Intratracheal Gene Transfer of Adrenomedullin Using Polyplex Nanomicelles Attenuates Monocrotaline-induced Pulmonary Hypertension in Rats

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Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by progressive PAH and right ventricular failure. Despite recent advances in therapeutic approaches using prostanoids, endothelin antagonists, and so on, PAH remains a challenging condition. To develop a novel therapeutic approach, we have established a nonviral gene delivery system of poly(ethylene glycol) (PEG)-based block catiomers, which form a polyplex nanomicelle with a nanoscaled core–shell structure in the presence of DNA. The polyplex nanomicelle from PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide) (PEG-*b*-P[Asp(DET)]), having ethylenediamine units at the side chain, showed ~100-fold increase in luciferase transgene expression activity in mouse lung via intratracheal administration with a minimal toxicity compared with the polyplex from linear poly(ethylenimine) (LPEI). The transfection activity was highest on day 3 after administration and remained detectable until day 14. PEG-*b*-P[Asp(DET)] polyplex nanomicelles were formulated with a therapeutic plasmid bearing the [Q2] human adrenomedullin (*AM*) gene and intratracheally administered to rats with monocrotaline-induced pulmonary hypertension. The right ventricular pressure significantly decreased 3 days after administration as confirmed by a notable increase of pulmonary human AM mRNA levels. Intratracheal administration of PEG-*b*-P[Asp-(DET)] polyplex nanomicelles showed remarkable therapeutic efficacy with PAH animal models without compromising biocompatibility.

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Idiopathic pulmonary arterial hypertension (PAH) is a rare disease characterized by a progressive increase in pulmonary vascular resistance, leading to right heart failure and death.[1] Recent advances in therapeutic approaches to PAH show promising targeting pathways believed to play critical pathogenic or pathophysiologic roles;[2] however, despite these findings, PAH remains a challenging condition.[3]

Adrenomedullin (AM), a peptide isolated from human pheochromocytoma,[4] has multiple beneficial effects on cardiovascular tissues, including a powerful hypotensive effect.[5] Moreover, AM is indicated for PAH because of its prodilatory effects and the abundance of AM receptors in the lung.[6] Inhalation of AM was reported to ameliorate PAH in animal models[7] as well as in PAH patients without inducing systemic hypotension, but this effect was transient.[8] To overcome these barriers, a new, efficacious, and long-lasting AM therapy for PAH is warranted.

Gene therapy is one of the strategic approaches to continuously supply therapeutic peptides or proteins to target tissues.[9] Gene delivery to the lung via inhalation can avoid many problems associated with intravenous delivery, such as immediate nuclease degradation in the blood stream and the difficulty associated with penetrating endothelial barriers. In this regard, AM-based gene therapy through intratracheal route for PAH may have a promise.[7] Successful gene delivery via inhalation strongly depends on the development of advanced gene vectors to protect the therapeutic plasmid, provide site-specific targeting, and effectively release these plasmids for the desired pharmacological effect.

To develop a novel gene therapy system, we utilized our established polymeric library consisting of poly(ethylene glycol) (PEG)-based block catiomers, which form core–shell polyplex nanomicelles with core sequestration of the therapeutic plasmid.[9,10]

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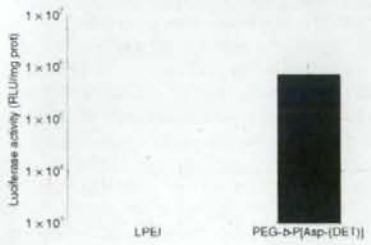
These polyplex nanomicelles are well dispersed even in aqueous media containing serum proteins and protect plasmid DNA from degradation by nuclease *in vivo*.[11–13] We recently developed P[Asp(DET)], a poly(aspartamide) derivative bearing an *N*-(2-aminoethyl)aminoethyl group as the side chain, that showed improved transfection efficiency and biocompatibility compared to linear poly(ethylenimine) (LPEI).[14] The PEG-based block cationomer with P[Asp(DET)] was applied *in vivo* to deliver therapeutic plasmids for a murine, skull bone defect model and a rabbit carotid artery with neointima model; its successful therapeutic efficacy with these mammalian studies provided the impetus for expanded application into the treatment of intractable diseases suited for gene therapy.[15,16]

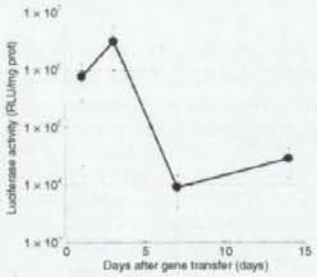
In this paper, we report advanced, pulmonary transfection efficiencies using intratracheally inhaled PEG-*b*-P[Asp(DET)] polymeric nanomicelles without compromising biocompatibility. The intratracheal administration of the *AM* gene by PEG-*b*-P[Asp-(DET)] polyplex nanomicelles reduced right ventricular pressure in PAH animal models without inducing inflammation, suggesting its suitability as a vector for translational research.

Reporter gene transfer using PEG-*b*-P[Asp(DET)] via intratracheal administration

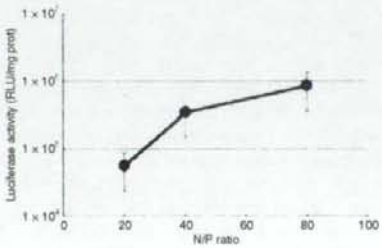
Plasmids bearing the luciferase reporter gene were formulated with PEG-*b*-P[Asp(DET)] (N/P = 80) and LPEI (N/P = 6) and were sprayed intratracheally into imprinting control region mice. Here, N/P ratio refers to the unit molar ratio of the amino group in the polymer to the phosphate group in the plasmid DNA. After 1 day, the mice were killed and the pulmonary tissues were harvested to quantify luciferase activity. PEG-*b*-P[Asp(DET)] polyplex nanomicelles showed nearly a 100-fold increase in luciferase levels than the LPEI controls (**Figure 1**). **Figure 2** shows the time-dependent changes of luciferase gene expression in the pulmonary tissue with PEG-*b*-P[Asp-(DET)] polyplex nanomicelles. Luciferase activity was highest on day 3, and remained detectable until day 14. To elucidate the effect of PEG-*b*-P([Asp(DET)]/pLuc N/P ratios on luciferase gene expression, a series of the nanomicelles formulated under the varying N/P ratios (20, 40, and 80) were also examined. **Figure 3** shows an ~50-fold increase in luciferase expression from N/P = 20 to N/P = 80 over a 3-day period. Next, PEG-*b*-P[Asp(DET)] polyplex nanomicelles loaded with plasmid DNA bearing the yellow fluorescence protein (YFP) gene (N/P = 80) or LPEI/pYFP polyplexes (N/P = 6) were sprayed intratracheally in imprinting control region mice. After 1 day, the pulmonary tissue was harvested and the YFP gene expression was visualized by fluorescence microscopy (**Figure 4**). Significantly higher fluorescence intensity was clearly seen in the lungs treated with the PEG-*b*-P[Asp(DET)] polyplex nanomicelle than the LPEI polyplexes; moreover, YFP fluorescence activity was distinctly visible for the animals treated with PEG-*b*-P[Asp(DET)] polyplex micelles in the secondary bronchi and lower pulmonary generations. To evaluate the toxicity, immunohistochemistry was conducted on the lung tissues after the transfection of LPEI/pLuc controls (N/P = 6) (**Figure 5a–c**) or PEG-*b*-P[Asp(DET)]/pLuc (N/P = 80) (**Figure 5d–f**). The lung administered with LPEI/pLuc showed moderate infiltration of neutrophils at the



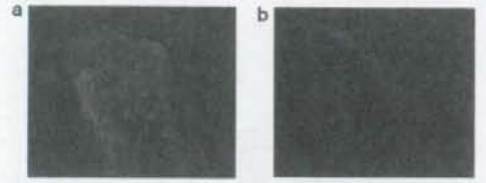
Samples of the polyplex and the polyplex nanomicelle were prepared before the administration and left for 1 day. The mice (five mice per group) were anesthetized and the polyplex or the polyplex nanomicelle was administered intratracheally. At 24 hour postadministration, the lung tissues were harvested, homogenized, and measured for luciferase activity (mean ± SEM, $N = 5$). LPEI, linear poly(ethylenimine); PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.



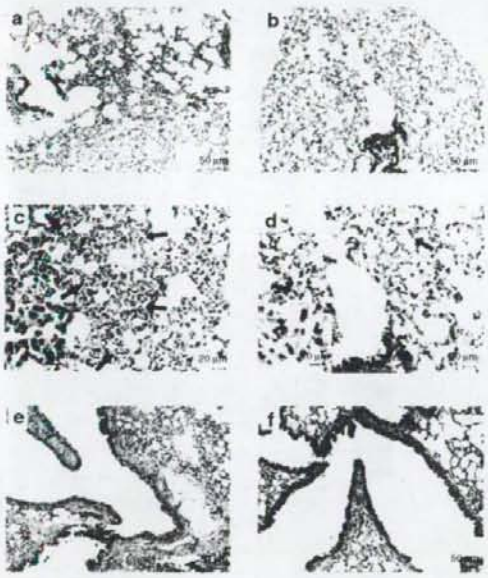
The mice (five mice per group) were anesthetized and the polyplex nanomicelle was administered intratracheally. After the indicated time, the lung tissues were harvested, homogenized, and measured for luciferase activity (mean ± SEM, $N = 5$). PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.



The mice (five mice per group) were anesthetized and the polyplex nanomicelle was administered intratracheally. At 3 days postadministration, the lung tissues were harvested, homogenized, and measured for luciferase activity (mean ± SEM, $N = 5$). PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.



(**a**) PEG-*b*-P[Asp(DET)] polyplex nanomicelle (N/P = 80), (**b**) LPEI polyplex (N/P = 6). The lung specimens were observed under a fluorescence microscope (SZX12; Olympus). LPEI, linear poly(ethylenimine); PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.



The terminal bronchiole and alveoli of the lungs administered with LPEI polyplex (**a**,**b**) and PEG-*b*-P[Asp(DET)] polyplex nanomicelle (**d**,**e**) are shown. The neutrophil infiltration is indicated with arrows in the photomicrographs with higher magnification (**b**,**e**). Each inset is the picture with higher magnification. The bronchus of the lungs administered with LPEI polyplex (**c**) and PEG-*b*-P[Asp(DET)] polyplex nanomicelle (**f**) are also shown. LPEI, linear poly(ethylenimine); PEG-*b*-P[Asp(DET)], PEG-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide}.

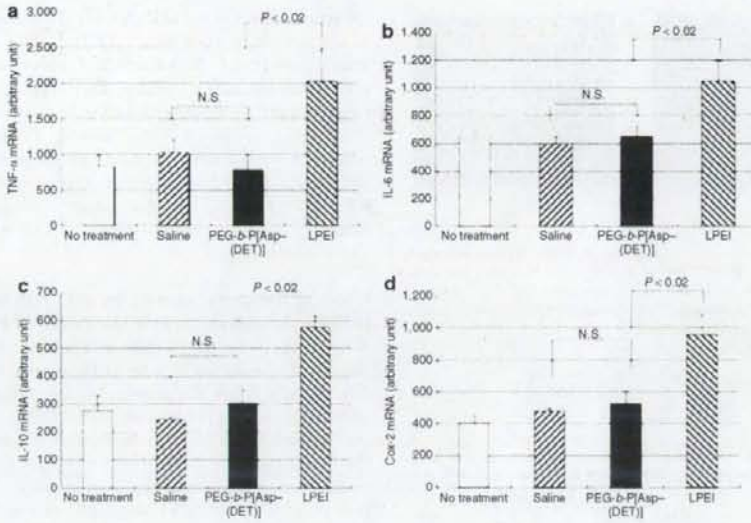
terminal bronchiole and alveoli as indicated (**Figure 5a,b**). However, the lung administered with PEG-*b*-P[Asp(DET)]/pLuc, neutrophilic infiltration was scattered and minimal or absent (**Figure 5d,e**). No apparent inflammatory infiltrate was observed in the bronchus of the both groups (**Figure 5c,f**). The findings thereby supported increased biocompatibility with the PEG-*b*-P[Asp(DET)] polyplex micelle. To further evaluate the toxicity of the gene carrier systems, mRNA levels of inflammatory cytokines in the pulmonary tissue were measured using real-time reverse transcriptase (RT)-PCR. A nontreated cohort was used as a control. Proinflammatory cytokine mRNA levels did not increase for intratracheally administered naked pLuc in saline or the PEG-*b*-P[Asp(DET)] polyplex micelles; however, LPEI/pLuc polyplexes revealed a twofold increase in TNF-α, interleukin (IL)-6, IL-10, and Cox-2 compared to the control (**Figure 6a–d**). Notably, PEG-*b*-P[Asp(DET)]/pLuc proinflammatory levels were statistically similar to the negative control cohorts in TNF-α, IL-6, IL-10, and Cox-2 mRNA levels.

Effect of *AM* gene transfer by PEG-*b*-P[Asp(DET)] polyplex nanomicelle in a rat model of PAH

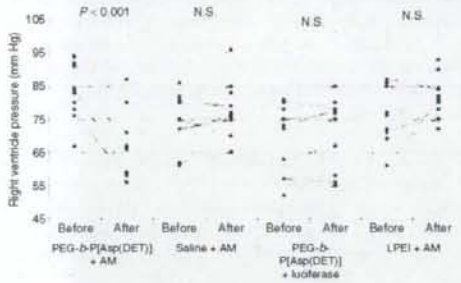
After 4 weeks of monocrotaline injection, right ventricular pressure was increased to twice of the normal value (**Figure 7**). Notably, right ventricular pressure was decreased significantly by an intratracheal spray of the PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with the expression vector of AM (N/P = 40). On the other hand, right ventricular pressure did not change significantly after administration of naked plasmid encoding the *AM* gene in saline or the LPEI polyplex loaded with the *AM* gene, or the polyplex nanomicelle loaded with the luciferase gene. The mRNA levels of human AM in the lung were measured by real-time RT-PCR (**Figure 8**). The lung transfected with the polyplex nanomicelle loaded with the expression vector of AM had high levels of AM mRNA. Alternatively, the levels were much lower in the lung transfected with the LPEI polyplex loaded with the expression vector of AM. The lung transfected with the polyplex nanomicelle loaded with the luciferase gene or with the naked *AM* gene in saline showed no expression of human AM.

DISCUSSION

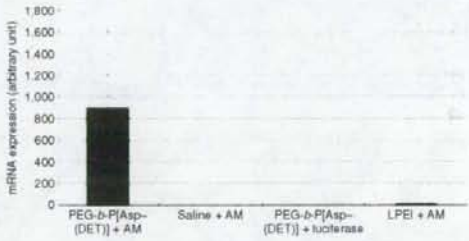
The large number of human diseases presenting poor prognoses and limited efficacy with current therapeutic regimens necessitates the advent of alternative approaches. PAH is such a disease without a highly efficacious therapeutic regimen.[17] PAH patients are currently treated with a variety of drugs including prostacyclin, prostacyclin analogues, calcium channel blockers, nitric-oxide inhalation, angiotensin-converting enzyme inhibitors, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors; in severe cases lung transplantation and subsequent immunosuppression are necessary.[17] However, promising alternative therapies for PAH have been recently reported. For example, Champion *et al.* reported that adenoviral gene transfer of endothelial nitric-oxide synthase to the lungs of endothelial nitric-oxide synthase knockout mice ameliorates the symptoms of PAH.[18] Champion *et al.* also reported that adenoviral gene transfer of calcium gene-related peptide attenuates the symptoms of PAH.[19] Nagaya *et al.* reported that transfection of human prostacyclin synthase using hemagglutinating virus of Japan-liposomes ameliorates monocrotaline-induced PAH.[20] However, in these attempts, viral or viral-related vectors were used for the delivery of therapeutic genes and these gene carriers have the potential for immunogenicity and inflammatory response. In diseases where a single dose can cure or provide palliative care, viral vectors may be suitable; however, PAH therapy requires repeated



(**a**) TNF-α, (**b**) IL-6, (**c**) IL-10, (**d**) Cox-2. IL, interleukin; LPEI, linear poly(ethylenimine); TNF, tumor necrosis factor; PEG-*b*-P[Asp(DET)], PEG-*b*-poly(*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide).



At 4 weeks after subcutaneous monocrotaline injection, a hemodynamic study was performed to measure the RV pressure indicated as "Before". The PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with AM expression vector; AM expression vector in saline; PEG-*b*-P[Asp(DET)] polyplex micelle loaded with luciferase gene; or the LPEI polyplex loaded with AM expression vector were sprayed intratracheally. Three days later, a hemodynamic study was performed again to measure the RV pressure indicated as "After". AM, adrenomedullin; LPEI, linear poly(ethylenimine); PAH, pulmonary arterial hypertension; PEG-*b*-P[Asp(DET)], PEG-*b*-poly(*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide); RV, right ventricular.



Rats were transfected intratracheally at 4 weeks after subcutaneous monocrotaline injection. Three days later, lungs were harvested, homogenized, and measured for AM mRNA using real-time RT-PCR (mean ± SEM, $N = 4$). AM, adrenomedullin; LPEI, linear poly(ethylenimine); PAH, pulmonary arterial hypertension; PEG-*b*-P[Asp(DET)], PEG-*b*-poly(*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide).

administrations for efficacy, hence the utility of viral or viral-based gene therapy is contraindicated.

Alternatively, nonviral gene carriers have been recognized with several advantages over viral vectors in terms of safety, immunogenicity, and ease of manufacture. To develop a method of gene therapy suitable for clinical translation, four primary factors must be clearly addressed: (i) the gene carrier, (ii) the therapeutic gene, (iii) the route of administration, and (iv) patient compliance. In this study, we chose to further explore the promising (i) PEG-*b*-P[Asp(DET)] polyplex nanomicelle nonviral gene carrier system based upon our previous findings. Next, we chose (ii) *AM* as the therapeutic gene because of its reported effectiveness in the transient treatment of PAH. For the route of administration, we selected (iii) intratracheal administration to avoid the rapid propensity of nuclease degradation in the blood compartment and also that we might exploit the lung, based upon its enormous surface area, for

use as a therapeutic bioreactor for AM production. Pulmonary administration is a promising therapeutic route of administration in the clinic for its (iv) high patient compliance with utilization of an inhaler or nebulizer.

Recently, we have demonstrated that PEG-*b*-P[Asp(DET)] polyplex nanomicelles achieved amplified *in vitro* and *in vivo* transfection activity with minimal cytotoxicity.[14-16,21] With regard to the transfection mechanism, P[Asp(DET)] possesses the ethylenediamine side chain, which undergoes two-step protonation from the mono-protonated gauche form at physiological pH to di-protonated anti form at acidic pH, thereby exhibiting an effective buffering function in the acidic endosomal compartment.[22] Also, we revealed the membrane destabilization effect of P[Asp(DET)] responding to acidic endosomal pH conditions by [Q9] hemolysis, leakage of cytoplasmic enzyme (lactate dehydrogenase assay), and confocal laser scanning microscopic observation.[22] Consequently, we observed the facilitated transport of Cy5-labeled plasmid DNA by the P[Asp(DET)] polyplexes from endo/lysosomal compartment into cytoplasm directly under the confocal laser scanning microscope in single cellular level.[22] Therefore, the increased transgene expression in **Figure 3** may be a result of the facilitated translocation of the polyplex nanomicelles from the endosome to the cytoplasm based on the buffering capacity (proton sponge effect) and/or endosomal membrane-destabilizing effect of P[Asp(DET)] segment. The reason why a relatively high N/P ratio was required for the efficient transfection (**Figure 3**) may be because the membrane-destabilizing effect of P[Asp(DET)] is dependent on the polymer concentration as previously reported. Nevertheless, the PEG-*b*-P[Asp(DET)] polyplex nanomicelles displayed minimal cytotoxicity even at a high N/P ratio, which may be due to the pH-sensitive properties of P[Asp(DET)] segment.[22] The highly transfectable but less cytotoxic properties of PEG-*b*-P[Asp(DET)] polyplex nanomicelles motivated us to apply them to the gene therapy of PAH animal models through the intratracheal administration in this study.

A number of nonviral vectors including polyplex and lipoplex have been applied for *in vivo* intratracheal transfection. Special notice for the pulmonary gene delivery via airways is that the lung has the features critically influencing the transfection efficiency, such as the presence of surfactant, alveolar macrophages, and mucocilliary clearance mechanisms. In the early 1990s, lipoplex was used by aerosol delivery or intratracheal instillation. However, cationic lipids were shown to have a decreased transfection efficiency due to the interaction with lung surfactant compared to cationic polymer like PEI.[23,24] To overcome the surfactant barriers, cationic emulsion was used and showed much higher transfection activity compared with lipoplexes, such as lipofectin, lipofectamine, and DMRIE/c.[25] However, even for the cationic emulsion, the luciferase activity was limited to 55 pg/mg protein, which is significantly lower than the value attained by the polyplex nanomicelles loaded with PEG-*b*-P[Asp(DET)] [3,000,000 relative length unit/mg protein (135 mg/mg protein)] as reported here. Polyplexes made from cationic polymer was reported to show higher transfection efficiency compared with cationic lipoplexes for pulmonary gene delivery via airways.[23,24] PEI or modified PEI has been shown to be one of the most effective agents for constructing gene delivery systems available today with high levels of pulmonary gene transfer by airways.[26,27] Intratracheal injection of polyplex loaded with 22 kd of LPEI (ExGen 500) showed up to 20,000–40,000 relative length unit/mg protein of luciferase activity in the lung by adjusting N/P ratio. Worth noting is that the nanomicelles achieved two orders of magnitude higher value in luciferase gene expression compared to the LPEI polyplex. Furthermore, no induction of cytokine responses is appealing for the nanomicelles over LPEI polyplex (**Figure 6**), which was reported to induce the activation of $CD8^+$ and $CD4^+$ T cells, and Fas ligand–mediated antigen-induced cell death.[28,29]

To ameliorate the symptoms of PAH in animal models, several genes have been identified including: endothelial nitric- [Q10] oxide synthase, inducible nitric-oxide synthase, PGIS, calcium [Q11] gene-related peptide, vascular endothelial growth factor, and hepatocyte growth factor.[18-20,30-36] We used AM as a therapeutic gene because of its high potency and long-term effectiveness as a vasodilator in the pulmonary vascular bed.[37] The effect of pulmonary vasodilation is mediated by cyclic adenosine [Q12] monophosphate–dependent and nitric oxide–dependent mechanisms.[38] PAH patients have elevated plasma AM concentrations, which increase in step with the disease's severity[39,40] often resulting in pulmonary hypotension. In previous studies, intravenous administration[41] and inhalation[8] of the AM peptide showed acute hemodynamic and hormonal efficacy in PAH patients. However, despite alternative routes of delivery, the small AM peptide was rapidly degraded *in vivo* displaying a poor pharmacokinetic profile with a temporal window of only 30–45 minutes. For the treatment of PAH, sustained effect of AM is required. In this report, the therapeutic indicator for successful nonviral AM delivery was a decrease in the right ventricular pressure. Indeed, for the polyplex nanomicelle/pAM formulation, the right ventricular pressure did decrease, but more importantly the persistence of *AM* gene expression continued within a therapeutic range for a minimum of 3 days. The results indicate that the therapeutic approach using the polyplex nanomicelle as a vector does not require chronic infusion or very frequent inhalation, which will make the therapy more clinically applicable.

In this study, we succeeded in delivering DNA to the lung via intratracheal administration using the polyplex nanomicelles, resulting in extremely improved transfection efficiency with concomitant high biocompatibility. More specifically, the lung transfected with the polyplex nanomicelle had much lower toxicity than that transfected with the LPEI polyplex, according to the histological findings and measurement of the mRNA levels of inflammatory cytokines (**Figure 6a–d**). We also developed an effective treatment for the PAH rat model by delivering the therapeutic *AM* gene with polyplex nanomicelles from PEG-*b*-P[Asp(DET)]. These results showed a significant increase in transfection efficiently *in vivo* with intratracheal administration. The PEG-*b*-P[Asp(DET)] polyplex nanomicelle delivery system clearly showed promising *in vivo* results of transgene and therapeutic AM expression, when coupled with the clear visual localization of the polyplex nanomicelles in the pulmonary tissue and the lack of proinflammatory responses. We posit that the PEG-*b*-P[Asp(DET)] nonviral gene carrier clearly shows those characteristics requisite for novel and advanced therapeutic systems ideally suited for translational research.

Materials. An expression vector for YFP (RIKEN, Tokyo, Japan) was amplified in competent HB101 *Escherichia coli* and purified by Plasmid Giga Kits (Qiagen, Hilden, Germany). An expression vector for luciferase with a CAG promoter was provided by RIKEN. An expression vector for human AM was constructed as follows. The *EcoRI/XhoI* fragment of the full-length human AM complementary DNA (cDNA)[12] was ligated into the *EcoRI/XhoI* site of pcDNA1 (Invitrogen, Carlsbad, CA). The restriction maps of expression vector for luciferase and human AM cDNA are listed in **Supplementary Figure S1a,b**, respectively. To confirm that pcDNA/AM
[Q13] encodes AM, pcDNA/AM was transfected into Chinese hamster ovary cells, and the medium and the cells were collected for the measurement of immunoreactive AM using an AM radioimmunoassay Shionogi (Cosmic, Tokyo, Japan).

Animals. Male imprinting control region mice weighing 25–30 g were administered the reporter gene intratracheally. Male Wistar rats weighing 100–120 g were used to make a model of PAH. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute (Osaka, Japan).

Synthesis and characterization of PEG-b-P[Asp(DET)]. PEG-*b*-P[Asp(DET)] was prepared as previously described.[14] Briefly, PEG-poly(β-benzyl-L-aspartate) (PEG-PBLA) diblock copolymer was synthesized by the ring-opening polymerization of β-benzyl-L-aspartate *N*-carboxyanhydride from the terminal primary amino group of α-methoxy-ω-amino PEG (M_n: 12,000; Nippon Oil and Fats, Tokyo, Japan). Gel-permeation chromatography confirmed that the copolymer was unimodal with a narrow molecular weight distribution (M_w/M_n: 1.23), and the number of benzyl-L-aspartate repeating units was calculated to be 68 by ^{1}H-NMR. The N-terminal amino group of PEG-PBLA was then acetylated using acetic anhydride in dichloromethane solution to obtain PEG-PBLA-Ac. The obtained polymer was dissolved in distilled *N,N*-dimethylformamide (Wako Pure Chemical Industries, Osaka, Japan) and reacted with diethylenetriamine (Tokyo Kasei Kogyo, Tokyo, Japan) for 24 hours at 40 °C in a dry argon atmosphere to undergo aminolysis of the benzyl side chain. After 24 hours, the solution was slowly dripped into a 10% acetic acid solution and dialyzed (Spectra/Por Membrane, 3,500 molecular weight cutoff; Spectrum Laboratories, Rancho Dominguez, CA) against 0.01 N HCl and subsequently against distilled water. The final solution was lyophilized to obtain PEG-*b*-P[Asp(DET)] as the hydrochloride salt form. ^{1}H-NMR confirmed the complete substitution of benzyl ester of the polymer with diethylenetriamine through the aminolysis reaction, as well as the chemical structure of the obtained PEG-*b*-P[Asp(DET)] block copolymer.

Preparation of polyplex nanomicelles. The PEG-*b*-P[Asp(DET)] block
[Q14] copolymer and plasmid DNA were separately dissolved in 10 mmol/l HEPES buffer (pH 7.4). Both solutions were mixed at the indicated nitrogen/phosphate ratios [=(total amines in cationic segment)/(phosphates in plasmid DNA)] and incubated overnight at room temperature to make PEG-*b*-P[Asp-(DET)] polyplex nanomicelle. LPEI (ExGen; Cosmo Bio, Tokyo, Japan) polyplexes were prepared by mixing plasmid DNA and LPEI according to the manufacturer's protocol.

In vivo *gene delivery by intratracheal administration.* Imprinting control region mice were anesthetized by intraperitoneal administration of pentobarbital (30 mg/kg) (Dainippon Sumitomo Pharma, Osaka, Japan). Tracheostomies were performed under sterile conditions for PEG-*b*-P[Asp-(DET)] polyplex nanomicelle or LPEI polyplex (10 μg of DNA for each mouse) in a 50 μl of solution administration by a microsprayer Model IA-1C (Penn Century, Philadelphia, PA). After the indicated time, the mice were killed by cervical dislocation and the pulmonary tissues harvested. To measure luciferase activity, the pulmonary tissues were homogenized in a lysis buffer using a polytron. The lysate was then centrifuged at 14,000*g* for 10 minutes at 4 °C, and 20 μl of the supernatant was analyzed for luciferase activity by a Luminous CT-9000D luminometer (Dia-Iatron, Tokyo, Japan), according to a previously described method.[15] Background of luciferase activity in the lung was measured from the lung of mice after administration of saline, which was <3% of the total activity of day 14. All the data of luciferase activity were obtained by subtraction of background data. To detect YFP expression, mice were killed by cervical dislocation and the lungs harvested. Frozen sections (5-μm thick) of the lung specimens were visualized by a fluorescence microscope (SZX12; Olympus, Tokyo, Japan). To examine the histological features of the lung tissue, the specimens were also fixed in 4% paraformaldehyde and embedded in paraffin. Sections (3-μm thick) were stained with hematoxylin.

Isolation of RNA and cDNA synthesis. Total RNA was extracted using the Trizol method (Gibco BRL Life Technologies, Breda, Netherlands) according to the protocol provided by the manufacturer. The RNA was dissolved in RNAse-free water and quantified by a spectrophotometer. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

Real-time RT-PCR. mRNA expression levels of TNF-α, IL-6, IL-10, Cox-2 and human AM were measured by quantitative real-time RT-PCR based on TaqMan chemistry (Applied Biosystems) using an ABI PRISM 7700 sequence detector (Applied Biosystems). The reaction mixture contained 0.5 μl of 5 μmol/l probe (final concentration, 100 nmol/l); 1 μl of 10 μmol/l forward primer and 1 μl of 10 μmol/l reverse primer (400 nmol/l final concentration of each primer); 12.5 μl of TaqMan Universal Mastermix, 5 μl of diethyl pyrocarbonate–treated water, and 5 μl of a cDNA sample. [Q15] Assay controls were performed in the same TaqMan plate with no-template controls to test for the contamination of any assay reagents. The thermocycling conditions were initiated at 50 °C for 2 minutes with an enzyme activation step of 95 °C for 10 minutes followed by 40 PCR cycles of denaturation at 95 °C for 15 seconds, and anneal/extension at 60 °C for 1 minute.

Hemodynamic studies. Hemodynamic studies were performed 4 weeks after gene transfer. Rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg) and placed on a heating pad to maintain body temperature 37–38 °C throughout the study. Under sterile conditions, a polyethylene catheter (PE-50; BD Biosciences, San Jose, CA) was inserted through the right jugular vein into the right ventricle to measure right ventricular pressure by a hemodynamic transducer (PowerLab 8/30; ADInstruments, Colorado Springs, CO).

Evaluation of gene transfer effect in a PAH rat model. Monocrotaline (60 mg/kg) was subcutaneously injected into male Wistar rats and left for 4 weeks to make a model of PAH. After 4 weeks, a hemodynamic study was performed to introduce a catheter into the right ventricle through the right jugular vein. The PEG-*b*-P[Asp(DET)] polyplex nanomicelle loaded with the AM expression vector (200 μg of DNA for each rat) in a 200 μl of solution was sprayed intratracheally. Three days later, a hemodynamic study was performed again and the gene transfer effect was evaluated. Pulmonary tissue specimens were frozen to measure *AM* gene expression by real-time RT-PCR.

Statistical analysis. All data are expressed as means ± SEM unless otherwise indicated. Comparisons of parameters among four groups were made by one-way analysis of variance, followed by Scheffe's multiple-comparison test. Paired *t*-test was applied for the comparison of the values before and after the gene transfection (**Figure 7**).

SUPPLEMENTARY MATERIAL

Figure S1. The restriction maps of expression vector for luciferase and human adrenomedullin cDNA.

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Fundamental Studies on Genetically Engineered Elastin Model Peptides for Biomaterials

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Elastin model peptide ((Val-Pro-Gly-Ile-Gly)$_{40}$; VPGIG$_{40}$) was designed and biosynthesized as injectable scaffold for cell transplantation therapies. In this report, the thermoresponsiveness and the potential as the base materials for injectable scaffold of VPGIG$_{40}$ expressed using genetic-engineering technique were explored.

Keywords: biomaterials, elastin model peptide, genetic engineering, injectable scaffold, thermoresponsiveness

Introduction

Recently, cell transplantation therapies are much attended with progress of regenerative medicine and stem-cell research. It is known that substantial effects can not be obtained only by injecting the cell suspensions and then feasible scaffolds are necessary for the cell transplantation therapies. Therefore, various biodegradable polymeric materials have been investigated as scaffolds actively. However, these scaffolds are not suitable for the cell transplantation therapy, because these bulk-type scaffolds require invasive surgery, and the cells inside the scaffold often necrotize. In order to solve these problems, photo-crosslinkable and thermoresponsible hydrogels have been investigated as injectable scaffolds. However, these non-biodegradable materials are not approved for the clinical use yet.

Elastin, the major component of elastic fibers in basement membrane, provides the resilience or restorative force to tissues. Noteworthy, soluble elastin-related polypeptides, such as tropoelastin, α-elastin and synthetic elastin model peptides, indicate the characteristics of temperature-depending phase transition. Therefore, we are interested in the potential of elastin model peptides as injectable scaffolds. In this report, we try to biosynthesize and characterize an elastin model peptide ((Val-Pro-Gly-Ile-Gly)$_{40}$; VPGIG$_{40}$) and develop the gel matrices consisting of VPGIG$_{40}$ to apply as injectable scaffold.

Results and Discussion

The elastin model peptides with repetitive sequence, VPGIG$_{40}$, were biosynthesized using *E. coli* [BL21(DE3)pLysS] which was transformed with an

expression-vector encoding VPGIG sequence [pET-28ap ($VPGIG_{40}$)] [1]. The insert region in pET-28ap ($VPGIG_{40}$) was confirmed by direct PCR of *E. coli* using primers which were flanking sequence of the insert region. By agarose electrophoresis, a band was observed at approximately 650 bp corresponding to the DNA length for the insert region of $VPGIG_{40}$. Protein expression was induced with the addition of β-isopropyl thiogalactoside (IPTG) and was allowed to continue for 24 hours. Protein purification was performed using the thermoresponsible property of $VPGIG_{40}$, namely, $VPGIG_{40}$ was purified with temperature control over/below the transition temperature. By SDS-PAGE, it is confirmed that crude $VPGIG_{40}$ was gradually purified by repeating this method and a clear band was indicated at approximately 18 kDa (Fig. 1). The purified $VPGIG_{40}$ was also confirmed by MALDI-TOF/MS analysis.

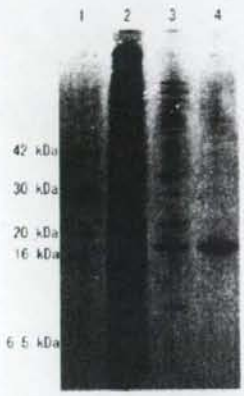

Fig. 1. SDS-PAGE (12%) for elastin model peptide $VPGIG_{40}$ at each purification step. (lane 1; marker, lane 2; bacterial lysate, lane 3; peptide purified once, lane 4; peptide purified twice)

Furthermore, the cloud points of purified $VPGIG_{40}$ were 28.2, 23.6 and 21.9 °C at 0.02, 0.05 and 0.1 w/v%, respectively. The mechanism for the temperature-depending phase transition of $VPGIG_{40}$ in solution has been suggested that the peptides are aggregated by hydrophobic interaction with conformational change from random-coil to β-spiral [2, 3].

The results suggest that genetically engineered $VPGIG_{40}$ has high potential as injectable scaffold for cell transplantation.

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幹細胞分離法とポピュレーション解析

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5 幹細胞分離法とポピュレーション解析

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5.1 はじめに

次世代医療として着目されている再生医療は，生体の細胞や組織が有している再生能力を人為的に利用して，失われた機能や構造の再生を促す治療法である。細胞ソースとしては，患者自身から採取した間葉系幹細胞などの自己幹細胞が広く検討され，その臨床利用も始まっている。このような再生医療がさらに一般的な治療法となるためには，安全性が担保された均一な幹細胞ポピュレーションの調製が必須であり，組織の中でさまざまな細胞と混在している幹細胞を，未分化状態を維持したまま効率的に分離することが求められる。さらに，これらの体性幹細胞，あるいは，近年注目されているES細胞やiPS細胞を体外で目的の細胞に分化させて利用する場合には，分化誘導の過程でさまざまな分化ステージの細胞が出現するために，ここでも，均一な細胞ポピュレーションのみを抽出する必要がある。つまり幹細胞の単離・分離法は，再生医療の治癒効果の向上や安全性を担保するためにも極めて重要な操作となる。本項では再生医療で利用が期待されている幹細胞の分離法に焦点を絞り，その現状と最新の研究成果について紹介する。

5.2 再生医療における幹細胞

幹細胞研究の急速な発展は，再生医療や細胞移植治療の可能性を大きく広げた。1998年Thomsonらにより報告されたヒトES細胞樹立[1]，1999年のPittengerらによる間葉系幹細胞の分離と分化能についての報告[2]，2002年のVerfaillieらによる間葉系幹細胞の可塑性についての発見[3]，2007年Yamanakaらによる人工多能性幹細胞（iPS）の作製の成功[4]は，再生医療における細胞ソースの可能性として大きなインパクトを与えた。しかし，分子生物学分野における幹細胞の発見や分化に関する研究成果は，直ちに再生医療へと応用できるものではない。組織から採取した幹細胞を移植細胞ソースとして利用する場合，副作用がなく安定な治療効果を達成するためには，その純度，分化の均一性，感染の可能性を十分に考慮する必要がある。表1には種々の幹細胞の特徴を示した。未分化な細胞であれば種々の疾患に対して応用することができ，その中でも，自家細胞移植は最も安全性が担保しやすいものと考えられている。

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表1　幹細胞ソースとしての特徴

分化度	細胞	利点	欠点
未分化	胚性幹細胞	多分化能	テラトーマ形成 倫理的な問題
細胞の分化度	人工多能性幹細胞	倫理的問題の回避	テラトーマ形成
	間葉系幹細胞	組織より採取可能 自家移植が可能	単離・同定が困難
	造血幹細胞		分化できる組織が限定
成　熟			

5.2.1　胚性幹細胞（ES細胞）

1998年，Thomsonらのグループはヒト ES細胞の樹立を報告した[1]。胚盤胞に形成される細胞塊から作製されるES細胞は，神経系細胞[5]，血球系細胞[6]，心筋細胞[7, 8]などさまざまな組織細胞へと分化が可能である。しかし，ES細胞の移植を考えた場合，分化能力が高い反面，目的以外の組織細胞へ分化する可能性も含まれており，奇形腫（テラトーマ）形成の危険性が危惧されている。このことから，ES細胞を試験管内で目的組織へと分化させてから移植する必要がある。また，ES細胞は受精卵より形成される胚盤胞から調製されるため，免疫拒絶や倫理的な問題も指摘されている。

5.2.2　人工多能性幹細胞（iPS細胞）

Yamanakaらのグループによって，ヒト繊維芽細胞に対して4つの遺伝子（Oct3/4，Sox2，Klf4，c-Myc）を発現させることでES 細胞様の未分化な幹細胞へとリプログラミングできることが報告された[4]。作製された細胞はiPS細胞として報告され，遺伝子の発現パターンや成熟細胞への分化，テラトーマ形成といったES細胞特有の性質をiPS細胞も有していることが示されている。その後，ガン関連遺伝子であるMycの発現を必要としなくてもiPS細胞を作製できることが同グループより報告されている[9]。細胞移植ではテラトーマ形成が問題となるものの，患者自身の繊維芽細胞から幹細胞が作製可能であることから，倫理的な問題をクリアーできる技術として期待されている。

5.2.3　間葉系幹細胞（MSC）

Pittengerらのグループにより1999年に報告されたMSCに関する研究[2]では，骨髄中に含ま

れる接着細胞ポピュレーションに骨，軟骨，脂肪へと分化が可能な幹細胞の存在が示された。しかし，この手法で得られる接着細胞ポピュレーションのすべての細胞が分化能力を有しているわけではなく，数%程度が幹細胞として機能すると考えられている。ここで報告された手法は，比較的簡便に幹細胞ポピュレーションを分離できることからGold Standardとして考えられている。また，VerfailleらによりMSCの可塑性についての研究も報告されており[2, 10]，MSCは血管内皮，神経細胞などさまざまな組織細胞への分化能力を有しているものと期待されている。細胞移植を考えた場合，テラトーマ形成の危険性がなく自家細胞移植できるMSCは，再生医療における有力な細胞ソースとして多くの臨床研究が進められている。

5.2.4 造血幹細胞（HSC）・血管内皮前駆細胞（EPC）

1961年血液学者McCullochと物理学者Tillらにより造血幹細胞（HSC）の存在が報告され，1996年Nakauchiらによりマウスの HSC[11]，1999年にはZieglerらのグループによりヒトのHSCが同定された[12]。この幹細胞は，表面マーカーが同定されているので，FACSなどにより純化した細胞を調製することが可能となる。特にHSCの中でも，長期間幹細胞としての機能を有する long-term HSCと，短期間しか機能を示さないshort term HSCという2つのポピュレーションが存在しており，この2つのポピュレーションも表面マーカーによる分離が試みられている[13, 14]。一方，1997年にはAsaharaらによりEPCが発見され[15]，血管を形成するための前駆細胞が同定された。これらの細胞は前述した幹細胞と比較して未分化度は低いものの，肝細胞や心筋細胞などへの可塑性についても報告されていることや，完全に細胞を同定できることから高純度な細胞ソースとして臨床応用が可能となる。

このように未分化度の高い細胞は，テラトーマ形成や，作製時における倫理的な問題点が危惧される。一方で，比較的成熟度の高い組織幹細胞では，同定や分離は容易であるが，分化できる組織が限定される。このような中，MSCは比較的多くの組織細胞へ分化することが可能であり患者自身の組織から容易に採取できるため，臨床応用の可能性が極めて高い幹細胞ソースであると考えられている。最も大きな問題は，組織から均一に，かつ未分化な状態を保持した状態でMSCを分離する手法の確立である。すなわち，MSCの分離法を確立することは自家移植が可能であり免疫拒絶が少ない細胞移植治療法の実現化へ向けて大きな進展をもたらすものと期待される。

5.3 細胞分離法

効率的な細胞移植療法のためには，治療効果のある幹細胞ポピュレーションを採取・分離する必要がある。ここで用いられる細胞分離法の定義には2つの異なるステージが存在するものと考えられる。1つは細胞とそれ以外の組織や物質を分けるための「細胞採取」であり，2つ目は，

ヘテロな細胞の中から目的の機能を有する細胞ポピュレーションを分けるための「細胞分離」である。組織幹細胞は細胞外マトリックスや他の成熟細胞とともに存在しているため，細胞成分を組織より採取し，その後幹細胞ポピュレーションを分離する必要がある。固形の臓器や組織から細胞を採取する場合，酵素や機械的切断により細胞懸濁液を調製する必要があるが，血液や骨髄などの液体組織では，吸引により比較的簡単に細胞懸濁液を調製できる。MSCは骨髄だけでなく脂肪組織にも存在することが報告されているが，患者から簡便に採取が可能な骨髄由来のMSCは細胞移植における有力な細胞ソースであろう。

最も重要なステップは，細胞懸濁液から移植に必要な細胞ポピュレーションのみを分離することであり，さまざまな手法が開発されている。目的の細胞ポピュレーションを分離するための細胞分離法を表2にまとめた。分離する場合の指標としては，細胞の物理的性質に基づくものと，生物学的性質に基づくものに分類されている[16]。また，細胞を分離する形式としてDigital型とContinuous型に分類した。Digital型とは，特定の指標に着目してヘテロな細胞懸濁液から1つの細胞ポピュレーションを分離する手法である。密度勾配遠心により採取される単核球細胞や，細胞表面マーカーの有無で磁気ビーズ法により分離される細胞ポピュレーションも，Digital型分離法として分類できる。これに対してContinuous型分離法とは，特定の指標に着目してその連続的な物理量により複数の細胞ポピュレーションを分離する手法である。この手法の研究例は少ないものの，連続磁場中において磁気ビーズで標識された細胞を標識量の違いに基づいて分離するデバイスや，細胞の電気的性質の違いにより細胞を分離する方法も報告されている。

表2　細胞分離技術

指　標	Force field	Type	名　称	内　容
物理的性質（細胞の大きさ，比重など）	Centrifngal	Digital	密度勾配遠心分離	単核球成分の分離
	Adhesion	Digital	Adhesion population	接着細胞分画の分離
	Electronic	Continuous	DEP (Dielectrophoresis)	電場の中で誘電率の違いによる分離
		Digital	FACS (Fluorescence activated cell sorting)	表面抗原を蛍光ビーズで標識して分離
生物学的性質（表面マーカー）	Magnetic	Continuous	DMFF (Dipole Magnet Flow Fraction)	磁場の中で標識に基づく細胞分離
		Digital	MACS (Magnetic activated cell sorting)	表面抗原を磁気ビーズで標識して分離
	Hydrodynamic	Continuous	Column system	リガンド固定化界面で細胞ローリングにより特定のマーカー密度をもつ細胞を分離
		Digital	Membrane	分離膜で特定の細胞を分離

Digital型分離法である密度勾配遠心や培養皿に対する接着細胞分画の分離では操作が非常に簡便である反面，厳密に特定細胞を分離することができない。分化度という観点から特定の幹細胞を高純度で分離する場合，細胞表面に提示される糖タンパク質（表面マーカー）など，細胞の生物学的な性質と関連がある因子に基づいて分離することが有効であると考えられる。

5.3.1 電場を利用した細胞分離

Fluorescence activated cell sorting (FACS) 法は特定の細胞表面マーカーに対して蛍光標識抗体を結合させることで目的細胞を識別し，識別された細胞を電場によりソーティングする。複数の表面マーカーを組み合わせることでより複雑なソーティングが可能になるが，移植に必要な大量の細胞を短時間で分離することは難しい。しかし，個々の細胞の表面マーカーや細胞のサイズ・内部構造を解析できること，複数の抗体染色や識別する閾値をさまざまに設定できることから，基礎研究分野においては極めて有効な手法となる。

連続的な電場において，細胞の電気的な性質の違いにより分離する手法も報告されている[17-19]。細胞に標識することなく分離できることが大きな特徴となるが，生物学的な性質の違いによる分離ではないため，現在ではその用途は限定されるものと思われる。

5.3.2 磁場を利用した細胞分離

抗体修飾磁気ビーズにより特定の細胞ポピュレーションを分離するMagnetic activate cell sorting (MACS) 法がある[20-22]。この手法は，抗体修飾磁気ビーズにより認識された細胞を磁場によりトラップし（あるいは不要な細胞をトラップし）特定の細胞を分離することができる。磁場により分離できるため，装置が簡便であることや，大量の細胞を一度に処理できることが大きな利点となっている。このため，臨床で使用できる装置も開発されており，このシステムを用いて移植細胞からT細胞を除去することによる移植片対宿主病の回避に関しても報告されている[23]。

磁気ビーズにより標識した細胞を連続的な磁場の中で分離する研究も報告されている[24]。この手法は，細胞に標識された磁気ビーズ量（表面マーカー量に相当する）に応じて細胞をソーティングするContinuous型の細胞分離システムである。

5.3.3 Hydrodynamicを利用した分離

この手法はHydrodynamicにより細胞に対してshear stressを与え，固定化された抗体と細胞表面レセプター（細胞表面マーカー）との相互作用や，パターン化された流路により目的細胞を分離するものである。Katoらは，polyethylene terephtalateフィルターに単核球細胞を流すことで，lineage$^-$CD34$^-$の細胞ポピュレーションを分離できることを報告している[25]。NagrathとSequistらは，血中で循環しているガン細胞を検出する目的でマイクロチップ内に固定化された抗体により細胞を分離し診断できるCTC-chip法を報告している[26]。これは，流路内に数100ミクロンの複数の柱を配置し，柱に固定化された抗体と細胞との相互作用で目的の細胞ポピュレ

ーションを分離するものである。同様のメカニズムとして，Changらの報告でもマイクロ流路内に配置した柱にリガンドを固定化し，細胞を流すことでリガンドと結合した細胞を分離できることが報告されている[27]。このような手法は，特定の細胞を基材上に補足することで分離を達成するため，Digital型分離法として分類することができる。

これに対して筆者らのグループでは，固定化リガンドと細胞表面マーカーとの連続的な相互作用を利用して細胞ポピュレーションを分離する手法を開発している。これは，Hydrodynamicsにより細胞をリガンド固定化界面へ流すことでリガンドと特異的に結合した細胞がshear stressを受け回転運動（ローリング）し，細胞表面レセプター量に応じた細胞分離が達成されるものである。このローリング速度は表面レセプター量に応じて変化するため，Continuous型の細胞分離として分類することができる。詳細は後述する。

他にも，FACSを利用してHoechst 33342の染色挙動から分離されるSP（side population）細胞を採取して幹細胞を分離する方法がある。細胞の表面マーカーが同定されているHSC, EPCの場合では，比較的容易に均一な細胞ポピュレーションを分離することが可能となる。しかしMSCの場合では，未分化度が高くさまざまな表面マーカーの発現が報告されているにも関わらずコンセンサスな結果が得られていない。成熟した細胞の場合では特定の表面マーカーの発現の有無により細胞ポピュレーションが規定されるが，未分化な細胞の場合，表面マーカーの発現量は連続的に変化するものと思われる。MSCやHSCにおいてCD34の発現は，*in vitro*の培養期間や個体の年齢によって一定に発現しないことを示すデータも報告されている[28, 29]。このような観点から筆者らのグループでは，細胞表面マーカーの発現量に応じて連続的に細胞ポピュレーションを分離可能な細胞分離カラムシステムの開発を進めている。

5.4　細胞分離カラムの開発

これまでに筆者らのグループでは，表面マーカー発現量に応じて細胞を分離する細胞分離システムを開発している。これは表面マーカーに対する抗体を平面基板上へ固定化し，検体となる細胞をローリングさせることで，細胞表面マーカーと固定化抗体との相互作用を誘起し目的細胞ポピュレーションを分離する。この相互作用は，血管内で炎症部位付近に白血球が集積する場合に観察される白血球ローリング現象として知られており[30]，セレクチンとの相互作用により白血球のローリング速度は著しく減少する[31, 32]。この原理を応用してGreenbergらは，表面マーカーの有無によるローリング速度の差を利用して細胞分離する手法を報告している[33]。また，Langerらは平面基板上へリガンドを2次元に固定化し，作製したパターン化リガンド上で細胞ローリングさせることにより目的の細胞ポピュレーションを分離するマイクロチップを開発して

いる[34]。

細胞ローリングにおけるリガンドとレセプターとの吸脱着反応に関するメカニズムの定量的な解析は，Hammerらのグループを中心に多数報告されている[35～39]。特に細胞ローリング速度に関しては，表面マーカーの有無のみならずその発現密度にも依存することが示されている[40]。つまり，マーカーの発現密度が高い細胞は，低い細胞より相互作用の頻度が高くなるため，ローリング速度は減少する（図1）。そこで筆者らのグループでは，この相互作用により細胞の表面マーカー発現密度に応じて細胞を分離できるカラムを開発している。

この手法の利点は，表面マーカーの発現密度により細胞を分離できること，分離される細胞自身を抗体等で標識する必要がないため回収される細胞を直ちに移植できること，カラムに固定化する抗体あるいはリガンドを選択することできまざまな表面マーカーに基づく分離が達成できることが挙げられる。この分離システムの有効性を評価するため，マウス骨髄細胞から培養皿へ接着する細胞分画を採取し，CD34抗体を固定化したカラム上で分離した（図2）。リガンドが固定化されていないカラムの場合，細胞はフラクション2-3付近ですべて溶出するのに対して，CD34抗体固定化カラムを用いた場合では溶出時間が遅延するブロードな溶出ピークが示された（図3）。この溶出ピークに存在する細胞のCD34発現密度は溶出時間に応じて変化していたことから，このシステムにより表面マーカー量の異なる細胞を分離できているものと考えている。

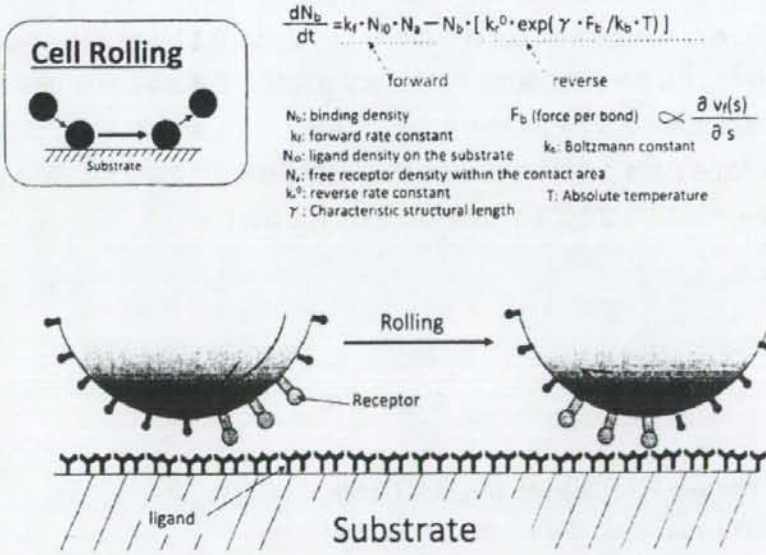

図1　細胞ローリングのメカニズム

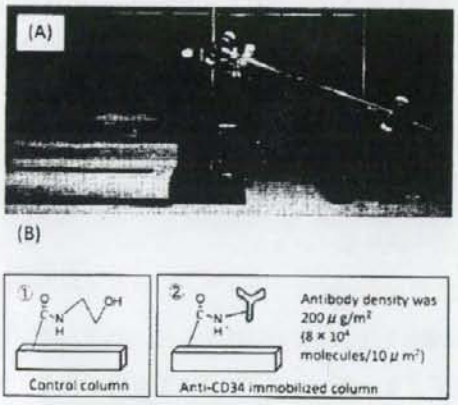

図2　(A) 細胞分離カラムシステムの外観
(B) 分離カラムの構造 (①コントロールカラム、②抗体固定化カラム)

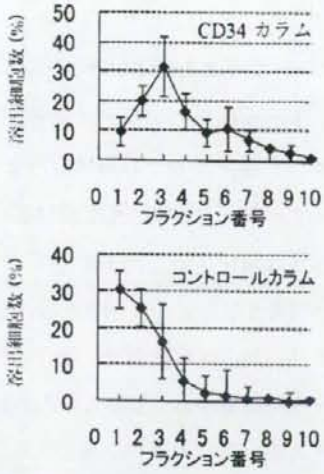

図3　マウス由来MSCを抗CD34抗体修飾細胞分離カラムにより溶出させた時のプロファイル

5.5　おわりに

細胞懸濁液から幹細胞を分離する場合，大きく分けて2つのアプローチが存在しているものと考えている。1つは高純度で均一なMSCを分離する手法，2つ目としてはMSC成分を含む細胞ポピュレーションを分離する手法である。MSCの機能やその性質を本質的に理解するためには，高純度で得られたMSCを用いて分化能や表面マーカーの発現量を詳細に検討する必要がある。一方で，MSCによる治療効果を検討する場合，大量のMSCが必要となるので，簡便な操作により大量に細胞を分離できることが望まれる。今後，治療メカニズムの検証や安全で効率的な移植治療法の実現化を目指すためにも組織再生を担う幹細胞を高純度で分離できるデバイスの開発や新たな分離メカニズムの開発はますます重要になるものと思われる。

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