configuration than that resulting from the binding of pioglitazone. This three-dimensional change in protein configuration may prevent the association of PPAR-γ with the PPAR-γ responsive element previously reported.

What do stimulatory effects of telmisartan depend on? It is postulated that telmisartan may bind and activate PPAR-α. PPAR-α is expected to be a crucial transcriptional factor for adiponectin gene expression, because in the assay system used in this study bezafibrate, a PPAR-α agonist, was found to stimulate adiponectin transcription (data not shown). It has also been reported that administration of bezafibrate increased both the mRNA and plasma level of adiponectin [34,35].

The present study indicates that telmisartan stimulates adiponectin transcription by a PPAR-γ independent mechanism. Identification of this mechanism, could lead to the development of a new drug for hypoadiponectinemia, which is commonly seen in metabolic syndrome and type 2 diabetes. Precise understanding of this molecular mechanism will require further investigation.

Acknowledgments

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ORIGINAL ARTICLE

Interaction of penton base Arg-Gly-Asp motifs with integrins is crucial for adenovirus serotype 35 vector transduction in human hematopoietic cells

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Most subgroup B adenoviruses (Ads), including adenovirus (Ad) serotype 35 (Ad35), bind to human CD46 as a receptor; however, the infection processes of subgroup B Ads following attachment to CD46 remain to be elucidated. Subgroup B Ads possess Arg-Gly-Asp (RGD) motifs in the penton base, similarly to subgroup C Ad serotypes 2 and 5. In this study, we examined the role of penton base RGD motifs in Ad35 vector-mediated transduction in human hematopoietic cells. Inhibition of interaction between integrins and the RGD motifs by divalent cation chelation and a synthetic RGD peptide reduced the transduction efficiencies of Ad35 vectors; however, the amounts of cell-associated vector DNA of Ad35 vectors at 4 or 37°C were not decreased by divalent cation chelation or the RGD peptide. Mutation of penton base RGD motifs reduced the transduction efficiencies of Ad35 vectors, although the amounts of cell-associated vector DNA of Ad35 vectors at 4 or 37°C were not altered by mutation of penton base RGD motifs in Ad35 vectors. Furthermore, preincubation with several types of anti-integrin antibodies significantly inhibited Ad35 vector-mediated transduction. These results suggest that interaction between integrins and penton base RGD motifs plays a crucial role in Ad35 vector-mediated transduction in hematopoietic cells, probably in the post-internalization steps.
Gene Therapy advance online publication, 6 September 2007; doi:10.1038/sj.gt.3303019

Introduction

Human adenoviruses (Ads) are nonenveloped, double-stranded DNA viruses currently containing 51 serotypes, which are classified into six distinct subgroups (A–F).[1] Among these serotypes, adenovirus (Ad) vectors based on Ad serotype 5 (Ad5), which belongs to subgroup C, are the most commonly used for gene therapy and have been the most extensively studied, including studies of their transduction mechanisms. As the first step for the entry of Ad5 vectors into cells, the fiber knob binds to the primary receptor, coxsackievirus-adenovirus receptor (CAR). After binding to CAR, Arg-Gly-Asp (RGD) motifs located in the penton base interact with cellular integrins, including $\alpha_v\beta_1$,[2] $\alpha_v\beta_3$, $\alpha_v\beta_5$,[3,4] $\alpha_5\beta_1$,[5] and $\alpha_M\beta_2$,[6] leading to the internalization of Ad5 vectors via clathrin-coated pits. In these transduction processes, binding to CAR is the most crucial determinant of the transduction efficiency of Ad5 vectors, and Ad5 vector-mediated gene

transfer to cells lacking CAR expression is inefficient. Regrettably, several important target cells for gene therapy, including hematopoietic stem cells,[7] dendritic cells[8] and malignant tumor cells,[9,10] express low or negligible levels of CAR.

On the other hand, most subgroup B Ads, including Ad serotype 11 and 35 (Ad35), attach to human CD46 as a receptor.[11,12] Human CD46 is ubiquitously expressed in almost all human cells, leading to the broad tropism of subgroup B Ad vectors in human cells, one of the subgroup's advantages. In particular, hematopoietic cells, which are important targets for gene therapy but resistant to Ad5 vectors, are susceptible to subgroup B Ads.[13–15] Infection of subgroup B Ads is initiated by the attachment of the fiber knob to short consensus repeats 1 and 2 of human CD46.[16–18] However, the infection process of subgroup B Ads following binding to CD46 is poorly understood. Elucidation of the transduction mechanism would lead to more effective applications and improvements of subgroup B Ad vectors. Previous studies demonstrated that subgroup B Ads possess an RGD motif in the penton base similarly to Ad5,[19–21] and that CD46 is associated with some of the integrins on the cell surface,[22] which caused us to hypothesize that interaction between penton base RGD motifs and integrins plays an important role in subgroup B Ad vector-mediated transduction.

In this study, we examined the involvement of interaction between integrins and penton base RGD motifs in subgroup B Ad35 vector-mediated transduction in human bone marrow-derived CD34⁺ cells and human leukemia cell line K562 cells, both of which have been shown to be highly susceptible to Ad35 vectors.[15,23,24] Human bone marrow-derived CD34⁺ cells are an important target for gene therapy, because the CD34⁺ cells are a fraction that contains hematopoietic stem cells. We found that inhibition of the interaction between integrins and penton base RGD motifs did not alter total amounts of cell-associated vector DNA of Ad35 vectors at 4 or 37 °C; however, it significantly reduced the transduction efficiencies of Ad35 vectors, suggesting that interaction between integrins and penton base RGD motifs is largely involved with Ad35 vector-mediated transduction, probably in postinternalization steps.

Results

Several types of integrins are expressed on human bone marrow-derived CD34⁺ cells and K562 cells

First, we performed flow cytometric analysis to examine which types of integrins are expressed on human bone marrow-derived CD34⁺ cells and K562 cells. Flowcytometric analysis demonstrated that the CD34⁺ cells and K562 cells both expressed high levels of integrins α_5 (95%; CD34⁺ cells, 100%; K562 cells) and β_2 (99%; CD34⁺ cells, 86%; K562 cells) (Figure 1). Integrins α_4, α_6 and β_1 were also expressed in the CD34⁺ cells, but hardly detected in K562 cells. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are well known as second receptors for Ad5 vectors,[3,4] were expressed in low levels in both cells ($\alpha_v\beta_3$; 13 and 20% in CD34⁺ cells and K562 cells, respectively; $\alpha_v\beta_5$; 2 and 17% in CD34⁺ cells and K562 cells, respectively).

Divalent cations are required for Ad35 vector-mediated transduction

Next, to examine involvement of integrins in Ad35 vector-mediated transduction, the hematopoietic cells were transduced with Ad35 vectors in the presence of EDTA or divalent cations. The affinity between integrins

and their ligands is regulated by divalent cations.[25–27] Notably, Mn²⁺ is reported to largely promote the binding of ligands to integrins. Transduction efficiencies of an Ad35 vector expressing an enhanced green fluorescence protein (GFP) (Ad35GFP) in K562 cells were significantly reduced in the presence of EDTA (Figure 2a). Treatment with 5 mM EDTA decreased GFP expression by 80%. On the other hand, Mn²⁺ significantly increased the transduction efficiencies of Ad35GFP in K562 cells, while exposure to Mg²⁺ or Ca²⁺ did not affect the transduction efficiencies of Ad35GFP (Figure 2b). These results suggest that integrins are involved with Ad35 vector infection. Transduction experiments using the CD34⁺ cells were also tried; however, pretreatments with EDTA or divalent cations largely reduced the viability of the CD34⁺ cells (data not shown).

To investigate the mechanisms of EDTA-mediated decrease and the Mn²⁺-mediated increase in transduction efficiencies of Ad35 vectors, cellular binding and uptake of Ad35GFP in the presence of EDTA or Mn²⁺ was assessed by real-time PCR. Exposure to 5 mM EDTA or Mn²⁺ did not increase the total amounts of Ad35 vector genome associated with K562 cells at 4 °C 1.5 h following infection, indicating that cellular binding of Ad35GFP is not affected by divalent cations (Figure 3). In addition, the cells treated with EDTA or Mn²⁺ also showed no change in the amounts of cell-associated Ad35 vector DNA at 37 °C (the sum of cellular binding and uptake of Ad35GFP). These results suggest that postinternalization steps in Ad35 vector-mediated transduction are affected by EDTA and Mn²⁺.

Penton base RGD motifs are involved with Ad35 vector-mediated transduction

To examine whether the RGD motifs in the Ad35 penton base are involved with Ad35 vector-mediated transduction in the CD34⁺ cells and K562 cells, the cells were transduced with Ad35GFP in the presence of synthetic RGD peptide. Transduction with Ad35GFP was significantly suppressed by the RGD peptide in a dose-dependent fashion in the human CD34⁺ cells and K562 cells (Figure 4). The GFP expression levels by Ad35GFP in the CD34⁺ cells and K562 cells were respectively
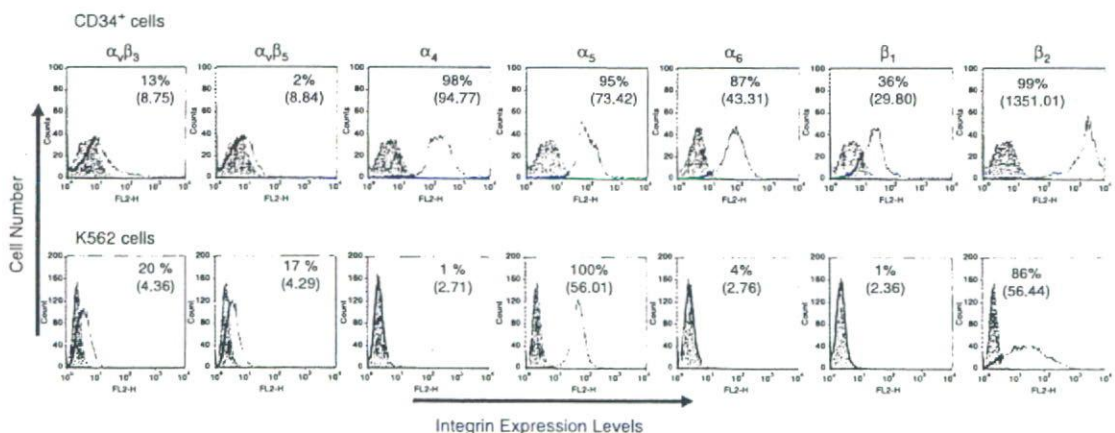

Figure 1 Flow cytometric profiles of integrin expressions on human bone marrow-derived CD34⁺ cells and K562 cells. The cells were stained with monoclonal anti-integrin antibodies, followed by a phycoerythrin-labeled secondary antibody, and subsequently analyzed by a flow cytometry (thick line). As a negative control, cells were incubated with an isotype control antibody (shaded histogram). Percentages of positive cells (and mean fluorescence intensities) are shown by number in the upper right-hand corner of each profile.
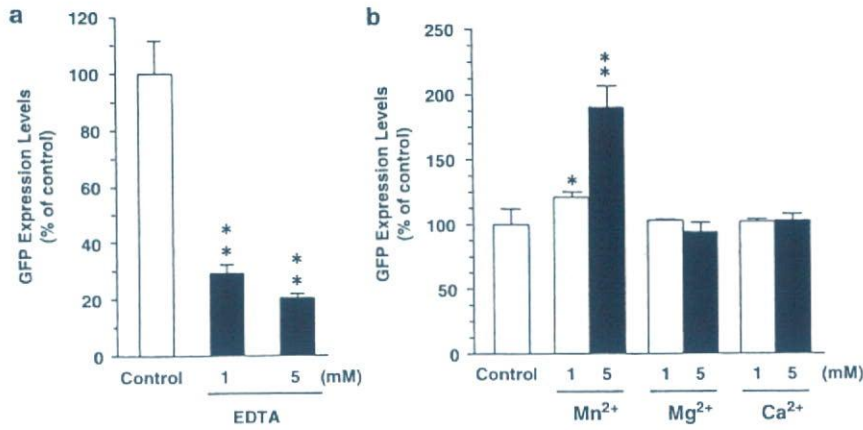
Figure 2 Effects of divalent cations on Ad35 vector-mediated transduction. (a) Ad35GFP-mediated transduction in the presence of EDTA. K562 cells were transduced with Ad35GFP at 1000 VP per cell for 1.5 h in the presence of EDTA at the indicated concentrations. GFP expression levels were measured using flow cytometry 48 h after transduction. The GFP expression levels (mean fluorescence intensity; MFI) in the absence of EDTA (control) and the mock-infected cells were 107.6 and 2.4, respectively. (b) Ad35GFP-mediated transduction in the presence of Mn^{2+}, Mg^{2+} or Ca^{2+} ions. K562 cells were preincubated in TBS buffers containing $MnCl_2$, $MgCl_2$ or $CaCl_2$ at indicated concentrations for 30 min and then transduced with Ad35GFP at 1000 VP per cell. The transduction experiments were performed as described above. The GFP expression level (MFI) in TBS buffer (control) and the mock-infected cells were 119.3 and 2.3, respectively. The data were normalized to the GFP expression levels (MFI) in K562 cells, which were preincubated in TBS buffer prior to transduction (control). Data are expressed as means ± s.d. ($n = 3$ or 4). $*P < 0.05$, $**P < 0.001$ in comparison with the control. TBS, Tris-buffered saline.
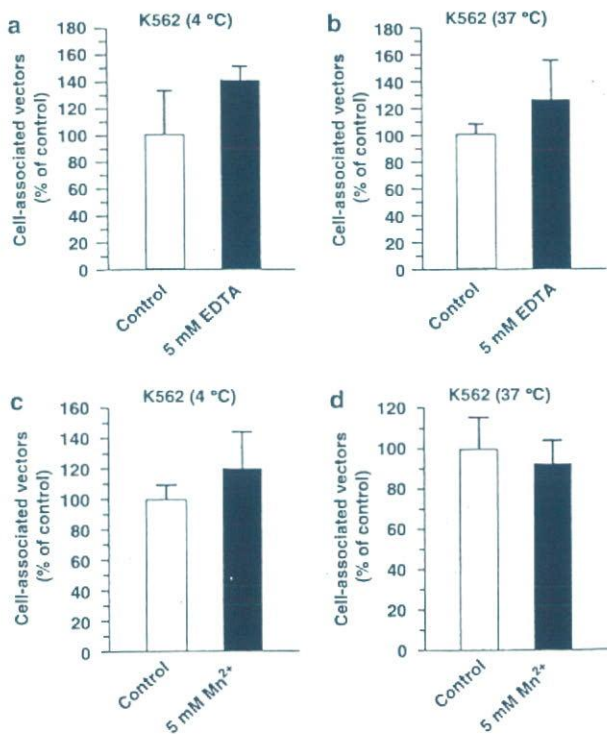

Figure 3 Effects of divalent cations on cellular binding and uptake of Ad35 vectors in K562 cells. K562 cells were incubated with Ad35GFP in the presence of 5 mM EDTA (a, b) or 5 mM Mn^{2+} (c, d) as described in Figure 2. After incubation, the cells were washed and total DNA, including Ad vector DNA, was recovered. The vector copy number was quantified using real-time PCR. The data were normalized to the vector copy number when transduced with Ad35GFP in Tris-buffered saline buffer. Data are expressed as means ± s.d. ($n = 4$). GFP, green fluorescence protein.

decreased by 57 and 24% in the presence of 200 µg ml^{-1} of the RGD peptide, compared with the presence of the control RGE peptide.

Next, to clarify why coincubation with the RGD peptide decreases the transduction efficiencies of Ad35 vectors, we evaluated cellular binding and uptake of Ad35 vectors in the presence of the RGD peptide. As shown in Figure 5, the RGD peptide did not decrease the total amounts of cell-associated vector DNA of Ad35GFP at 4 or 37 °C in the CD34$^+$ cells and K562 cells, suggesting that the interaction between integrins and penton base RGD motifs are crucial for Ad35 vector-mediated transduction; however, this interaction would not be largely involved with the cellular binding or uptake of Ad35 vectors in the CD34$^+$ cells and K562 cells.

Mutation of penton RGD motifs in Ad35 vectors significantly reduces the transduction activity of Ad35 vectors

To further examine the involvement of penton RGD motifs with Ad35 vector-mediated transduction, we constructed Ad35 vectors containing the RGE sequence instead of RGD in the penton base (D343E) (Ad35RGE-GFP) or a deletion of the RGD motifs in the penton base (Ad35ΔRGD-GFP) (Table 1). Final yields of Ad35RGE-GFP and Ad35ΔRGD-GFP were comparable to those of Ad35GFP (data not shown). The CD34$^+$ cells exhibited approximately 80% reduction in GFP expression following transduction with Ad35RGE-GFP and Ad35ΔRGD-GFP at 6000 vector particles (VP) per cell (Figure 6a). In K562 cells, Ad35RGE-GFP and Ad35ΔRGD-GFP mediated approximately 35% reduced GFP expression compared with Ad35GFP at 3000 VP per cell (Figure 6b). Transduction with each Ad35 vector at lower doses resulted in similar transduction profiles to those shown here in the CD34$^+$ cells and K562 cells (data not shown),
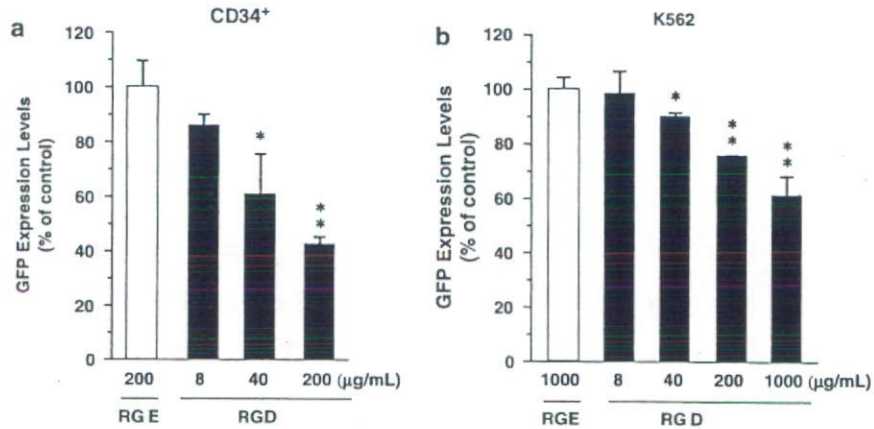
Figure 4 Inhibition of Ad35 vector-mediated transduction by synthetic RGD peptide. Human CD34+ cells (a) and K562 cells (b) were transduced with Ad35GFP at 3000 VP per cell for 3 h (human CD34+ cells) or at 1000 VP per cell for 1 h (K562 cells) at 37 °C in the presence of RGD peptides. GFP expression levels were measured 48 h after transduction by flow cytometry. Data were normalized to the GFP expression levels (MFI) in the presence of control RGE peptide. The GFP expression levels (MFI) in the presence of control RGE peptide (CD34+ cells; 200 μg ml⁻¹, K562 cells; 1000 μg ml⁻¹) were 198.2 (CD34+ cells) and 748.9 (K562 cells). The GFP expression levels (MFI) in the mock-infected cells were 2.3 (CD34+ cells) and 2.9 (K562 cells). The Data are expressed as means ± s.d. ($n = 3$). $*P < 0.05$, $**P < 0.001$ in comparison with control RGE peptide. MFI, mean fluorescence intensity; RGD, Arg-Gly-Asp.
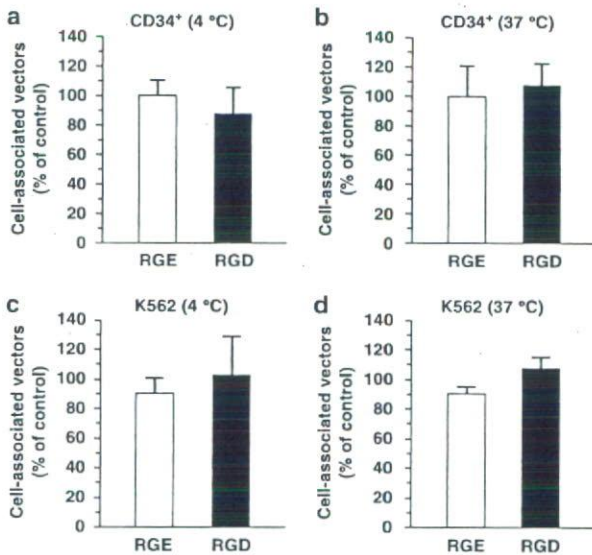

Figure 5 Cellular binding and uptake of Ad35GFP in the presence of RGD peptide. Human CD34+ cells (a, b) and K562 cells (c, d) were incubated with Ad35GFP in the presence of RGD peptide (200 μg ml⁻¹) as described in Figure 4. After incubation, the cells were washed, and total DNA, including Ad vector DNA, was recovered. The vector copy number was quantified using real-time PCR. The data were normalized to the vector copy number of Ad35GFP in the presence of control RGE peptide. Data are expressed as mean ± s.d. ($n = 4$). GFP, green fluorescence protein; RGD, Arg-Gly-Asp; RGE, Arg-Gly-Glu.

Table 1 Mutation in the penton base of Ad35 vectors

Ad35 vectors	Amino-acid sequence of penton base
Ad35GFP (conventional Ad35)	—NAGEVRGDNFAPT—
Ad35RGE-GFP (an amino acid substitution)	—NAGEVRGENFAPT—
Ad35ΔRGD-GFP (deletion of RGD motif)	—NAGEV --------- NFAPT—

Abbreviations: Ad, adenovirus; GFP, green fluorescence protein; RGD, Arg-Gly-Asp.

Next, we compared the cellular binding and uptake of Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP in the CD34+ cells and K562 cells by real-time PCR analysis to examine why the mutation of penton base RGD motifs decreased the transduction efficiencies. We found that 3 h (CD34+ cells) or 1.5 h (K562 cells) following infection, the Ad35 vector genome levels associated with both cells were comparable to those of Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP at 4 and 37 °C (Figure 7), although the amount of Ad35ΔRGD-GFP associated with the CD34+ cells at 37 °C was significantly higher than those of the other vectors. These results indicate that penton base RGD motifs play an important role in postinternalization steps.

Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ and α_5 are involved with Ad35 vector infection

To determine which types of integrins participate in Ad35 vector infection in the CD34+ cells and K562 cells, we performed an infection-blocking experiment using several anti-integrin antibodies. Among the several anti-integrin antibodies, incubation with anti-$\alpha_v\beta_3$ antibody significantly reduced the GFP expression level by 41% in the CD34+ cells (Figure 8a). The other types of anti-integrin antibodies did not inhibit Ad35 vector-mediated

suggesting that the transduction efficiencies of the Ad35 vectors at these doses would not be saturated. Apparent toxicities were not observed in the cells after transduction at these doses (data not shown). These results indicate that penton base RGD motifs are largely involved with Ad35 vector-mediated transduction in both cells.
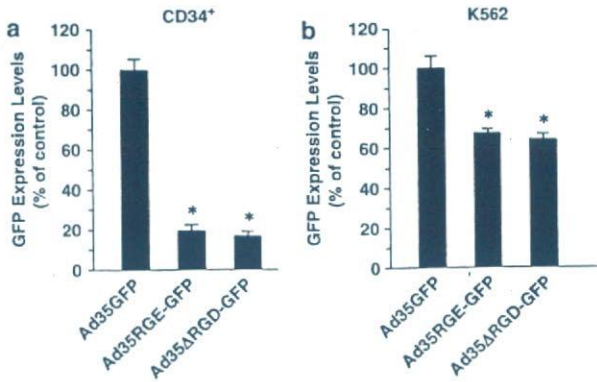
Figure 6 Effects of modification of penton RGD motifs on transduction efficiencies of Ad35 vectors. Human CD34^{+} cells (**a**) and K562 cells (**b**) were transduced with Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP at 6000 VP per cell for 6 h (human CD34^{+} cells) or at 3000 VP per cell for 1.5 h (K562 cells) at 37 °C. GFP expression levels (MFI) were measured 48 h after transduction using flow cytometry. The GFP expression levels of Ad35GFP in the CD34^{+} cells and K562 cells were 432.4 and 3232.7, respectively. The GFP expression levels in the mock-infected cells were 3.2 (CD34^{+} cells) and 3.3 (K562 cells). The data were normalized to the GFP expression levels by Ad35GFP. Data are expressed as means ± s.d. ($n = 3$). *$P < 0.001$ in comparison with Ad35GFP. GFP, green fluorescence protein; MFI, mean fluorescence intensity; RGD, Arg-Gly-Asp.
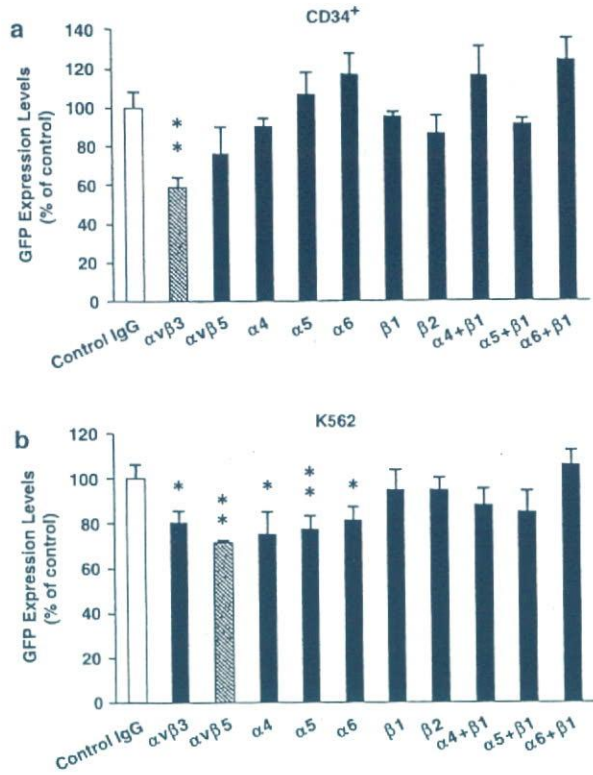

Figure 7 Effects of modification of penton RGD motifs on cellular binding and uptake of Ad35 vectors. Human CD34^{+} cells (**a, b**) and K562 cells (**c, d**) were incubated with Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP as described in Figure 6 at 4 or 37 °C. After incubation, the cells were washed five times with ice-cold phosphate-buffered saline, and total DNA, including the Ad vector DNA, was extracted. The vector copy numbers were quantified by real-time PCR. The data were normalized to the vector copy number of Ad35GFP. Data are expressed as means ± s.d. ($n = 4$). *$P < 0.05$ in comparison with Ad35GFP. GFP, green fluorescence protein; RGD, Arg-Gly-Asp.

Figure 8 Inhibition of Ad35 vector-mediated transduction by monoclonal anti-integrin antibodies. Human CD34^{+} cells (**a**) and K562 cells (**b**) were transduced with Ad35GFP at 3000 VP per cell for 3 h (human CD34^{+} cells) or at 300 VP per cell for 0.5 h (K562 cells). GFP expression levels were measured 48 h after transduction using flow cytometry. The GFP expression levels (MFI) in the presence of control IgG were 427.0 (CD34^{+} cells) and 24.9 (K562 cells). The GFP expression levels in the mock-infected cells were 2.8 (CD34^{+} cells) and 2.7 (K562 cells). The data were normalized to the GFP expression levels (MFI) in human CD34^{+} cells and K562 cells in the presence of control mouse IgG. Data are expressed as means ± s.d. ($n = 3$). *$P < 0.05$, **$P < 0.01$ in comparison with control IgG. GFP, green fluorescence protein; IgG, immunoglobulin G; MFI, mean fluorescence intensity
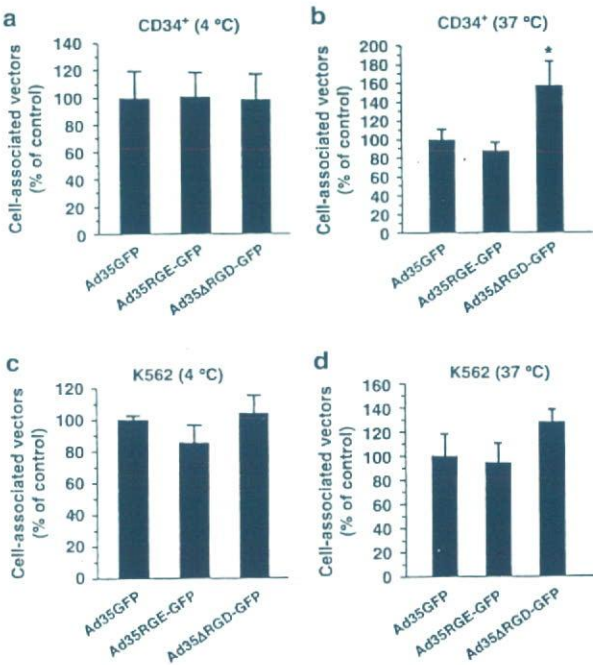
transduction in the CD34^{+} cells. In K562 cells, Ad35GFP showed 20, 30 and 22% reduced levels of GFP expression in the presence of anti-$\alpha_v\beta_3$, -$\alpha_v\beta_5$ and -α_5 antibodies, respectively (Figure 8b). Pretreatment with anti-α_4 and anti-α_6 antibodies in K562 cells also significantly decreased GFP expression by Ad35GFP, in spite of undetectable expression levels of these integrins in K562 cells (see Figure 1). These results indicate that the integrins described above are involved with Ad35 vector-mediated transduction in the CD34^{+} cells or K562 cells.

Discussion

The goal of this study was to evaluate the involvement of interaction between penton base RGD motifs and integrins on the cell surface with Ad35 vector-mediated transduction in human hematopoietic cells. To this end, we performed transduction with Ad35 vectors in hematopoietic cells in the presence of EDTA, divalent

6

cations and RGD peptide. Ad35 vectors with a mutation in the penton base were constructed, and transduction properties of the mutant Ad35 vectors were examined. Furthermore, Ad35 vectors transduced hematopoietic cells in the presence of several types of anti-integrin antibodies.

The interaction of penton base RGD motifs with α_v integrins is well known to facilitate the internalization of Ad5 vectors via clathrin-mediated endocytosis.[28] Several types of integrins, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_1$, mediate efficient transduction with Ad5 vectors.[2–6] The structures of the Ad5 fiber and penton base allow easy access of penton base RGD motifs to integrins on the cell surface. Ad5 possess a long (22 β-repeats), and flexible fiber shaft.[29,30] The RGD-containing hypervariable loop of the Ad5 penton base is longer than those of other serotypes,[20,21] and protrudes above the penton base.[31] In contrast, the fiber shaft of Ad35 is short (7 β-repeats).[30] The hypervariable loop of the Ad35 penton base is 19 amino-acid shorter than that of Ad5. These properties of Ad35 suggest that the RGD motifs in the Ad35 penton base might be less easily associated with integrins on the cell surface. The present study indicates that the interaction of penton base RGD motifs in Ad35 with integrins is not largely involved with the cellular binding and uptake of Ad35. Segerman et al.[32] and Tuve et al.[33] also demonstrated that the presence of divalent cations, which increases the affinity of integrins to their ligands, did not affect the cellular binding of subgroup B Ads. Properties of CD46 also contribute to the lack of necessity of interaction between penton base RGD motifs and integrins for uptake of Ad35. Gaggar et al.[11] suggested that CD46 is not only an attachment receptor, but also a receptor for internalization of Ad35. The cytoplasmic tail of CD46 possesses a signal sequence for endocytosis.[34]

The experimental method used in this study does not allow exact evaluation of the internalized Ad35 vector copy numbers, because the amounts of the vector copy numbers at 37 °C contains both the virus particles attached on the cell surface and those truly internalized into the cells. However, it is unlikely that the amounts of Ad35 vector particles bound on the cell surface are dramatically different between 4 and 37 °C, because CD46 expression levels on the cell surface did not decrease at the Ad35 vector dose used in this study (data not shown). Therefore, we can roughly estimate that the amounts of the internalized Ad35 vector particles are not dramatically different in the presence or absence of EDTA, Mn^{2+} or the RGD peptide, assuming that the amounts of the Ad35 vector particles bound on the cell surface at both temperatures are the same.

However, interaction of penton base RGD motifs with integrins might facilitate the internalization rates of CD46-utilizing Ad vector particles into cells, especially shortly after virus infection. Deletion of penton base RGD motifs in chimeric Ad5 vectors containing the Ad35 fiber knob showed 15-min delays in the postinfection uptake of the vectors.[35] Ad serotype 41, which lacks an RGD motif on the penton base, undergoes delayed uptake in A549 cells.[21]

On the other hand, inhibition of interaction between the RGD motifs and integrins significantly decreased the transduction activity of Ad35 vectors, indicating that binding of the RGD motifs to integrins is largely involved with the transduction process of Ad35 vectors.

Our group also confirmed that fiber-substituted Ad5 vectors bearing the fiber shaft and knob of Ad35 with deletion of penton base RGD motifs showed reduced transduction efficiencies compared with the parent fiber-substituted Ad5 vectors (data not shown). Shayakhmetov et al.[35] also demonstrated that mutation of penton base RGD motifs in chimeric Ad5 vectors containing the fiber knob of Ad35 significantly decreased the transduction efficiencies. These results indicate that penton base RGD motifs are important for transduction with not only subgroup B Ad vectors, but also CD46-utilizing chimeric Ad5 vectors. It remains unclear which process is facilitated by interaction between Ad35 penton base RGD motifs and integrins. However, the present study revealed that interaction of the RGD motifs with integrins did not significantly enhance cellular binding and uptake of Ad35, suggesting that postinternalization steps, such as escape from endosomes/lysosomes, would be accelerated by the interactions. This hypothesis is supported by the study by Shayakhmetov et al.,[35] which demonstrated that deletion of penton RGD motifs in chimeric Ad5 vectors possessing the fiber knob of Ad35 decreased escape from the endosomes/lysosomes. The interaction of $\alpha_v\beta_5$ integrins with penton RGD motifs was reported to enhance the escape of Ad5 from endosomes.[4]

Although significant decreases in the transduction activities were found in Ad35 vectors with a mutation of the RGD motifs, Ad5 vectors with deletion of penton base RGD motifs exhibited approximately comparable levels of transgene expression in CAR-positive cells with normal Ad5 vectors.[35,36] The difference between Ad5 and Ad35 vectors in their need for penton base RGD motifs for efficient transduction might be due to the difference in their ability to escape from endosomes. Ad5 vectors are released from endosomes immediately after endocytosis; in contrast, chimeric Ad5 vectors possessing the fiber knob of Ad35, which are supposed to show similar intracellular trafficking to Ad35 vectors, remained longer in late endosomes/lysosomes than conventional Ad5 vectors.[37]

Blocking studies using anti-integrin antibodies revealed that integrins involved with Ad35 vector infection are $\alpha_v\beta_3$ in the CD34+ cells and $\alpha_v\beta_3$, $\alpha_v\beta_5$ and α_5 in K562 cells (anti-α_4 and anti-α_6 antibodies showed statistically significant inhibition in Ad35 vector-mediated transduction; however, expressions of α_4 and α_6 integrins are below detectable levels, as commented in the result section). Before the experiments, we had speculated that $\alpha_5\beta_1$ integrin was mainly involved with Ad35 vector-mediated transduction, because human bone marrow CD34+ cells expressed integrins α_5 and β_1. In addition, the affinity of ligands to integrins is influenced by the amino acid following the RGD sequence while the RGDN (Arg-Gly-Asp-Asn) sequence, which is contained in the Ad35 penton base, has a high affinity for $\alpha_5\beta_1$ integrin.[38] However, the efficiencies of Ad35GFP transduction did not decrease in the presence of anti-α_5 or anti-β_1 antibodies in the CD34+ cells, although preincubation with anti-α_5 antibody reduced transduction of Ad35GFP in K562 cells. It remains unclear why $\alpha_5\beta_1$ integrin is not involved with Ad35 vector infection in the CD34+ cells. In addition, only the anti-$\alpha_v\beta_3$ antibody inhibited transduction with Ad35 vectors in the CD34+ cells, and anti-integrin antibodies used in this study showed low levels of inhibition in both cells. Other types

of integrins which were not tested in this study, or unknown receptor(s), might be involved with Ad35 infection.

In summary, we demonstrated that the interaction between penton base RGD motifs and integrins are important for Ad35 vector-mediated transduction in hematopoietic cells. The results of our study contribute to a better understanding of the mechanism of Ad35 vector-mediated transduction and may offer valuable information for the development of more efficient Ad vectors.

Materials and methods

Cells and antibodies

Human bone marrow-derived CD34+ cells (Cambrex Bio Science Inc., Walkersville, MD, USA) were recovered from the frozen stock 16–18 h before transduction, and suspended in StemSpan 2000 containing the cytokine cocktail StemSpan CC100 (human Flt-3 ligand (100 ng ml⁻¹), human stem cell factor (100 ng ml⁻¹), human interleukin-3 (20 ng ml⁻¹) and human interleukin-6 (20 ng ml⁻¹)) (StemCell Technologies Inc., Vancouver, BC, Canada). K562 cells (human chronic myelogeneous leukemia from blast crisis) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Purified function-blocking monoclonal antibodies to integrins $\alpha_v\beta_3$ (LM609), $\alpha_v\beta_5$ (P1F6), α_5 (P1D6), β_1 (P4C10) and β_2 (P4H9-A11) were purchased from Chemicon International (Temecula, CA, USA). Monoclonal antibodies to integrin α_4 (HP2/1) and α_6 (GoH3) were obtained from Immunotech (Marseille, France) and R&D Systems Europe (Abingdon, UK), respectively.

Plasmids

Ad35 vector plasmid pAdMS18 was constructed as follows. The SbfI/AscI fragment of pAdMS1,[15] which is the left end of the Ad35 genome (1–7930 bp), was ligated with the SbfI/AscI fragment of pFS2,[15] resulting in pFS2-Ad35-12. pFS2-Ad35-12 was cut by SnaBI and PacI, and ligated with oligonucleotides 1 (5'-TAACTATAACGGTC CTAAGGTAGCGA*ATTTAAAT*ATCTATGTCGGGTGCG GAGAAAGAGGTAATGAAATGGCAAT-3') and 2 (5'-T GCCATTTCATTACCTCTTTCTCCGCACCCGACATAG AT*ATTTAAAT*TCGCTACCTTAGGACCGTTATAGTTA-3') (I-CeuI, SwaI and PI-SceI recognition sequences are noted by underlining, italics and bold, respectively), resulting in pFS2-Ad35-13, which contains I-CeuI, SwaI and PI-SceI sites in the E1 deletion site of the Ad35 genome. The I-CeuI/AscI fragments of pFS2-Ad35-13 and pAdMS4[23] were ligated, resulting in pAdMS18. pAdMS18 has I-CeuI, SwaI and PI-SceI sites in the E1 deletion region (ΔE1:450–2912 bp).

Vector plasmids pAdMS19 and pAdMS20, which were used to generate Ad35 vectors containing an amino-acid substitution in the penton RGD sequence (D343E) or a deletion of the RGD motif in the penton base, respectively, were constructed using the following procedures. pFS5-Ad35-2 was constructed by ligation of the AscI/ EcoRI fragment (7930–21 944 bp) of the Ad35 genome and the AscI/EcoRI fragment of pFS5. pFS5 was constructed by ligation of XbaI/SacI-digested pHM5[39] with the oligonucleotides containing the multicloning sites. A shuttle plasmid pFS4, which contains the

multicloning site, PmeI/AscI/NheI/Bst1107I/Csp45I/ PacI/NotI, was digested with SphI/Csp45I and ligated with the SphI/Csp45I fragment of pFS5-Ad35-2, resulting in pFS4-Ad35-1. pFS4 was constructed by ligation of SphI/SalI-digested pHM5 with oligonucleotides containing the multicloning site. pFS4-Ad35-2, which has the Ad35 genome (14 409–15 544 bp), was constructed by ligation of Bst1107I/Csp45I fragments of pFS4-Ad35-1 and pFS4. Then, pFS4-Ad35-2 was cut by PvuII and PstI, and ligated with oligonucleotides 3 (5'-CTGCTGCA GAAGCTAAGGCAAACATAGTTGCCAGCGACTCTAC AAGGGGTTGCTAACGCTGGAGAGGTC<u>AGAGGAGAG</u> AATTTTGCGCCAACACCTGTTCCGACTGCA-3') and 4 (5'-GTCGGAACAGGTGTTGGCGCAAAATTCTCTCCTC TGACCTCTCCAGCGTTAGCAACCCTTGTAGAGTCGC <u>T</u>GGCAACTATGTTTGCCTTAGCTTCTGCAGCAG-3') (the RGE sequence is underlined), resulting in pFS4-Ad35-5. pFS4-Ad35-5 was digested with SphI/PvuII and ligated with the SphI/PvuII fragment of pFS4-Ad35-1, resulting in pFS4-Ad35-6. pFS4-Ad35-7, which was constructed by ligation of the I-CeuI/BlpI fragment of pFS5-Ad35-2 and I-CeuI/BlpI-digested pFS4-Ad35-6, were cut by SgrAI/PacI and ligated with the SgrAI/PacI fragment of pFS5-Ad35-2, resulting in pFS4-Ad35-9. Finally, pAdMS19 was constructed by homologous recombination of the AscI/PacI fragment of pFS4-Ad35-9 with Bst1107I-digested pAdMS18 in Escherichia coli BJ5183. pAdMS20 was similarly constructed using oligonucleotides 5 (5'-CTGCTGCAGAAGCTAAGGCA AACATAGTTGCCAGCGACTCTACAAGGGTTGCTAA CGCTGGAGAGGTCAATTTTGCGCCAACACCTGTTCC GACTGCA-3') and 6 (5'-GTCGGAACAGGTGTTGGCG CAAAATTGACCTCTCCAGCGTTAGCAACCCTTGTA GAGTCGCTGGCAACTATGTTTGCCTTAGCTTCTGCA GCAG-3').

Virus

The Ad35 vectors were prepared by means of an improved in vitro ligation method described previously.[23,39,40] GFP-expressing Ad35 vector plasmids (pAdMS18CA-GFP, pAdMS19CA-GFP and pAdMS20CA-GFP) were constructed by ligation of I-CeuI/PI-SceI-digested pAdMS18, pAdMS19 and pAdMS20, respectively, and I-CeuI/PI-SceI-digested pHMCA5-GFP. pHMCA5-GFP was constructed by insertion of the GFP gene, which was derived from pEGFP-N1 (Clontech, Mountain View, CA, USA) into pHMCA5.[41]

To generate the virus, pAdMS18CA-GFP, pAdMS19-CA-GFP and pAdMS20CA-GFP were digested with SbfI and purified by phenol–chloroform extraction and ethanol precipitation. Linearized plasmids were transfected into 293E1B cells[42] with SuperFect (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Determination of virus particle titers was accomplished spectrophotometrically by the method of Maizel et al.[43]

Flow cytometric analysis of integrin expressions

Human CD34+ cells and K562 cells suspended in staining buffer (phosphate-buffered saline buffer containing 1% bovine serum albumin) were incubated with mouse anti-human integrin $\alpha_v\beta_3$, $\alpha_v\beta_5$, α_4, α_5, α_6, β_1 and β_2 antibodies for 1 h. Subsequently, the cells were reacted with phycoerythrin-labeled secondary anti-mouse IgG antibody (Pharmingen, San Diego, CA, USA). After washing

8

with the staining buffer, the stained cells (10^4 cells) were analyzed using FACSCalibur and CellQuest software (Becton Dickinson, Tokyo, Japan).

Transduction experiments

Human CD34$^+$ cells and K562 cells were seeded at 1×10^4 cells per well into a 96-well plate. The cells were transduced with Ad35GFP, Ad35RGE-GFP or Ad35ΔRGD-GFP at 6000 VP per cell for 6 h (CD34$^+$ cells) or 3000 VP per cell for 1.5 h (K562 cells). After a 48 h culture period, GFP expression levels were measured using flow cytometry as described above.

To examine the effects of divalent cations on Ad35 vector-mediated transduction, K562 cells were preincubated in Tris-buffered saline buffer containing $MnCl_2$, $MgCl_2$ or $CaCl_2$ at indicated concentrations for 30 min at 4 °C prior to transduction. Subsequently, the cells were transduced with Ad35GFP at 1000 VP per cell for 1.5 h at 37 °C. After a 1.5 h incubation, the cells were washed and resuspended in a fresh medium. GFP expression levels were measured using flow cytometry following a total of 48 h of the incubation as described above. In blocking experiments using the RGD peptide, human CD34$^+$ cells and K562 cells were preincubated in the medium containing synthetic RGD peptide (GRGDSP, GRGESP; TaKaRa, Osaka, Japan) at 4 °C for 1 h. Subsequently, the cells were transduced with Ad35GFP at 3000 VP per cell for 3 h (CD34$^+$ cells) or 1000 VP per cell for 1 h (K562 cells) at 37 °C after which the cells were washed, resuspended in a fresh medium and incubated at 37 °C GFP expression levels were measured 48 h after transduction as described above. Blocking experiments were similarly performed using monoclonal anti-human integrin antibodies. Following incubation with anti-human integrin antibodies (50 μg ml^{-1}), the cells were transduced with Ad35GFP at 3000 VP per cell for 3 h (CD34$^+$ cells) or 300 VP per cell for 0.5 h (K562 cells). GFP expression levels were evaluated as described above.

Cellular binding and uptake of Ad35 vectors

For evaluation of effects of divalent cations on cellular binding and uptake of Ad35 vectors, K562 cells, which were seeded at 3×10^4 cells well^{-1} into a 48-well plate, were incubated with Ad35GFP in the presence of 5 mM EDTA or Mn^{2+} for 1.5 h at 4 or 37 °C as described above. The cells were then recovered and washed five times with ice-cold phosphate-buffered saline, and total DNA, including the Ad vector DNA, was extracted from the cells using DNeasy Tissue Kit (Qiagen). Quantitative real-time PCR was performed as described previously.[23] In transduction-blocking experiments using the RGD peptide, human CD34$^+$ cells and K562 cells were incubated with Ad35GFP at 4 or 37 °C in the presence of 200 μg ml^{-1} of the RGD peptide for 3 h (CD34$^+$ cells) or 1.5 h (K562 cells) as described above. Following incubation, the cells were washed and the vector genome numbers were measured by real-time PCR analysis, as described above. The amounts of vector genome of Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP were similarly evaluated following incubation with Ad35GFP, Ad35RGE-GFP and Ad35ΔRGD-GFP at 6000 VP per cell for 3 h (CD34$^+$ cells) or 3000 VP per cell for 1.5 h (K562 cells) at 4 or 37 °C.

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OPINION

"想像力"と"創造力"

早 川 堯 夫

ダヴィンチやガウディは, 自然現象に, 絶妙に統合化された均衡や生物機能の発現に合目的性を持つよう造形された形態があることを洞察し, "想像力"と"創造力"を発揮して創作に投影した. 天才が自然の摂理に学んだ創造物は時空を超えて普遍的な人類の遺産となった.

医療分野では, 生命が直接対峙し, 学ぶ対象である. 天才によらずとも, 生命現象の本質や原理, 合目的性について洞察し, "想像力"と"創造力"を発揮すれば, 必然的に人類共通の資産である医薬品などを効果的に創出できる機会は多くなると期待される.

生命現象のミクロな有機的機能単位は, 細胞, 細胞外マトリクスおよび生体内機能分子の動的連携的な相互作用で成り立っている. 正常な生命現象が営まれている際には, これらの要素に関する適切な場(環境)の形成ならびに量や時間的制御が行われ, いわば内在性のDDSが成立している, との見方が出来る. このホメオスタシスが破綻すれば病的状態となる.

薬物療法や医療技術は, 疾病原因や疾病機構を制御し, 破綻を修復することにより正常な生命現象への回復と維持を図ることを目標としている. その際, 最終製品レベルで人為的に操作しうる最も効果的な手段がDDSである. DDSの基本的コンセプトは, 有効成分などが生体のバリアを通過し, 標的部位に選択的に到達し, その場で, 必要な濃度で必要な時間その機能を発揮する方策を付与することである. このコンセプトをさらに広げ, 標的部位での内在性のDDS状態(正常な生命現象)をミミックする複合的方策を目指せば, DDSの医療における活用範囲は一段と広がり, 意義は高くなる.

たとえば, 再生医療では外部から目的細胞を移植する試みが一般的だが, 生体に残存している内在性の目的細胞やその前駆細胞の増殖・分化・機能発現(再生)を促すために, その場の周辺環境整備に必要な要素(機能分子のカクテルやクロストークに必要な細胞など)を送達するというDDSも試みられてよい. 各がん細胞に特異的ながんワクチンの組合せによる免疫細胞群動員戦略や, 特定細胞にトロイの木馬を導入して, つぎの指令で細胞での効果を目指すDDSもありうる.

新たなDDS技術の開発に併せて, 適正な評価技術の開発が不可欠なのはいうまでもない.

ゲノム科学や幹細胞学の進展を背景に, 新規遺伝子や蛋白質, 細胞, それらの制御分子など医薬シーズの探索・開発競争が熾烈である. この段階でもわが国なりの成果が望まれるが, 究極の課題はDDSにより医薬シーズを患者にいかに効果的に適応させるかである.

わが国のDDSは高いレベルにあり, 世界に伍して医薬振興を図っていくうえでの基幹技術としてきわめて重要な位置を占めていると考えられ, 今後ますますの進展が期待される.

開発研究を実用化に結実させるには, 産・学・官の関係者が, 一日でも早い患者の治療が共通の目標であるとの認識のもと, みずから担う役割と目標の関係について "想像力"と"創造力"を発揮し, それぞれの立場でベストを尽くし, かつ緊密に連携をすることが肝要である. 関係者が適所で光を放ち, その英知の結晶が一つでも多く, わが国独自に生まれることを切望している.

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バイオ医薬品等をめぐる
最近の動向と話題

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1―はじめに

バイオ医薬品や医療機器には、①細胞基材より生産されるタンパク質性医薬品、②遺伝子治療用医薬品、③細胞・組織利用医薬品・医療機器、④トランスジェニック動物や植物由来の製品、⑤核酸医薬品、などが挙げられる。これらがより望ましい形でより早く世に出て、疾病治療等に適正に供されるには、基礎研究、開発研究、実用化研究、承認審査、適正使用、市販後の製品管理や情報収集などがそれぞれ合理的、効率的、効果的に推進、運用されるとともに、相互の密接な連携のもと各活動や要素が相乗的に作用することが必要である[1]。これらをめぐる最近の動向と話題をいくつかとりあげてみる。

2―細胞基材由来タンパク質性医薬品

1) 細胞基材由来タンパク質性医薬品は依然として開発の主役

大腸菌、酵母、ヒト及び動物細胞由来のタンパク質性医薬品は、1980年代に初めて上市されて以来、バイオ医薬品の代名詞になった。これらは現在もなお最も盛んなバイオ医薬品開発の対象となっている。わが国では最近5か年に新規酵素及びその制御剤6種類、新規ホルモン及びホルモン誘導体5種類、組換えインターフェロンサブタイプ3種類、改変型エリスロポエチン1種類、ヒト化抗体及びヒト抗体4種類、融合タンパク質（可溶性TNF受容体Fc）1種類、ヒト血清アルブミンなどが承認されている。このうち、ヒト化抗IL6受容体抗体、アルブミン、トロンボモデュリンなどがわが国オリジナルで、開発者のご努力には深甚なる敬意を払いたい。しかし、世界全体の開発数に占める割合は国力からみるとかなり低い。現在、わが国で申請中のものは、適応追加を中心に約11品目、申請準備中が2品目である。フェーズ1以上のものは、適応や剤型追加も含めて50品目以上にのぼるが、わが国オリジナルなものは数例でしかない。近年の新有効成分開発の中心は、抗体医薬品であり、その開発は初期のマウス抗体からキメラ抗体、ヒト化抗体、ヒト抗体と

進化し、さらにはFc部分と他の機能分子を融合したタンパク質も開発されてきている。承認については米が先行し、EUがその7割近くであるが、わが国は4割程度である。欧米にあるものがわが国にないドラッグラグは患者さんの利益からすると重大であり解消される必要があるが、この問題は徐々に解決の兆しがみえる。ちなみに、第1世代のインスリン、エリスロポエチン（EPO）、インターフェロン、ヒト成長ホルモン（hGH）などのアミノ酸配列改変、糖鎖改変、PEG化など改変型タンパク質性医薬品については7割を越える程度にドラッグラグは解消してきている。一方で抗体医薬品の場合、わが国オリジナルなものがただ1例（数％）であり、それも欧米市場に進出していないことは残念である。さらに現在世界中のフェーズ1以上のバイオ医薬品開発状況をみるとき、わが国オリジナルなものが約3％に過ぎないことは深刻に憂慮すべき問題である。

2) 後続バイオ製品、捲土重来を期待

後続バイオ製品の規制に関する論議が盛んである。著者も、最近6か月の間に4つの国際シンポジウムに招かれ、後続バイオ製品に関する見解を述べた（Biosimilar2007：2007年9月ワシントンDC、第2回PMDA国際シンポ：2008年1月東京、WCBP2008：2008年1月ワシントンDC、Biogenerics2008：2008年3月ボストン）。要約中の要約を図1に示す。本問題は、先発品の上市以来約20年に及ぶ知見、蓄積を踏まえた上で品質、安全性、有効性の保証に焦点を絞って論議を進めていけば、科学的にはさほど解決困難なものではない[1]。しかし、欧米では、法律、経済、先発企業や地域の思惑などの要素がさまざまにからまった中で問題が論じられ、複雑化している。わが国は、2003年にICHで製法変更に伴う製品の同等性・同質性評価（Q5E：コンパラビリティ）の課題を取り上げる際、科学的観点から後続品もスコープに入れるべく強く提案したが、米・EUの反対にあって容れられなかった。Q5E終了後の2005年に改めてICH新規課題として提案したが、欧米産官は製法問題がより優先するとした。その
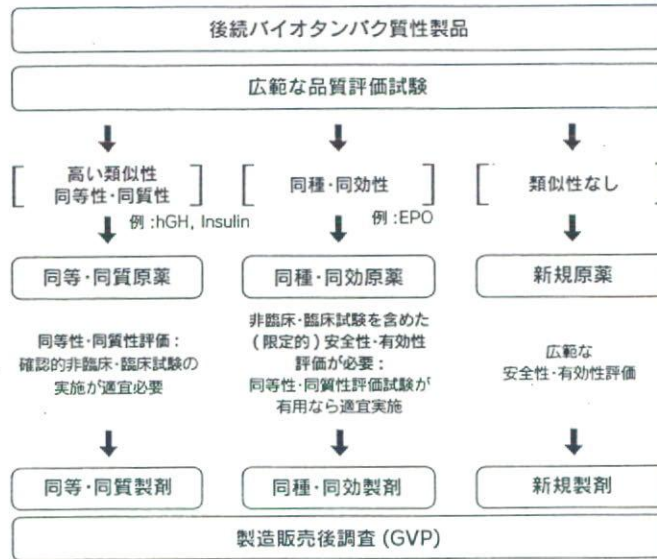
図1　後続タンパク質性医薬品の評価

際わが国が独自の指針作りなどに踏み出すチャンスがあったが機を逸した。その間、EUは後続品をBiosimilarと称していくつかの指針を出した。これらには域内の先発保護の要素も含まれ、科学的不合理さもあるが既成事実の積み重ねは大きい。WHOもEUに類似した指針の制定を準備中とのことである。ところで十数年前わが国のバイオ医薬品開発への試みは非常に活発であったが、その勢いは特許問題等によってブレーキがかけられ、多くのリソースが散逸した。著者はわが国のバイオ医薬開発の復活と振興を願い、その一環として開発目標が明確な後続バイオ製品の開発を契機に、残されたリソースや潜在力を結集して、やがては新薬の開発にも及ぶような活動を開始することを5、6年前より提言してきた。しかし現在のところ、欧米企業の先行を許している状況である。EUでは2006年にhGH2製品、2007年にEPO3製品を承認し、現在EPO2製品が承認見込みという。このままでは後続バイオ製品も外来品にほとんど専有されかねない。チャンスはまだある。わが国独自の取組みの強化を切望したい。

3) タンパク質性医薬品固有の安全性問題に対する取組み

　タンパク質性医薬品の安全性確保上の主要問題の一つに抗原性の問題がある。これについては、有効成分とその他の成分による抗原性に分けて考えた方が合理的である。その他成分については製造過程で極力低減化に努めること及び最終製品への存在許容量を理化学試験法で規定することが合理的であると思われる。有効成分によって引き起こされる抗原性については、動物を用いた非臨床段階で予測することはほとんど困難であり、臨床試験

で注意深くモニターする以外に適切な方策はない。しかし、限られた数の患者や期間では明らかな異常は検出できても、幅広い患者に長期間投与した際の影響は観察できない。したがって、製造販売承認後の安全性モニター計画を綿密に立て、一定期間詳細な観察を続けることが合理的方策であると考えられる。

　もう一つは、2006年に抗CD28スーパーアゴニスト抗体のP1試験（First in Man：FIM）においてみられた重篤な副作用発生事件の教訓を今後にいかに生かすかである。特に留意すべき事項は、①薬物の特性や生物学的作用の特徴、作用機構などに関するデータを極力収集し、これらを踏まえてヒト体内で起こりうる現象を想定し、非臨床データからヒトでの反応がどの程度予測できるか、ヒトに重篤な副作用を生じる可能性があるかを、慎重に評価すること、②FIM試験における開始用量の設定に関し、表1に例示したような明らかなリスクを有する

表1　明らかなリスクを有する薬物の例示

1	生体系に重篤な生理学的撹乱を引き起こす薬物
2	アゴニストあるいは亢進作用を有する薬物
3	先行する事例のない新規の作用機序を有する薬物
4	高い種特異性によって前臨床でのリスク評価が困難/不可能であるような薬物
5	天然のリガンドよりも高いポテンシーを有する薬物
6	多機能性の薬物（例：二価抗体、Fc結合）
7	正常な制御機構をバイパスする薬物
8	免疫系を標的とする薬物
9	In vivoでの生物学的増幅系に作用する可能性のある薬物

薬物については、動物での無毒性量に基づく算出法にのみ依存するのではなく、例えば、ヒト細胞や霊長類等での「最小の生物学的作用を生じる用量」を考慮するなど幅広いアプローチを採択すべきこと、③FIM試験では投与方法を緩徐なものとすること、投与間隔を十分に空けて投与後の有害事象の発生の有無を観察すること、④副作用発症後の処置等を含めて慎重に吟味された試験デザインを作成すること、⑤被験者の急変に備えた緊急体制、ICUの確保、活用等に万全を期すること、⑥免疫系に作用する医薬品のように作用が全身に及ぶことが予測される場合には、FIM試験には、患者を用いることを考慮すること、などである。また、別に、規制当局間での情報共有化のための非公開データベースの構築や審査プロセスの充実、FIM試験の実施環境の整備や人材の育成も望まれるところである。

4）科学に基づく効果的・効率的で柔軟な品質規制を各国に適した形で

タンパク質性医薬品の品質をめぐる問題については、国際レベルではICHQ5A～E、Q6B及びCTD（Common Technical Document）において基盤的な問題についての科学的な留意事項に関する調和ガイドラインが示されている。これらに盛り込まれた医薬品の品質確保の目的や本質、科学的基本原則を踏まえ、個々の事例への適用を柔軟で合理的なものにするという前提で考え、解釈運用を適切に行えれば、患者さんに速やかに優良な医薬品を提供するという目標に対する品質関連の規制環境はほぼ整備されていると考えられる。ただ、バイオ医薬品の製造関連事項については、CTDの説明にはあるが、対応する技術的要件に関する正式な指針がICHガイドラインとしてはない。そこで2年半ほど前よりバイオ医薬品の製造問題をICHの課題としてとりあげる機運が欧米を中心に高まり、一旦はわが国の立場、意見も取り込んだコンセプトペーパーが6極の専門家の同意を得て完成した。ところが、品質問題について総合的に新たな概念と方向性を掲げて進めたいとする欧米の産官、特に化学薬品関係者からの強い要望によって、バイオ医薬品も渦中におかれ、独自の活動は中断したままになっている。新たな概念と方向性とは、筆者の理解が及ぶ範囲で要約すれば、知見や経験の蓄積を最大限活用し、医薬品のライフサイクルにわたってリスク管理と科学を根拠として効率的、効果的に品質の改善改良を統合的にかつ柔軟に行うことが肝要であるという概念や体系を産官ともに認識し、共有してことを進めるというものかも知れない。これは、ことの本質から製造、工程管理、工程評価/検証、規格設定など個々の要素はもとより、品質確保全体戦略にも適用される。とはいえ具現化する際には、製品毎、会社毎に適用の仕方は異なり、同等・同質製品を対象にした場合にもアプローチは異なることになる。肝要なのは申請者側が最終的に採用した方策の妥当性を明らかにする

ことであり、規制側にとってはその方策を十分に理解した上で評価するということであろう。こうした動きの背景の一つには現行の欧米の審査のあり方が深く関わっている。欧米では、基本的にCTDの第3部が承認対象であり、それをベースに審査する。そのため、業界にとっては膨大な資料の作成が必要である。必然的に、承認後の一部変更も膨大な作業量と人的/時間的リソースを要することになる。それは審査当局にとっても同様の作業量やリソースを要することを意味している。わが国より一桁あるいは二桁近くの人員を要する米国やEUをもってしても、現行のままでは立ち行かない。そこで、より高い科学的達成度を目指しつつ、一方でリソースの合理的活用や柔軟な対応に向けての方向性の提示やある種の制度的改革を必要とすることになった。それ自体はそれぞれの国や地域の事情に応じた政策であり、コメントの余地はない。しかし問題は、世界的企業が世界同一文書による承認を究極には目指して、また、欧米が地域の政策的統合のためのテコとなるよう、あるいは汎欧米主義を意識して、これをICHの場に持ち出し、しかも特殊な用語を概念や方向性を示すキーワードとして政策的に用いている事であろう。その用語とはQbD（Quality by Design）であり、Design Space（DS）などである。また、その背景となるガイドラインはQ8、Q9、Q10ということである。ところで1月末に開催されたWCBP2008では多くのFDA担当官をはじめ約450名の関係者が集まり、口々にQbDやDSを唱えたが、議論は混迷を極めた。QbDやDSに対するそれぞれの理解や解釈が非常にまちまちであったからである。また、一口に製品の製造や品質確保といっても、課題の対象を、①医薬品候補物質の探索段階、②治験に入る前の段階、③承認申請のための最終データ整備段階、④承認申請・審査、⑤市販後の改善改良・一変のいずれの段階に焦点をあてて議論しているのか、何をどの程度、科学的目標課題とし達成度とするのか、それぞれの段階で課題に取り組む主体が誰なのかが明確に定義されないまま議論が行われているところにも混乱の原因があると思われる。この筆者の見解にFDAやPhRMAのICH代表は全く異議をはさまなかった。バイオ医薬品の製造に関して、筆者はICHでは産・官に最も共通する上記④に焦点を絞り、その上で前後を見渡すべきと考える。また、ICHが技術的要件の調和をはるかに越えてある国や地域の政策やシステムの変更、世界戦略の展開の具にすることは避けるべきと思う。加えて、彼の地でさえ関係者間で共通の理解や認識が得られない政策的キーワードを共通の理解や認識が最も核心をなすICHという場に持ち込むべきではないと思う。適宜、適所で共通・共有の情報媒体となる平易な表現を用いて必要な科学的事項を記述すればすむことである。審査官の人数等が圧倒的に少なく、第2部に第3部のエッセンスを要約し、第2部のエッセンスを第1部の承認事項としていて、

科学的・効果的・効率的で柔軟な規制制度を有している
わが国と、第3部をベースにした承認事項の合理化や柔
軟な取り扱い、人的資源スリム化を図ろうとしている欧
米とは事情が異なる。FDA等の目指す理想の究極に日本
型モデル（図2）があるのではないかとの問いに、うなず
くFDAの専門家も多い。ところでQ8の一部、Q9、Q10は
現在のところオプションである。それらには多くの学ぶ
べきこともあり、必要に応じて適宜活用するのは望まし
いことといえる。一方で、化成品とバイオ医薬品原薬製
造に関する共通ガイドラインをQ8、Q9、Q10をバックボ
ーンにしながら作成しようとする動きがある。そのよう
な形で技術的要件のガイドラインができてしまうと、そ
れを通してオプションであるはずのQ8の一部、Q9、Q10
が要求事項になってしまうことに思い至る必要がある。
ICHは各国に適用できる形で必要な科学的要件の調和を
図るべきであろう。

3—遺伝子治療薬や核酸医薬品はこれから

遺伝子治療はまだ安全性・有効性が確立されていない
ため、重篤な遺伝子疾患、がん、その他の生命を脅かす
疾患又は身体の機能を著しく損なう疾患を対象としてい
る。臨床開発初期段階のものが多く、日米欧では未承認
であるが、中国等では承認例も出ている。わが国におけ
る遺伝子治療研究はいまだ20余例で米国の約840例や欧
の約320例に比較して少ない。しかしわが国独自の導入
遺伝子としてHGF、ベクターとしてセンダイウイルスベ

クターやその構成要素を用いた膜融合リボソームの開発
などが試みられており、今後の発展に期待したい。遺伝
子治療は現在のところ、X連鎖重症複合免疫不全症（X-
SCID）、アデノシンデアミナーゼ欠損症、慢性肉芽腫症、
パーキンソン病などの単一遺伝子疾患で、著効あるいは
一定の効果が得られているが、癌に対する効果は限定的
である。一方、X-SCID遺伝子治療でT細胞白血病様症状
発症（治療可能）やアデノウイルスベクターの不適切な
大量投与による異常免疫反応等の重篤な副作用も見ら
れ、安全性などに慎重な検討が必要である。遺伝子治療
用医薬品においては、①複製（増殖）性ウイルスの検出
方法、存在許容量と管理方法、②ウイルスタンパク質に
よる抗原性に対する留意と軽減方策、③目的外の細胞・
組織への遺伝子導入の回避と投与量の軽減のための方
策、④染色体への遺伝子組み込みに伴う遺伝毒性、がん
原性発現への慎重な対処と回避策、などが安全性確保上
の重要課題であるといわれており、ICHのワーキンググ
ループでも検討対象となっている。

最近ウイルス療法が、がんの治療で遺伝子治療よりも
高い効果が期待されるとして注目を浴びてきた。これは、
正常細胞内では増殖できず、標的とするがん細胞内での
み選択的に増殖可能な腫瘍溶解性ウイルス（変異又は組
換え単純ヘルペスウイルスや組換えアデノウイルスな
ど）を用いたがんの新しい治療法である。わが国では4
例ほどの実施例がある。

核酸医薬品は、一般にゲノム医学を背景に、特定の遺

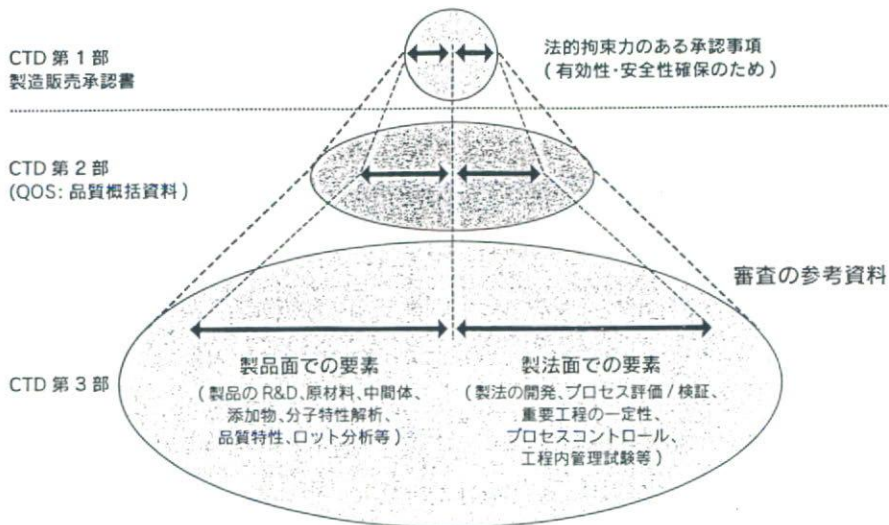
図2　科学に基づく効果的・効率的で柔軟なわが国の品質に関する規制

伝子発現を制御するよう設計した塩基配列を有するものである。アンチセンス、リボザイム、デコイ、siRNA（small interfering RNA）などがこの範疇に含まれる。海外ではサイトメガロウイルス性網膜炎を対象にしたアンチセンスが医薬品となっている。さらに、海外では少なくとも18品目以上のアンチセンス医薬品について臨床試験が行われている。SiRNA医薬品は、海外では少なくとも7品目以上の製品について臨床試験が行われている。リボザイムについてはVEGF受容体、EGF受容体、HCVをターゲットにしたものなどが臨床試験の段階にある。DNAワクチンについては海外で、エイズ、B型肝炎、インフルエンザなどの感染症や、がんを対象とした臨床試験が進められている。

4—日、米、EU、韓国でしのぎをけずる細胞治療

　ヒトまたは動物より分離した細胞や組織を培養、加工し、直接患者に投与することによりさまざまな疾患の治療を行うことを一般に細胞治療あるいは再生医療と称している。細胞治療や再生医療に用いられる細胞と対象疾患の関係は、例えば、ガンには活性化リンパ球や樹状細胞等、神経疾患には神経幹細胞、熱傷・創傷等には表皮細胞/線維芽細胞、心筋梗塞には骨格筋芽細胞/心筋芽細胞/血管内皮細胞、リウマチには軟骨芽細胞、骨粗しょう症には骨芽細胞、再生不良性貧血には血液幹細胞、糖尿病にはランゲルハンス細胞、重篤な肝疾患には肝細胞、筋ジストロフィーには筋芽細胞等がある。当面は他の治療法では治療困難な疾患が対象である。わが国では、最近、培養皮膚が製造販売承認を得た。また、外傷性軟骨欠損症などを適応とする軟骨細胞が治験を終了し、さらに心機能改善のための骨格筋芽細胞や造血幹細胞移植時の移植片対宿主病を適応とするヒト間葉系幹細胞が治験準備中である。いわゆる確認申請済みあるいは申請中のものは合わせて6品目である。一方、医師主導型で臨床研究が行われていると推定される例は200例を越える。

　最近、再生医療の一層の推進を目指して、確認申請やその審査（先端的治療に用いられる細胞・組織製品における品質・安全性をヒトに投与する前に確認するという、いわゆる上乗せ部分の申請・審査）のあり方についての再検討を含めて、関係指針の見直しが行われている。改訂指針案の概略を表2に示す。

表2　ヒト由来細胞・組織加工医薬品等の品質
及び安全性確保に関する改訂指針案の概略

1　自己由来のものと同種（他家）由来のものに分け、それぞれの製品における品質及び安全性確保のために必要な基本的要件を明確にすること。

2　基本的要件は承認申請をも念頭においたものであるのに対して、確認申請とは治験を開始するに当たって支障となる品質、安全性上の問題があるか否かを確認するためという趣旨を踏まえて、基本的要件のうち確認申請までにどの程度の試験や評価をするべきかを明確にすること。

3　従来は必要な試験や評価に関する科学的考え方及び申請に際して必要な情報や記載すべき事項が1つの指針に盛り込まれていたが、確認申請の記載要領に関することは別記事項として明確にする。

4　指針の記述は理解しやすいものとするとともに、Q＆Aにより、必要な背景説明を行うこと。

5　細胞・組織加工医薬品等の種類や特性、臨床上の適用法は多種多様であり、また、本分野における科学的進歩や経験の蓄積は日進月歩である。本指針を一律に適用したり、本指針の内容が必要事項すべてを包含しているとみなすことが必ずしも適切でない場合もある。従って、個々の医薬品等についての試験の実施や評価に際しては本指針の目的を踏まえ、その時点の学問の進歩を反映した合理的根拠に基づき、ケース・バイ・ケースの原則で柔軟に対応することが必要であること。

6　最終製品の規格及び試験方法の設定、個別患者への適用ごとの原材料の品質管理、製造工程の妥当性の検証と一定性の維持管理の他、中間製品の品質管理を細胞・組織加工医薬品等の品質管理全体方策の要素ととらえ、これらを相補的、合理的に組合わせて全体として品質管理の目的が達成されるとの観点に立つこと。

7　製品の特性及び適用法から評価が必要と考えられる安全性関連事項について、技術的に可能であれば、科学的合理性のある範囲で、適切な動物を用いた試験又はin vitroでの試験を実施すること。ヒト由来の試験用検体は貴重であり、また、ヒト由来製品を実験動物等で試験して必ずしも意義ある結果が得られるとは限らない。合理性のない試験の実施を求める趣旨ではないという前提で、製品の特性等を考慮して適切な試験を検討すること。

8　技術的に可能かつ科学的に合理性のある範囲で、実験動物、細胞等を用い、適切に設計された試験により、製品の機能発現、作用持続性、医薬品・医療機器として期待される効果を検討すること。

　再生医療分野では、世界の再生医療技術の3分の1を日本発にというスローガンでその推進を図ろうとしている。例えば、角膜再生ではわが国が先行している。皮膚再生、心筋再生、神経再生、膵島再生でわが国は米国あるいはEU等と並んでいる。一方、再生骨・軟骨や体性幹細胞による肝細胞再生技術等では、米国、EUや韓国の後塵を拝している。

　ところでヒト胚性幹細胞（ES細胞）はあらゆる細胞に分化誘導可能な万能細胞として注目を浴びてきたが、科学技術的な問題はもとより、倫理的な問題が大きなネックとなってきた。最近、皮膚など正常な組織（細胞）から適切な遺伝子群の導入と適切な増殖因子を組合せる培養により、再プログラム化された人工多能性幹細胞（iPS細胞）が得られることが明らかになった。iPS細胞は、さまざまな基礎研究の対象としてきわめて重要な意義を持つ。その一方で、各種の目的細胞・組織に分化・誘導して、基礎・応用研究に必要な細胞を得ることや、さらに細胞治療・再生医療用の素材としてもきわめて大きな期待を集めている。実用化には、より効率的・効果的なiPS細胞作成技術の開発とその確実性、品質・安全性の確保、iPS細胞の大量獲得技術、各種目的細胞への確実かつ安全な分化・誘導技術と目的細胞の大量生産技術、各段階での細胞の確実な特性解析と最終製品での品質・安全性確保のための評価技術の開発、臨床評価等、切り拓くべき課題は多い。しかし、わが国の本分野の英知を結集して取り組むに価する課題であることは明かである。最終ゴールへの道を明確に示し、推進を図る規制環境のさらなる整備も充実していく必要がある。

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A New Role of Thrombopoietin Enhancing *ex Vivo* Expansion of Endothelial Precursor Cells Derived from AC133-positive Cells*

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We previously reported that CD31^{bright} cells, which were sorted from cultured AC133^{+} cells of adult peripheral blood cells, differentiated more efficiently into endothelial cells than CD31^{+} cells or CD31^{−} cells, suggesting that CD31^{bright} cells may be endothelial precursor cells. In this study, we found that CD31^{bright} cells have a strong ability to release cytokines. The mixture of vascular endothelial growth factor (VEGF), thrombopoietin (TPO), and stem cell factor stimulated *ex vivo* expansion of the total cell number from cultured AC133^{+} cells of adult peripheral blood cells and cord blood cells, resulting in incrementation of the adhesion cells, in which endothelial nitric oxide synthase and kinase insert domain-containing receptor were positive. Moreover, the mixture of VEGF and TPO increased the CD31^{bright} cell population when compared with VEGF alone or the mixture of VEGF and stem cell factor. These data suggest that TPO is an important growth factor that can promote endothelial precursor cells expansion *ex vivo*.

Neovascularization is an important adaptation to rescue tissue from critical ischemia. Postnatal blood vessel formation was formerly thought to be primarily due to the migration and proliferation of preexisting, fully differentiated endothelial cells, a process referred to as angiogenesis. Recent studies provide increasing evidence that circulating bone marrow-derived endothelial progenitor cells (EPCs)[2] contribute substantially to adult blood vessel formation (1–5). Cell therapy using EPCs is widely performed to rescue tissue damaged due to critical ischemia.

Although EPCs have been thought to be derived from many kinds of cells, cells characterized as CD34^{+} (6), AC133^{+} (7, 8), and CD14^{+} (9) are also thought to differentiate to EPCs. The main role of EPCs has been thought to be the release of angiogenic factors such as interleukin-8 (IL-8), granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor, and vascular endothelial growth factor (VEGF) (9). To obtain a sufficient number of EPCs for the treatment may be very important in cell therapy for critical ischemia.

On the other hand, EPCs are mobilized from bone marrow by many substances such as G-CSF (10), granulocyte macrophage-colony stimulating factor (GM-CSF) (5), VEGF (3), erythropoietin (11–13), and statins (14, 15) *in vivo*. To get as many EPCs as possible without unduly burdening the patient, it is desirable to establish efficient expansion methods for EPCs *in vitro*.

Thrombopoietin (TPO), initially identified as the primary regulator of platelet production (16), plays an important and nonredundant role in the self-renewal of and expansion methods for hematopoietic stem cells (17–19). Recently, TPO has been found to exert a proangiogenic effect on cultured endothelial cells (20). The mechanism by which hematopoietic cytokines support revascularization *in vivo*, however, remains unknown. TPO has increased the number of colony-forming units-granulocyte-macrophage (21) and of burst-forming units-erythroid (22) *in vivo* and leads to a redistribution of colony-forming units-erythroid from marrow to spleen. Moreover, TPO acts in synergy with erythropoietin to increase the growth of burst-forming units-erythroid and the generation of colony-forming units-erythroid from marrow cells (21, 23, 24).

In our previous study (25), we isolated AC133^{+} cells and examined their endothelial differentiation *in vitro*. CD31(PECAM-1)[+] and CD31^{bright} cells appeared at an early stage of the *in vitro* differentiation of AC133^{+} cells, and CD31^{bright} cells derived from AC133^{+} cells were identified as the precursors of endothelial cells because CD31^{bright} cells had differentiated more efficiently to endothelial cells than others. Therefore, we conclude that CD31^{bright} cells derived from AC133^{+} cells possess the typical character of EPCs. In this study, we analyzed the effects of TPO on the appearance of CD31^{bright} cells from AC133^{+} cells, and we show that TPO plays an important role in *in vitro* EPC expansion.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant TPO and recombinant stem cell factor (SCF) were kindly provided by Kirin-Amgen Inc. (Thousand Oaks, CA). Recombinant human VEGF was purchased from Strathmann Biotec AG (Hamburg, Germany). The AC133

[2] The abbreviations used are: EPCs, endothelial precursor cells; VEGF, vascular endothelial growth factor; FN, fibronectin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PE, phycoerythrin; TPO, thrombopoietin; SCF, stem cell factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; PI3K, phosphatidylinositol 3-kinase; VEcad, vascular endothelial cadherin; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; STAT, signal transducers and activators of transcription; JAK, Janus kinase; KDR, kinase insert domain-containing receptor.

magnetic cell sorting kit and phycoerythrin (PE)-conjugated anti-CD133/2 antibody were from Miltenyi Biotec (Gladbach, Germany). Allophycocyanin-conjugated anti-CD110 (TPO receptor) antibody, fluorescein isothiocyanate (FITC)-conjugated anti-CD31 monoclonal antibody, FITC-conjugated anti-CD34 monoclonal antibody, and anti-STAT3 monoclonal antibody were from Pharmingen. Phycoerythrin-conjugated vascular endothelial cadherin (VEcad/CD144) antibody was from Beckman Coulter (Marseilles, France). Anti-vascular endothelial growth factor receptor-2 (Flk-1/KDR) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-human endothelial nitric oxide synthase (eNOS) rabbit polyclonal antibody (Cayman Chemical, Ann Arbor, MI) were obtained. Anti-phospho-Akt (Ser-473) antibody, anti-Akt antibody, and anti-phospho-STAT3 (Tyr-705) antibody were from Cell Signaling Technology (Beverly, MA). Fibronectin (FN)- and type IV collagen-coated dishes were purchased from Iwaki Co., Tokyo, Japan. Phycoerythrin-conjugated anti-CD14 antibody was from DakoCytomation (Glostrup, Denmark).

Preparation of Peripheral Blood Mononuclear Cells—Human cord blood was kindly supplied by the Metro Tokyo Red Cross Cord Blood Bank (Tokyo, Japan) with informed consent. The buffy coat fraction was prepared from voluntary donated human blood of Saitama Red Cross of Japan (Saitama, Japan). The blood sample was diluted with phosphate-buffered saline (PBS) containing 2 mM EDTA and was loaded on a Lymphoprep™ tube (Axis-Shield PoC AS, Oslo Norway) (density = 1.077). After being centrifuged for 20 min 800 × g at 18 °C, mononuclear cells were collected and washed with sorting solution (PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin).

Flow Cytometric Analysis of AC133 and CD34 Expression in Mononuclear Cells—To eliminate the dead cells, dead cells were stained with 7-amino actinomycin D. Mononuclear cells were labeled with PE-conjugated anti-AC133 monoclonal antibody and FITC-conjugated anti-CD34 monoclonal antibody simultaneously at 4 °C for 30 min. After washing with the sorting solution, flow cytometric analysis was performed with a FACSCalibur (BD Biosciences).

Magnetic Cell Sorting of AC133⁺ Cells—Mononuclear cells were labeled with magnetic bead-conjugated anti-AC133 antibodies according to the protocol directed by the manufacturer. After the brief wash with the sorting solution, the cells were separated by a magnetic cell separator (autoMACS, Miltenyi Biotec, Gladbach, Germany), and the positive cells were then collected.

Culture of AC133⁺ Cells—Isolated AC133⁺ cells were cultured in EBM-2 (Cambrex Corp., East Rutherford, NJ) medium containing 20% heat-inactivated FBS and 30 mg/liter kanamycin sulfate at 37 °C under moisturized air containing 5% CO_2 with 50 ng/ml VEGF as control medium. Control medium containing VEGF was added with TPO, SCF, or both. Cells were plated on FN- or type IV collagen-coated dishes at a cell density of ~10^6 cells/ml. We have previously shown that EPCs can tightly adhere to an FN-coated dish but weakly to type IV collagen-coated dish (25). Analysis of adherent EPCs was performed on FN-coated dish and that of suspended EPCs on type IV collagen-coated dish. Half of the medium was exchanged

once every 3–4 days with fresh medium. Adherent cells on FN-coated dish were fixed with ethanol chilled to −20 °C and then subsequently subjected to an immunostaining procedure or other treatments. Cells on type IV collagen-coated dish were subsequently subjected to flow cytometric analysis.

Immunostaining of Adherent Cells—After fixation with chilled ethanol (−20 °C), the cell layer was washed three times with PBS. Cells were incubated with 1% bovine serum albumin in PBS (−) for 1 h at 4 °C for blocking and then with each first antibody in 1% bovine serum albumin in PBS (−) for 1 h at 4 °C. After washing with PBS, the cells were incubated with FITC-conjugated anti-mouse IgG antibody or rhodamine-conjugated anti-rabbit IgG antibody for 1 h at 4 °C. Cells were washed with PBS and then examined using a Zeiss LSM 510 microscope with an excitation wavelength of 488 nm and an emission of 530/30 nm for FITC or 570/30 nm for rhodamine.

In every experiment, we used nonspecific immunoglobulin corresponding to the first antibody species as a control and confirmed that the cells were not stained with control immunoglobulin. The fluorescence intensity of 20 randomly selected cells was calculated using the Scion Image program within the linear range for quantitation.

Analysis of Cytokines in the Supernatant of CD31^bright and CD31⁺ Cells—The expression of CD31 on cultured AC133⁺ cells was determined with a flow cytometer. After AC133⁺ cells were cultured for several days on either FN-coated or collagen type IV-coated dishes, both adherent and nonadherent cells were collected. The collected cells were labeled with FITC-labeled anti-CD31 antibody for 15 min at 4 °C. After a brief wash with 0.5% bovine serum albumin in PBS, flow cytometric analysis was performed. CD31^bright and CD31⁺ cells were sorted from cultured AC133⁺ cells with FACSAria (BD Biosciences). Sorted cells of both populations were subsequently cultured in EBM-2 supplemented with 20% FBS in the absence of any cytokines. After 5 days, the collected supernatant of cells was frozen at −20 °C. Cytokines were measured by a BD™ cytometric beads array Flex set system (BD Biosciences) according to the manufacturer's protocol.

Flow Cytometric Analysis of Various Cell Surface Markers in Cultured AC133⁺ Cells—After AC133⁺ cells were cultured for the indicated period, cells were co-stained with FITC-labeled anti-CD31 antibody and PE-labeled anti-CD14 antibody or PE-labeled VEcad antibody. Cells were also stained with FITC-labeled anti-CD31 antibody, allophycocyanin-labeled anti-CD110 antibody, and PE-labeled anti-AC133 antibody triply and then subjected to flow cytometry. Dead cells were eliminated by staining with 7-amino actinomycin D.

Calculation of the Absolute Number of CD31^bright Cells—The absolute number of CD31^bright cells was multiplied by the total cell number of each well, and the ratio of CD31^bright cells was analyzed by fluorescence-activated cell sorter.

Preparation of Cell Lysates and Immunoblotting—After cell sorting, AC133⁺ cells were suspended in 20% FBS-EBM2 and cultured for 3 days in the presence of VEGF and TPO. Cells were collected and incubated in 2% FBS-EBM2 for 1 h. Cells were stimulated by 50 ng/ml TPO, 50 ng/ml VEGF, or both for 15 min. Cells (1 × 10^6) were collected and lysed in lysis buffer containing 1% Triton X-100, 10 mM K_2HPO_4/KH_2PO_4 (pH