxicity and the cellular uptake of them were investigated using Raw264 cell lines. The cell viability was not influenced whether the presence of the PVA–DNA nanoparticles. Further, the nanoparticles internalized into cells were observed by fluorescent microscope. These results indicates that the PVA–DNA nanoparticles prepared by UHP technology showed be useful as drug carrier, especially for gene delivery.

Keywords: Ultra high pressure; Hydrogen bond; Nanoparticles; Biocompatibility; Gene delivery; Polyvinyl alcohol

1. Introduction

Pressure processing technology has been used in many fields. The range of pressure is varied in each method from 1 to 100,000 atmosphere (atm) (9810 MPa). In the field of chemistry and biology, the pressure of over 6000 atm is thought as ultra high pressure (UHP). It is well known that the hydrogen bond is strengthens than electrostatic and hydrophobic interactions under the UHP condition [1–3]. From this fact, we recently reported that UHP is one of powerful tools for manipulatory inter- or intra-molecular interaction triggered by hydrogen bond [4]. We have shown some evidence of this hypothesis by using polyvinyl alcohol (PVA), which is synthetic hydrogen bonding polymers having simple hydrogen bonding structure, associated each other to form nanoparticles via hydrogen bond by UHP processing [5]. Among various fields of application, we focused on the usage of the nanoparticle as drug and gene delivery system.

Nanoparticles as gene carrier are able to enhance intracellular gene delivery in vitro and in vivo due to protection of DNA from nuclease cleavage [6–10]. Many types of them, such as cationic compounds [6–8], biodegradable polymers

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[9,10] have been developed. Nanoparticles containing DNA have been formed by electrostatic interaction between negative charge of phosphate groups of DNA and positive charge of cationic compounds or encapsulation. However, it was reported that such cationic substances has the essential problem of the cytotoxicity, and the difficulty of controlling of DNA release from nanoparticles.

In the present study, we report the preparation of novel nanoparticles of plasmid DNA and PVA via hydrogen bond using UHP technology and their application for gene delivery. The interaction force of nanoparticle formation is hydrogen bond between PVA and DNA, because DNA is one of typical hydrogen bonding polymer as well as PVA. Further, the biocompatibility and neutral charge nature of PVA allows the low cytotoxicity. The cellular uptake of them was investigated in order to evaluate the nanoparticles as biocompatible gene carriers.

2. Materials and methods

2.1. Preparation of PVA–DNA nanoparticles by UHP method

PVAs having different molecular weights and degree of saponifications were supplied from Kuraray (Osaka, Japan) (Table 1). Plasmid DNA encoding green fluorescent protein under cytomegalovirus promoter (pEGFP-C1) was obtained from BD Biosciences Clontech (Tokyo, Japan). PVA solutions (0.0001–0.1 w/v%) and pEGFP-C1 solution (0.02 w/v%) were mixed in water and treated under 10,000 atm at 40 °C for 10 min (UHP method) using high-pressure machine (Dr. Chef, Kobe Steel, Kobe, Japan).

2.2. Characteristics of PVA–DNA nanoparticles

At 0.0001–0.01 w/v% of PVA concentration, PVA and pEGFP-C1 mixture solutions treated with UHP were analyzed by agarose gel electrophoresis (1.0 w/v%, 100 V, 1 h). At 0.025–0.1 w/v% of PVA concentration, after centrifugation of the UHP-treated mixture solutions at 5000 rpm for 5 min, the supernatant was collected and the precipitation was washed by water. This procedure was carried out twice. The precipitation was melted by heat treatment for 10 min. They were electrophoresed though 1.0 w/v% agarose gels at 100 V for 1 h. The gels were stained with ethidium bromide. The shape and size of structures were observed by scanning electron microscope (JSM-6301F, JEOL, Tokyo, Japan).

Table 1
Various polyvinyl alcohols used

PVA	DP[a]	DS[b]	Mw
PVA205	500	88	22,000
PVA105	500	98.5	22,000
PVA117	1700	99.3	74,800
PVA140	4000	99.8	176,000

[a] Degree of polymerization.
[b] Degree of saponification.

2.3. Cytotoxicity of PVA–DNA nanoparticles

Mouse macrophage cell lines of Raw264 cells were cultured in a complete modified eagle medium (DMEM, Invitrogen, Tokyo, Japan), supplemented with non-inactivated 10% fetal calf serum (FCS), 50 IU/ml of penicillin, 50 μg/ml of streptomycin (ICN Biomaterials, Ohio, USA). To evaluate the cytotoxicity of PVA–DNA nanoparticles, 2.0×10^4 cells incubated with PVA–DNA nanoparticles at 37 °C for 20 h in the present of FCS and the number of viable cells was assessed using a Cell Counting Kit-8 (Dojindo Laboratory, Tokyo, Japan) according to the manufacturer's instruction.

2.4. Cellular uptake of PVA–DNA nanoparticles

To investigate the cellular uptake of PVA–DNA nanoparticles, pEGFP-C1 labeled with rhodamine by Label It kit (Panvera, WI, USA) was added on 2.5×10^5 cells of Raw264 cells cultured in the present of non-inactivated FCS and incubated at 37 °C for 20 h. The cells were observed under fluorescent microscope.

3. Results and discussion

Fig. 1 shows the microscopic observation of the mixture solutions of pEGFP-C1 and various PVAs at 0.1 w/v% concentration treated with UHP after centrifugation at 5000 rpm for 5 min. The mixture solution of PVA205 remained as clear solution as well as pEGFP-C1. However, a little precipitation was observed in PVA105 and the white precipitation was observed in the case of PVA117 and PVA 140 (Fig. 1(A)). When DNA solution mixed with PVA117 at different concentration were pressurized under UHP condition, the amount of white precipitation was decreased with decreasing PVA concentration, and the precipitation was not observed at 0.01 w/v% of PVA117 (Fig. 1(B)). These results indicate that the size of particle obtained varied in each molecular weight and concentration of PVAs used, and that the higher molecular weights of PVA tended to form particles. This phenomena was observed even when the PVA solution without DNA was treated with UHP (data not shown). Fig. 2 shows SEM images of the UHP treated mixture solutions of DNA in the presence of (A) 0.01 w/v% or (B) 0.025% of PVA. Nanoparticles having average diameter of about 200 nm were observed in 0.01 w/v% of PVA concentration. At 0.025 w/v% concentration, the nanoparticles aggregated each other. It became clear that the precipitation formation at higher PVA concentration under UHP condition due to the aggregation of nanoparticles of PVA or PVA/DNA mixture.

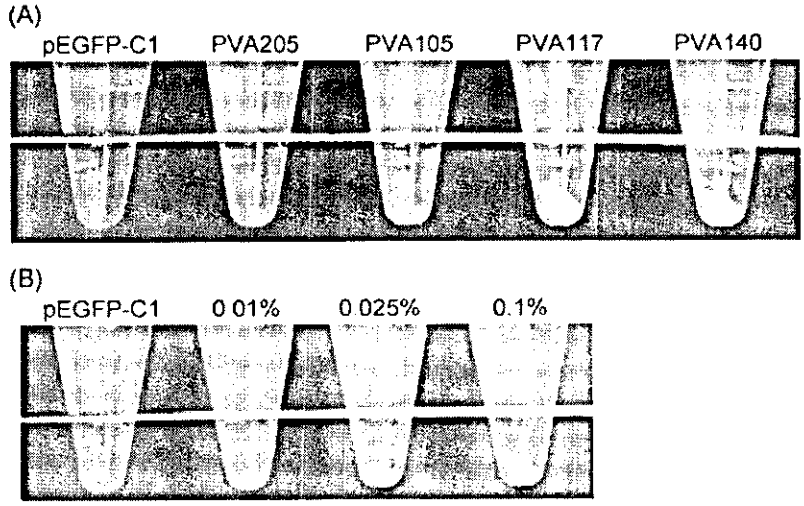

Fig. 1. Microphotographs of mixture solutions of DNA and (A) various PVAs of 0.1% concentration and (B) PVA117 of different concentration treated by UHP.

To confirm whether the nanoparticles contain DNA, the mixture solutions of DNA and PVA140 at less than 0.01% concentration treated with UHP were analyzed by agarose gel electrophoresis (Fig. 3(A)). The DNA bands in the non-treated mixture solutions were observed at the same pattern of pEGFP-C1, which contains circular, linear and super coiled form, irrespective of that concentration. On the other hand, the smear bands of DNA–PVA nanoparticles appeared at each concentration, indicating the nanoparticles consisting of DNA and PVA, but not PVA only. The heat melted aggregates of nanoparticles were

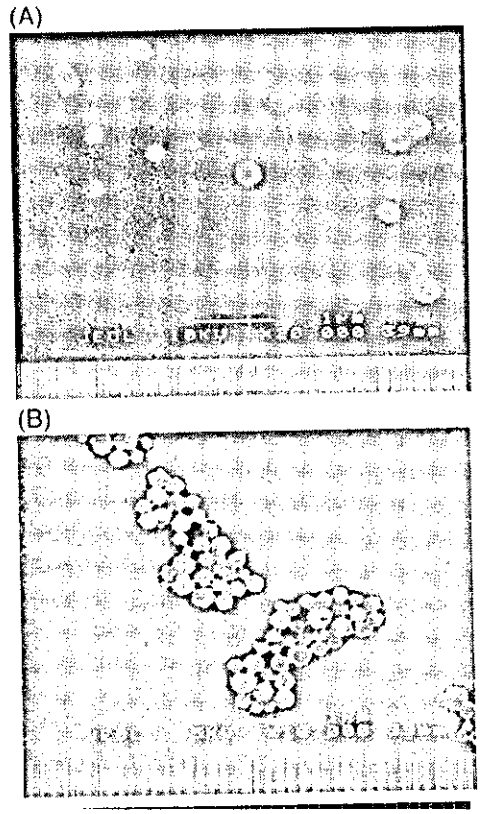

Fig. 2. SEM images of PVA–DNA nanoparticles. PVA117 was used.

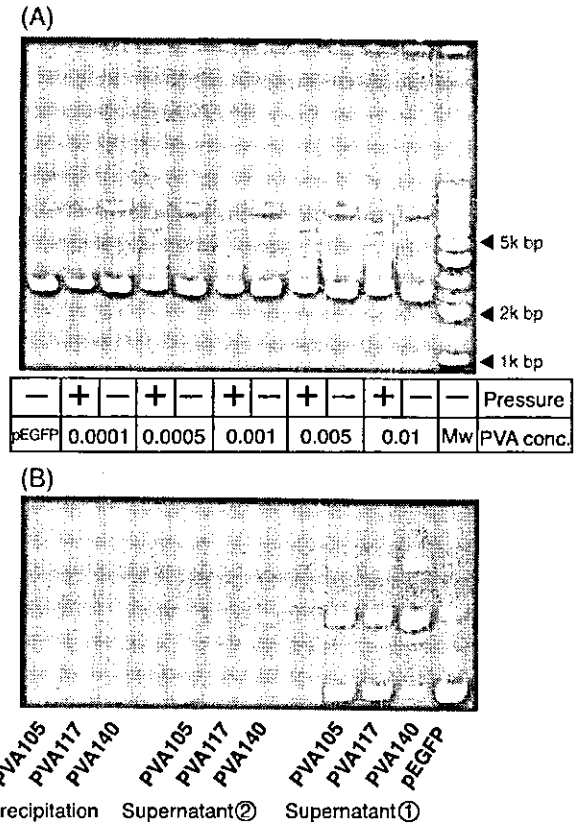

Fig. 3. Agarose gel electrophoresis of (A) PVA117-DNA nanoparticles prepared at 0.01% concentration and (B) the aggregates of PVA–DNA nanoparticles at 0.1% concentration after heat treatment.

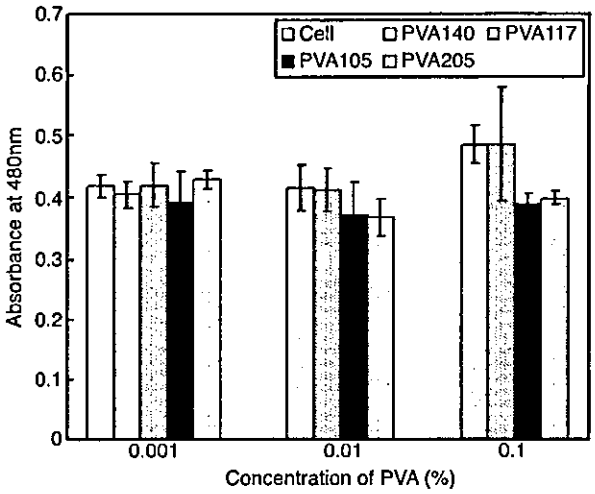

Fig. 4. Cytotoxicity of PVA–DNA nanoparticles.

also electrophoresed with agarose gel after twice washing procedure (Fig. 3(B)). The bands of DNA were observed not only in first and second supernatants but also in the collected precipitation. It was clear that the nanoparticles consisting of PVA and DNA.

Fig. 4 shows the result of the toxicity test of PVA–DNA nanoparticles. The result of high viability of Raw264 cells incubated with PVA–DNA nanoparticles was obtained irrespective of the molecular weights of PVA used. This result indicates that PVA–DNA nanoparticle is non-toxic. Conventionally, cationic polymers were widely used for gene delivery due to complex formation with DNA by electrostatic interaction, however, the cell damage for cationic nature of them was pointed out. Yamaoka et al. [11] reported that the cytotoxicity decreased with decreasing the charge density of polycations. Fischer et al. [12] suggests the necessity of optimizing the balance between the cytotoxicity and the biocompatibility of cationic polymers used as gene carrier. Therefore, it is considered that non-charged PVA permitted the low cytotoxicity of PVA–DNA nanoparticles formed by hydrogen bond.

In order to investigate cellular uptake of the PVA–DNA nanoparticles, the nanoparticles of PVA and pEGFP-C1 labeled with rhodamine were added to Raw264 cells in the present of FCS. In Fig. 5, a lot of red fluorescence spots

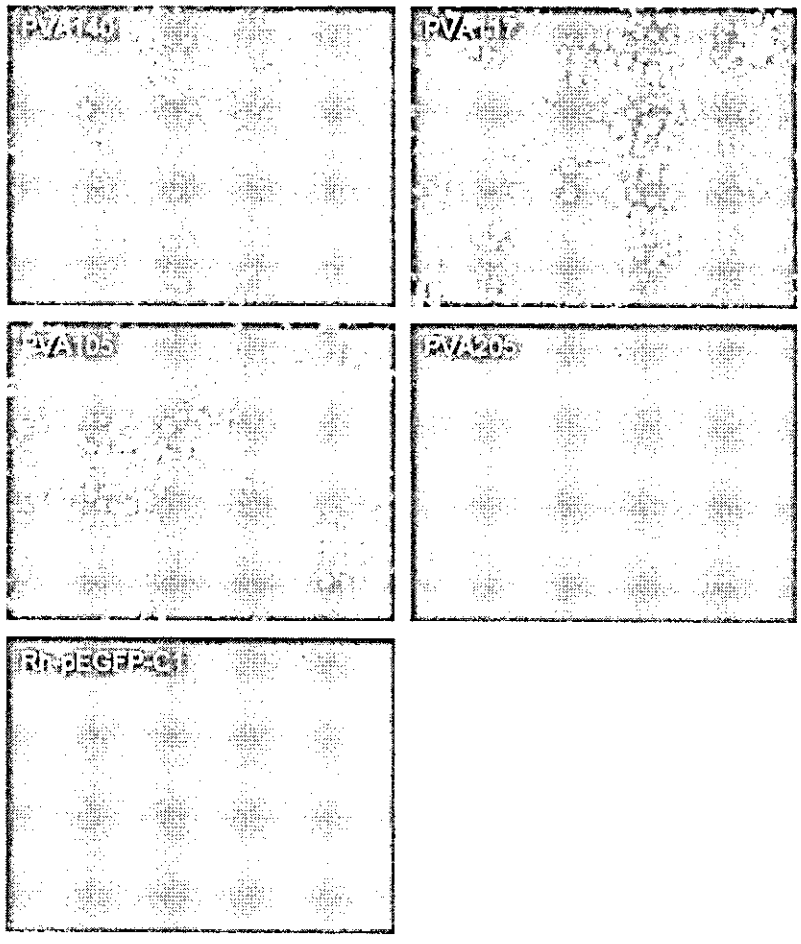

Fig. 5. Fluorescent images of Raw264 incubated with the nanoparticles of PVA and rhodamine-labeled pEGFP-C1 for 24 h.

in many cells were brightly observed in the case of PVA105, PVA117 and PVA140 except for pEGFP-C1 and PVA205. This result suggests that the significant internalization of DNA which means that the PVA–DNA nanoparticle was incorporated into cells. In the case of PVA205, as PVA–DNA particles formation was insufficient, low incorporation result was obtained. These results suggest that PVA–DNA nanoparticles have favorable characteristics for gene delivery system, are non-cytotoxic and high gene transfer into cell. The uptake of PVA–DNA nanoparticles by cells is probably achieved by complement activation because it is well-known fact that PVA activates complement system.

4. Conclusion

We have developed nanoparticles consisting DNA and PVA via hydrogen bonds using UHP technology. The average nanoparticle diameter was 200 nm. The nanoparticle formation could be controlled by the molecular weight of PVA used. Cell viability studies following incubation with the nanoparticles confirmed the lack of toxicity of PVA. The ability of the nanoparticles to delivery DNA into cells was also shown, and PVA–DNA nanoparticles are considered as a potential candidate for a gene carrier.

Acknowledgements

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Stem Cell Review Series

Endothelial progenitor cells: past, state of the art, and future

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Abstract

Recent evidences suggest that endothelial progenitor cells (EPCs) derived from bone marrow (BM) contribute to *de novo* vessel formation in adults occurring as physiological and pathological responses. Emerging preclinical trials have shown that EPCs home to sites of neovascularization after ischemic events in limb and myocardium. On the basis of these aspects, EPCs are expected to develop as a key strategy of therapeutic applications for the ischemic organs. Such clinical requirements of EPCs will tentatively accelerate the translational research aiming at the devices to acquire the optimized quality and quantity of EPCs. In this review, we attempt to discuss about biological features of EPCs and speculate on the clinical potential of EPCs for therapeutic neovascularization.

Keywords: endothelial progenitor cell (EPC) - vasculogenesis - angiogenesis - therapeutic neovascularization - cardiovascular disease - cell therapy

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Introduction

The identification of EPCs derived from BM was an outstanding event of stem cell biology in the field of vascular biology. This unique cell population existing in peripheral blood mononuclear cells (PBMNCs) derived from BM shares a similar profile to that of hematopoietic stem cells (HSCs) and incorporates into foci of physiological or pathological neovascularization in response to various angiogenic growth factors. Considering the importance of blood vessel formation on embryonic organogenesis, the development of tissue and organ regeneration could not be able to be realized without understanding the biological mechanisms of vasculogenesis by EPCs. This review provides an update of EPC biology as well as highlighting their potential utility for therapeutic neovascularization.

Post-natal vasculogenesis

EPCs, HSCs related descendants, have been isolated from human adult PBMNCs [1, 2]. Flk-1 and CD34 antigens were used to detect putative EPCs [3]. 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 'hemangioblast'.

In vitro, EPCs 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 [4–7] using equivalent or different methodologies. Recently, similar studies with EPCs isolated from human cord blood have demonstrated their analogous differentiation into ECs in vitro and in vivo [8, 9]. These findings, together with other recent studies [10, 11], are consistent with the notion of postnatal "vasculogenesis", which is de novo vessel formation by *in situ* incorporation, differentiation, migration, and/or proliferation of BM-derived EPCs [3] (Fig. 1).

Several studies have demonstrated that BM-derived EPCs functionally contribute to vasculogenesis during wound healing [12], limb ischemia [1, 3, 13–17], postmyocardial infarction [18, 19], endothelialization of vascular grafts [2, 12, 20, 21], or physiological cyclic organogenesis of endometrium [3] under the influence of appropriate cytokines, growth factors and/or hormones through the autocrine, paracrine, and/or endocrine systems.

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 BM (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 [22] or growth factor-induced neovascularization [23]. Also, in the tumor neovascularization, the range is approximately 35-45% higher than the former events [24]. The degree of EPC contribution to post-natal neovascularization is predicted to depend on each vessel formation event or disease.

More recently, Tamaki et al. reported that tissue specific stem/progenitor cells with the potency of differentiation into myocytes or ECs were isolated in skeletal muscle tissue of murine hindlimb, although the origin remains to be clarified [25]. This studies have introduced the concept that the origin of EPCs may not be limited to BM, *e.g.* tissue specific stem/progenitor cells possibly provide '*in situ* EPCs' as other sources of EPCs than BM. (Fig. 1)

Profiles of EPCs in adults

Since the initial report of EPCs [1][2], a number of groups have set out to define this cell population more profoundly. Because EPCs and HSCs share many surface markers, and no simple definition of EPCs exists, various methods of EPC isolation have

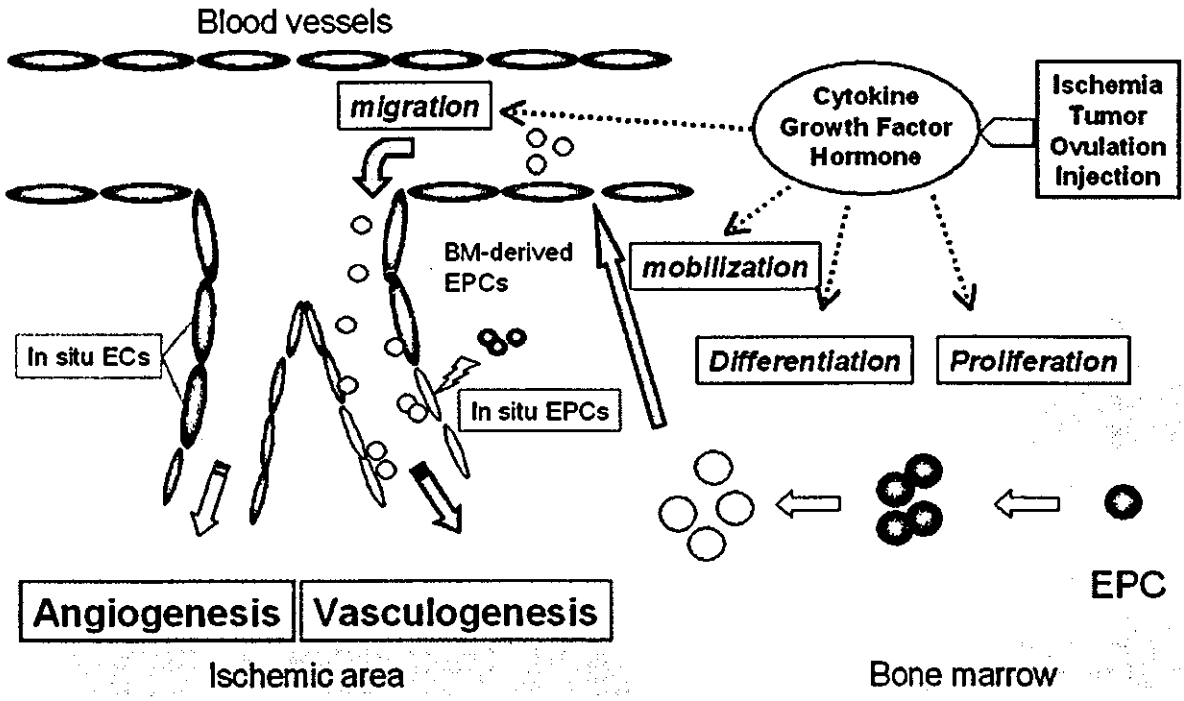

Fig. 1 Post-natal neovascularization in the physiological or pathological events is consistent with neovessel formation contributed by angiogenesis and vasculogenesis at the various rates between their two mechanisms. Angiogenesis and vasculogenesis are due to the activations of *in situ* ECs and BM-derived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

been reported [1, 2, 4, 6–9, 15, 16]. The term of EPC may therefore encompass a group of cells that exist in a variety of stages ranging from hemangioblast to fully differentiated endothelial cell (EC).

Under the current status, it is impossible to differentiate 'immature EPCs' from primitive HSCs, as those cells share common surface markers, i.e. CD133, CD34, or VEGFR2 (KDR). In circulation, the cell population with the capacity of differentiation to EPCs is considered to be included in the cell population expressing CD133 and VEGFR2 markers in the subset of CD34 positive cells [7]. Circulating EPCs are constitutively expressing stem/progenitor markers, i.e. CD34 or VEGFR2 except CD133, and start expressing endothelial lineage specific markers, VE cadherin or E-selectin. On the other hand, following the commitment and differentiation to hematopoietic stem/progenitor cells, the surface markers of CD133 and VEGFR2 are extinguished. Such stem/progenitor cell markers do not express on the differentiated hematopoietic cells. Alternatively, kinds of surface markers are expressed to characterize individual hematopoietic cell populations. CD133 is a marker to differentiate immature EPCs or primitive HSCs from circulating EPCs. To differentiate EPCs from hematopoietic stem/progenitor cells, VE cadherin or E-selectin are useful. Accordingly, circulating EPCs may be isolated via selection by the antigenicity of CD34, VEGFR2, and/or VE cadherin and also circulating immature EPCs by CD133 (Fig. 2).

In adult human body, there is a strong evidence to suggest that impaired neovascularization results in part from diminished cytokine production. However, endogenous expression of cytokines is not the only factor leading to impaired neovascularization. Diabetic or hypercholesterolemic animals-like clinical patients-exhibit the evidence of dysfunction in mature endothelial cells. While the cellular dysfunction does not necessarily preclude a favorable response to cytokine replacement therapy, the extent of recovery in limb perfusion in these animals fails to reach that of control animals; this suggests another limitation imposed by a diminished responsiveness of EPCs/ECs. Recently Vasa *et al.* have further investigated EPC kinetics and their relationship to clinical disorders, showing that

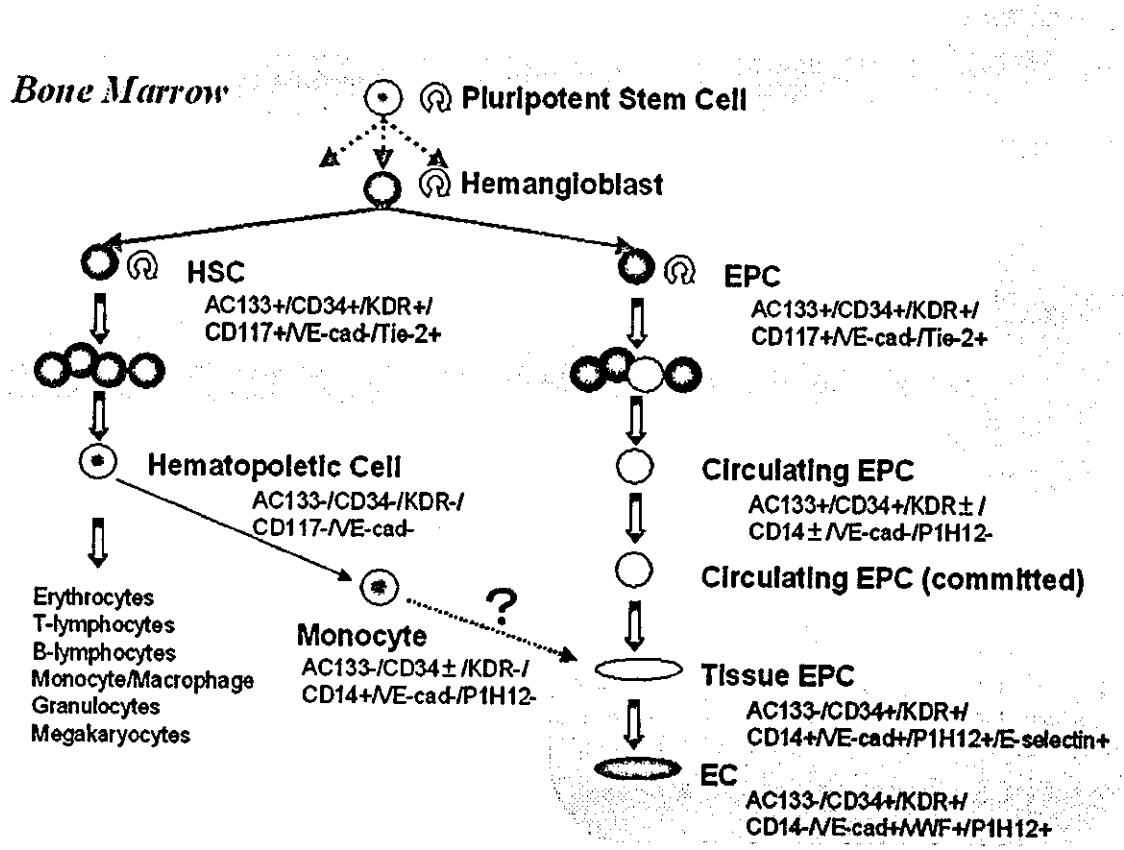

Fig. 2 Putative cascade and expressional profiles of human bone marrow-derived endothelial progenitor cell differentiation. (+: positive, –: negative).

the number and migratory activity of circulating EPCs inversely correlate with risk factors for coronary artery disease, such as smoking, family history and hypertension [26]. Tepper *et al.* reported that proliferation and tube formation of EPCs were down regulated in patients with type 2 diabetes compared with normal subjects [27]. Valgimigli *et al.* indicated that circulating EPCs decreased in patients with severe heart failure (HF) [28]. On the basis of these findings, monitoring of BM-derived EPC kinetics in the patients with vascular diseases is expected to be valuable in the evaluation of lesion activity and/or therapeutic efficacy.

The aging characterized by impaired neovascularization might be also associated with dysfunctional EPCs and defective vasculogenesis. Indeed, preliminary results from our laboratory indicated that the 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. 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 disclosed a significant reduction in the number of circulating EPCs before and after VEGF165 gene transfer; 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.

Regulation of EPC Mobilization

EPC kinetics in adults

Given the result of common antigenicity, BM has been considered the origin of EPCs as HSCs in adults. The BMT experiments have demonstrated the incorporation of BM-derived EPCs into foci of physiological and pathological neovascularization [3]. Wild-type mice were lethally irradiated and transplanted with BM 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 BM 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 [3].

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, the recent data have shown that mobilization of BM-derived EPCs constitutes a natural response to tissue ischemia. The former murine BMT model presented the direct evidence of enhanced BM-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 -galactosidase in the corneas of mice with, versus those without, an ischemic limb [17]. This finding indicates that circulating EPCs are mobilized endogenously in response to tissue ischemia, following the incorporation of EPCs into the foci neovascularization to promote tissue repair. Moreover, such concept were also reflected in clinical findings of EPC mobilization in patients with coronary artery bypass grafting, burns [12], and acute myocardial infarction [19].

EPC mobilization by endogenous agents

Having demonstrated the potential for endogenous mobilization of BM-derived EPCs, we considered that artificial 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) is well known to stimulate hematopoietic progenitor cells and myeloid lineage cells, but has recently been shown to exert a potent stimulatory effect on EPC kinetics. The delivery of this cytokine induced EPC mobilization and enhanced neovascularization of severely ischemic tissues and de novo corneal vascularization [17].

Among other growth factors, vascular endothelial growth factor (VEGF), critical for angio/vasculogenesis in the embryo, has recently been shown to be the critical factor for vasculogenesis and angiogenesis. Our studies carried out first in mice [13] and subsequently in patients undergoing VEGF gene transfer for critical limb or myocardial ischemia [29] established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is in part by mobilizing BM-derived EPCs. Similar modulation of EPC kinetics has been observed in response to other hematopoietic stimulators, such as granulocyte-colony stimulating factor (G-CSF), angiopoietin-1 [30], stroma-derived factor-1 (SDF-1) [31], and erythropoietin [32], or endogenous hormone, estrogen [33, 34].

EPC mobilization by exogenous agents

This potent 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 [35]. Recently, we [36] and Dimmeler and colleagues [37] demonstrated a novel function for HMG-CoA reductase inhibitors that contributes to postnatal neovascularization by augmented mobilization of BM-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.

On the other hand, some antiangiogenic agents, *i.e.* angiostatin or soluble flk-1, have been shown to inhibit BM-derived EPC kinetics, leading to tumor regression, as BM-derived EPC kinetics is a critical factor for tumor growth, in terms of tumor neovascularization [38].

Therapeutic potential 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. When placed *in vivo*, these cells are then provided with the proper milieu that allows them to reconstitute organ systems. 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 and colleagues.

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.

Indications of EPC transplantation

Three kinds of clinical states could be currently applied to indications of EPC transplantation, (1) Critical limb ischemia such as arteriosclerosis obliterans (ASO) or Burger disease, (2) Post myocardial infarction which is excluded from percutaneous catheter intervention (PCI) or coronary artery bypass grafting (CABG), (3) Vascular graft as a means of improving biocompatibility.

(1) Our studies indicated that cell therapy with ex vivo expanded EPCs could 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 [15]. Not only did the heterologous cell transplantation improve neovascularization and blood flow recovery, but also led to important biological outcomes-notably, the reduction of limb necrosis and auto-amputation by 50% in comparison with controls. Murohara et al. reported similar findings in which human cord blood-derived EPCs also augmented neovascularization in a hindlimb ischemic model of nude rats, followed by *in situ* transplantation [9]. In addition, Shatteman *et al.* [16] 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. These findings provided novel evidence that exogenously administered EPCs rescue impaired neovascularization in an animal model of critical limb ischemia.

(2) A similar strategy with limb ischemia applied to a model of myocardial ischemia in the nude rat demonstrated that transplanted human EPCs localize 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 [39]. Kocher *et al.* 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 [40]. These strategies 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.

(3) 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 [20]. 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 [21].

Cell source and modification of EPC for transplantation

A critical limitation for the therapeutic application of postnatal EPCs is their low number in the circulation. Especially patients with cardiovascular risk factors, aging, or HF who are the candidate for cell therapy have been considered to possess lower EPCs.

Ex vivo expansion of EPCs cultured from PBMNCs of healthy human volunteers typically yields 5.0×10^6 cells per 100 ml of blood on day 7. Our animal studies [15] suggest that heterologous transplantation requires systemic injection of $0.5\text{-}2.0\times10^4$ 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 l may be necessary to obtain adequate numbers of EPCs to treat critical limb ischemia in patients.

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, cytokine, or drugs) to promote BM-derived EPC mobilization [13, 17], (3) enrichment procedures, *i.e.* leukapheresis or BM aspiration, or (4) enhancement of EPC function by gene transduction, (5) *ex vivo* expanded EPCs from self-renewable primitive stem cells in BM or other tissues, (6) allogenic EPCs derived from umbilical cord blood (Fig. 3).

These approaches of EPC modification to acquire the ideal quality and quantity of EPCs for EPC therapy have already been applied to clinical patients in some institutions and preliminary results are expected to come out in the near future.

In some cases, nonselected total BM cells or BM-MNCs including immature EPC population have also been investigated for their potential to induce neovascularization. Several experiments have reported that autologous BM administration into hindlimb ischemic model and myocardial ischemic model, and could augment neovascularization in ischemic tissue mainly through the production of angiogenic growth factors and less through the differentiation of a portion of the cells into EPCs/ECs *in situ*. Although there are no long-term safety and efficacy data for local delivery of such cell population mostly composed of inflammatory leukocytes, these strategies have already been investigated in some institutions.

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. 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.

We have recently shown for the first time that gene-modified EPCs rescue impaired neovascularization in an animal model of limb ischemia [14]. 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 [15]. Thus, EPC cell therapy combined with gene (*i.e.* VEGF) transduction may be one option to overcome the limited number and function of EPCs that can be isolated from peripheral blood in patients.

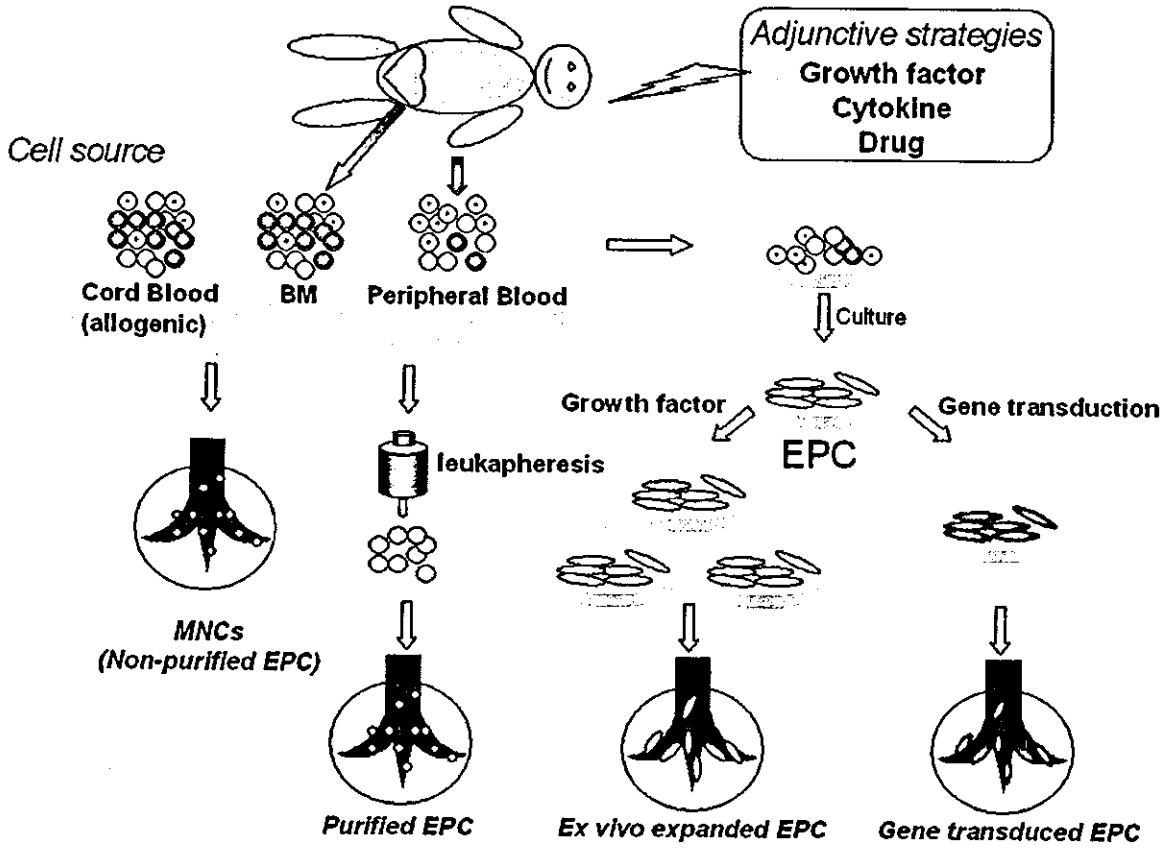

Fig. 3 Therapeutic application of EPCs for neovascularization.

EPC preview

EPCs have also been investigated in the cerebrovascular field. Embolization of the middle cerebral artery in Tie2/lacZ/BMT mice disclosed that the formation of new blood vessels in the adult brain after stroke involves vasculogenesis/EPCs. Similar data were reported using gender-mismatched wild-type mice transplanted with BM from green fluorescent protein-transgenic mice. 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. showed that BM-derived EPCs contribute to tumor neovasculature and that BM cells transduced with an anti-angiogenic gene can restrict tumor growth in mice. Lyden et al. recently used angiogenic defective, tumor resistant Id-mutant mice and showed the restoration of tumor angiogenesis with BM (donor)-derived EPCs throughout the neovessels following the transplantation of wild-type BM into these mice. These data demonstrate that EPCs are not only important but also critical to tumor neovascularization. Given the findings, 'anti-tumor EPC mediated gene therapy' by transplantation of EPCs transferred genes to inhibit tumor growth may be developed in the near future.

Pulmonary hypertension might also be included into EPC therapy candidates. Nagaya *et al.* [41] reported that transplantation of vasodilator gene-transduced EPCs derived from umbilical cord blood ameliorates pulmonary hypertension in rats.

Conclusion

BM-derived EPCs in adults possess numerous potentials as clinical tools for cardiovascular dis-

ease, tissue engineering, tumor, and so on. To acquire the more optimized quality and quantity of EPCs, several 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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