in hemophilia A mice (Fig. 6A). The percentages of lymph node-derived $CD25^+FoxP3^+$ cells of FVIII-IT mice (9.6% ± 2.2%) were significantly greater than those of B6 control (2.0% ± 1.6%), naïve (2.2% ± 0.7%), non-IT (1.8% ± 0.2%) or Alb-IT (1.9% ± 0.8%) mice (Fig. 6B). Moreover, the numbers of spleen-derived $CD25^+FoxP3^+$ cells of FVIII-IT mice (6.5% ± 1.7%) were also augmented as compared with those of B6 control (1.3% ± 0.4%), naïve (1.0% ± 1.3%), non-IT (0.9% ± 1.5%) or Alb-IT (1.2% ± 1.6%) mice. We also performed standard regulatory T-cell inhibition experiments, stimulating normal B6 T cells with anti-CD3 antibodies and adding the presumptive regulatory T cells at a variety of ratios (Fig. 6C). The $CD4^+CD25^+$ T cells isolated from all of these mice similarly inhibited proliferation of normal B6 mouse-derived $CD4^+CD25^-$ T cells in a dose-dependent manner, suggesting that the thymic tolerance of our system might not only depend on the induction of regulatory T cells. Nevertheless, our results show that intrathymic administration of FVIII antigen leads to the augmentation of FVIII-specific regulatory T cells in mice that had never previously been exposed to the antigen.

$CD4^+CD25^+$ T cells isolated from mice with intrathymic injection of FVIII inhibit the FVIII-immunized T-cell proliferative response.

We evaluated the effect of $CD4^+CD25^+$ T cells on the proliferation of antigen-presenting cell-mediated $CD4^+$ T cells of non-IT mice with stimulation with FVIII (Fig. 7A). The $CD4^+CD25^+$ T cells derived from B6, naïve, non-IT or Alb-IT mice could not suppress $CD4^+$ T-cell proliferation (Fig. 7B). By contrast, the FVIII-IT-mouse derived $CD4^+CD25^+$ T cells significantly blocked the proliferation of the non-IT-mouse-derived $CD4^+$ T-cells. Interestingly, we could not find any inhibitory effect in a subset of $CD4^+CD25^-$ T cells of FVIII-IT mice, suggesting that this form of thymic tolerance may be dependent on the generation of FVIII-specific $CD4^+CD25^+$ T cells. We also examined whether $CD4^+CD25^+$ T cells from mice injected during the neonatal period could induce *in vivo* immune tolerance in naïve hemophilia A mice that were challenged with subsequent stimulation of FVIII and Freund's adjuvant. As shown in Fig. 8, adoptive transfer with 0.5×10^6 cells per body of $CD4^+CD25^+$ T cells isolated from FVIII-IT mice significantly blocked the development of anti-FVIII antibodies as compared with $CD4^+CD25^+$ T cells from control non-IT mice (6.9 ± 4.1, $n = 9$ vs. 54.4 ± 11.8 BU mL^{-1}, $n = 10$, respectively; $P = 0.038$). These data support a possible role of regulatory T cells in mediating immune tolerance induction *in vivo*.

Discussion

The development of FVIII inhibitory antibodies represents the major complication in the treatment of patients with hemophilia A with FVIII products [2,25. We used a murine hemophilia A model, because multiple intravenous injections of human FVIII into these mice resulted in high titers of anti-FVIII antibodies that have similar characteristics to those of FVIII inhibitors in clinical cases (Figs 2B and 3A) [3,19,26]. The thymus plays a crucial role in the development of the self-immune system [27]. The presentation of antigen to the thymus may allow for precise modification of the immune repertoire. In several experimental models of organ transplantation, the inoculation of donor antigens into the recipient thymus promoted donor-specific tolerance and achieved prolongation of xenograft survivals [14,15,16. We demonstrated that the accurate administration of FVIII into the thymus under a high-resolution ultrasound system (Fig. 1) resulted in the induction of unresponsiveness to FVIII antigen in hemophilia A mice (Fig. 2D,E). These thymic tolerant mice, immunized with TT, developed high anti-TT antibody titers (Fig. 2H). Thus, our results indicate that the tolerance to FVIII exposure by direct thymic injection is antigen-specific, and that the ability to mount a humoral immune response to other T-cell-dependent antigens remains intact in mice with direct exposure to FVIII in the thymus.

The induction of FVIII-specific tolerance may require the $CD4^+$ T-cell subsets that promote FVIII inhibitors [28]. In addition, the IgG subclass of the antibody directed against an antigen is a good indicator of the $CD4^+$ subset contribution to the antibody response to the specific antigen. We showed that the IgG subclasses of anti-FVIII antibodies were mainly IgG_1, $IgG2_a$ and $IgG2_b$ in mice without thymic treatment; however, the production of each IgG subclass was significantly suppressed in hemophilia A mice with FVIII thymic injection (Fig. 3B). The Th1 immune response is known to be predominant in patients with inhibitors in the long term [29], and was also the predominant response in mice that developed antibodies after challenge in adulthood [30,31]. Furthermore, FVIII-deficient mice injected with FVIII into the thymus failed to develop T-cell proliferative response to FVIII antigen (Fig. 4C), and these $CD4^+$ T cells did not significantly increase their production of IL-2, IL-12 or IFN-γ in response to FVIII stimulation (Fig. 5E). Our observation is consistent with the finding of acquired thymic tolerance in experimental autoimmune encephalomyelitis, where intrathymic injection of myelin basic protein or its encephalitogenic peptide induced Th1 cell unresponsiveness and prevented peripheral expansion of antigen-specific $CD4^+$ T cells [32]. Thus, the thymic-treated mice showed reduced amounts of Th1 cytokines, which are important for antibody production. Intrathymic inoculation with antigen may provide a unique opportunity to study the generation of immunoregulatory T cells.

Several investigators showed that clonal deletion of antigen-reactive T cells might be predominantly responsible after thymic injection of antigen [33–35. Others showed that induction of acquired thymic tolerance occurred through the indirect recognition of donor peptides inoculated into the thymus [36,32,37. $CD4^+CD25^+$ T cells are known to be weakly reactive to antigenic stimulation and to be able to mediate suppression of $CD25^-$ naïve T cells [38,39]. We demonstrated that intrathymic administration of FVIII resulted in an enrichment of $CD4^+CD25^+FoxP3^+$ T cells

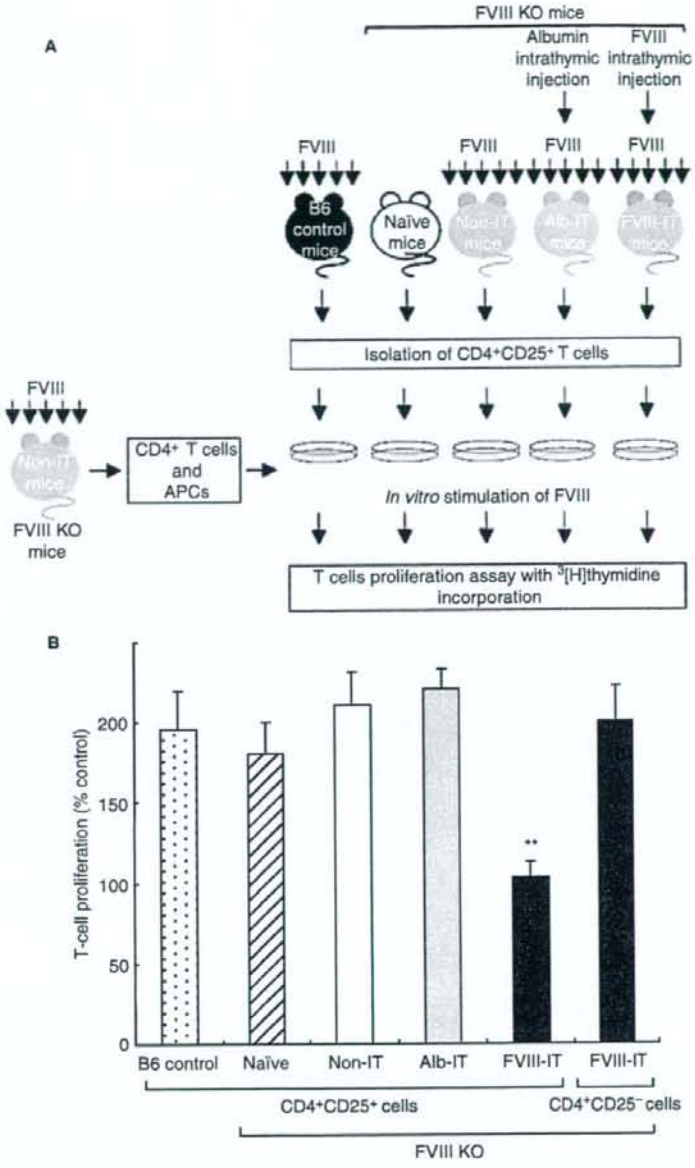

Fig. 7. $CD4^+CD25^+$ T cells isolated from thymus-injected mice inhibit the FVIII-immunized $CD4^+$ T-cell proliferative response. (A) $CD4^+CD25^+$ T cells were isolated from spleens of B6 control mice, FVIII-deficient mice without intrathymic administration of FVIII (non-IT mice), mice that had been given intrathymic injections of human albumin (Alb-IT mice), and mice that had been given intrathymic injections of FVIII (FVIII-IT mice), each of which had been stimulated with intravenous injections of FVIII, by cell sorting, as described in Materials and methods. The $CD4^+CD25^+$ T cells were also isolated from FVIII-deficient naïve mice. The $CD4^+$ T cells and antigen-presentingt cells (APCs) from non-IT mice with repeated intravenous stimulation with FVIII were separated by cell sorting, as described previously. The isolated cells were used for the T-cell proliferation assay with *in vitro* stimulation with FVIII. (B) The IT-mouse-derived $CD4^+$ T cells and APCs were cultured with $CD4^-CD25^+$ T cells isolated from B6 control mice ($n = 5$), naïve mice ($n = 5$), non-IT mice ($n = 6$), Alb-IT mice ($n = 5$) or FVIII-IT mice ($n = 6$) in the presence of 3 nmol L^{-1} FVIII for 72 h. The amounts of [^{3}H]thymidine incorporation were determined by scintillation counting. Data are shown as means ± standard deviations. $^{**}P < 0.03$ as compared with other groups.

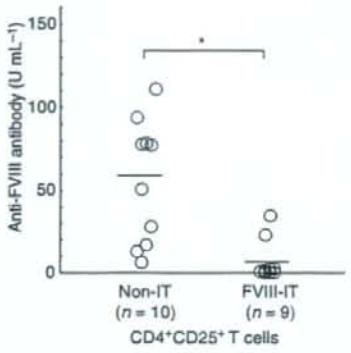

Fig. 8. Adoptive transfer of $CD4^+CD25^+$ regulatory T cells into syngeneic hemophilia A mice. The $CD4^+CD25^+$ T cells were prepared from the spleens of mice without intrathymic administration of FVIII (non-IT mice) or mice that had been given intrathymic injections of FVIII (FVIII-IT mice). A total of 0.5×10^6 $CD4^+CD25^+$ T cells per body in 100 μL of phosphate-buffered saline (PBS) was injected into naïve hemophilia A mice via the jugular vein. Each mouse was challenged with repeated intravenous stimulation of 0.05 u g^{-1} body weight FVIII every 2 weeks, and inhibitory antibody titers were measured after the fifth injection by Bethesda assay.

in peripheral lymphoid organs (Fig. 6A,B), suggesting that increased frequencies of antigen-specific regulatory T cells may be in favor of positive selection as the likely mechanism contributing to increased frequencies of antigen-specific $CD4^+CD25^+$ T cells upon recognition of the antigen in the thymus [40,41]. FoxP3 is thought to positively control the function of regulatory T cells in a binary fashion, as FoxP3 expression is sufficient to specify immune-suppressive activities in conventional T cells, and it is critically important for the development and function of regulatory T cells [42,43]. The results obtained with *ex vivo* expanded regulatory T cells in experimental models are worth noting, because infusion with *ex vivo* activated and expanded regulatory T cells significantly inhibited lethal graft-vs.-host disease in several murine models [44–46]. It is known that $CD4^+CD25^+$ T cells are not the only regulatory lymphocytes that have been found. $CD25^-$ regulatory T cells include the Th3 cells identified in the mucosal immune system and the Tr1 cells characterized *in vitro*. In particular, Tr1 cells could be generated after stimulation with a high concentration of IL-10 [47,48]. However, we could not find any inhibitory effect in a subset of $CD4^+CD25^-$ T cells (Fig. 7B). In addition, $CD4^+$ T cells isolated from FVIII-IT mice produced small amounts of IL-10 (Fig. 5E). Although Tr1 cells might not play an important role in the immune tolerance induction by thymic injection of FVIII antigen in our system, our study is limited to the role of $CD4^+CD25^+FoxP3^+$ regulatory T cells in inducing immune tolerance with thymic injection of specific antigens. Consequently, the antigen-specific regulatory T cells developed by inthrathymic injection of FVIII may efficiently prevent the formation of antibody against FVIII in our murine models.

The $CD4^+CD25^+$ regulatory T cells may be responsible for the translation of tolerance from an antigen-inoculated thymus to a mature but naïve peripheral immune system [38,39]. We showed that only $CD4^+CD25^+$ T cells from mice intrathmically treated with FVIII suppressed the antigen-presenting cell-mediated proliferative response of $CD4^+$ T cells under *in vitro* FVIII stimulation (Fig. 7B), and that the *in vivo* adoptive transfers with $CD4^+CD25^+$ T cells isolated from FVIII-IT mice blocked the development of anti-FVIII antibodies in naïve hemophilia A mice (Fig. 8). Several experiments involving the T-cell compartment have suggested that receptor editing and clonal anergy are involved in tolerance induction [49,50]. Although intrinsic processes of these cells are essential for survival of the organism, they are imperfect at times, and autoreactive T cells can be found even in the peripheral blood of immunologically competent animals and humans [51]. The $CD4^+CD25^+$ regulatory T cells, after thymic selection, are able to suppress proliferation of these autoreactive T cells [39]. Although the exact nature of the mechanisms driving regulatory T-cell generation in the thymus is the subject of intense scrutiny [52], our results delineate a simple means to generate immunoregulatory T cells in hemophilia A mice by central tolerance induction, and provide a relevant assay for their function *ex vitro*. The immune system of neonatal mice, which is more immature than that of newborns or infants, resembles more closely that of human fetuses, and the conclusions drawn from our study may not necessarily apply to patients suffering from hemophilia A. Further study of the precise mechanism of action and the events that determine whether a developing T cell will undergo deletion or will assume an anergic regulatory cell role will have the potential to define new strategies to induce stable tolerance in hemophilia A.

In conclusion, an understanding of the underlying mechanisms of T-cell tolerance induced by intrathymic inoculation of FVIII is essential for the development of this novel strategy for hemophilia A patients with inhibitors.

Addendum

S. Madoiwa designed and performed the research, analyzed data, and wrote the paper; T. Yamauchi, E. Kobayashi, Y. Hakamata, M. Dokai, N. Makino, Y. Kashiwakura and A. Ishiwata performed experiments; T Ohmori, J. Mimuro, and Y. Sakata analyzed data and revised the paper.

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Disclosure of Conflict of Interests

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Phenotypic Correction of Hemophilia A by Ectopic Expression of Activated Factor VII in Platelets

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Platelets are receiving much attention as novel target cells to secrete a coagulation factor for hemophilia gene therapy. In order to extend the application of platelet-directed gene therapy, we examined whether ectopic expression of activated factor VII (FVIIa) in platelets would result in an efficient bypass therapy to induce sufficient thrombin generation on platelet surfaces in mice with hemophilia A. Transduction of bone marrow cells with a simian immunodeficiency virus (SIV)-based lentiviral vector harboring the platelet-specific *GPIbα* promoter resulted in efficient transgene expression in platelets. FVIIa antigen was expressed in platelets by this SIV system; FVII transgene products were found to localize in the cytoplasm and translocate toward the sub-membrane zone and cell surface after activation. Although FVII antigen levels in platelets did not reach the therapeutic levels seen with FVIIa infusion therapy, whole-blood coagulation, as assessed by thromboelastography, was significantly improved in mice with hemophilia A. Further, we observed correction of the bleeding phenotype in mice with hemophilia A after transplantation, even in the presence of FVIII-neutralizing antibodies. Our results demonstrate that FVIIa-expressing platelets can strengthen hemostatic function and may be useful in treating hemophilia and other inherited bleeding disorders. These findings are comparable to the proven therapeutic effects of FVIIa infusion.

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INTRODUCTION

Platelets are differentiated anucleate cells whose functions are essential for hemostasis. Because platelets can circulate throughout the body, release a number of mediators on demand, and provide a scaffold for the coagulation cascade, the targeting of platelets as a circulating delivery system would seem a reasonable approach to genetic modification of hemostasis. The feasibility of such a platelet-directed approach was originally demonstrated by Poncz *et al.* in transgenic mice.[1] Platelet expression of urokinase-type plasminogen activator, using a platelet-specific *platelet factor-4* promoter, enabled urokinase-type plasminogen activator to be stored in platelets and then released within developing thrombi when the platelets became activated.[1] Further, platelet-specific expression of factor VIII (FVIII) can be achieved in a transgenic setting, with the resultant FVIII predominantly or exclusively stored in platelet granules rather than being released into the plasma.[2] In addition, Shi *et al.* have demonstrated that ectopically expressed FVIII in platelets can be used in the treatment of hemophilia with or without FVIII-neutralizing antibodies, and that targeted FVIII expression in platelets continues to support hemostasis even in the presence of high titers of FVIII-neutralizing antibodies.[3] We and others have applied this approach to gene therapy, and have demonstrated that transplantation of hematopoietic stem cells (HSCs) transduced with a lentiviral vector containing *human FVIII* driven by a platelet-specific promoter improves the hemostatic function of mice with FVIII-deficient hemophilia A, despite the levels of FVIII in their plasma being scant or undetectable.[4,5]

In order to further extend the application of platelet-directed gene therapy, we focused our attention on the extrinsic pathway initiated by tissue factor (TF). Assembly of TF and activated Factor VII (FVIIa) complexes on anionic phospholipids expressed on activated cell membranes is the most important initiation mechanism for blood coagulation.[6] Recently, recombinant human FVIIa (rhFVIIa: NovoSeven) has proven to be a highly successful alternative treatment for hemophilia patients.[7] Patients with a variety of other coagulation deficiencies that are characterized by impaired thrombin generation have been successfully treated with rhFVIIa.[8] In addition, liver-directed gene therapy with an adeno-associated virus vector equipped with hFVIIa achieved therapeutic plasma hFVIIa levels in a mouse model of hemophilia B, and phenotypic correction was observed when a murine FVIIa (mFVIIa) homolog was used.[9] In addition, it is possible that platelets that stably express FVIIa can efficiently induce hemostasis at the site of vascular injury in a variety of hemorrhagic disorders. In this study, we used gene therapy to examine whether platelet-specific FVIIa expression would result in an efficient bypass therapy for

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factor X activation, thereby generating sufficient thrombin on platelet surfaces in FVIII-deficient mice.

RESULTS

Enhanced green fluorescent protein expression in platelets after transplantation of HSCs transduced with a simian immunodeficiency virus lentiviral vector harboring the platelet *GPIbα* promoter

We have previously shown that transplantation of c-kit$^+$, sca-1$^+$, and lineage$^-$ (KSL) murine HSCs that are transduced with an simian immunodeficiency virus (SIV)-based lentiviral vector carrying enhanced green fluorescent protein (*eGFP*), driven by a platelet-specific *GPIbα* promoter, enables efficient expression of eGFP in platelets.[4] Because transplantation of KSL cells requires nontransduced bone marrow cells, engraftment by the transduced cells is no >40–55% after transplantation.[4] In order to obviate the need for competitor cells, we validated the transplantation procedure using unfractionated bone marrow cells. As shown in Figure 1, transplantation using unfractionated bone marrow cells resulted in more efficient gene targeting to platelets. eGFP expression in platelets was sustained for at least 3 months after transplantation (Figure 1b), and we found that 0.60–2.78 vector copies/genome had integrated into the cells of the mice that had received the transplants (Figure 1c).

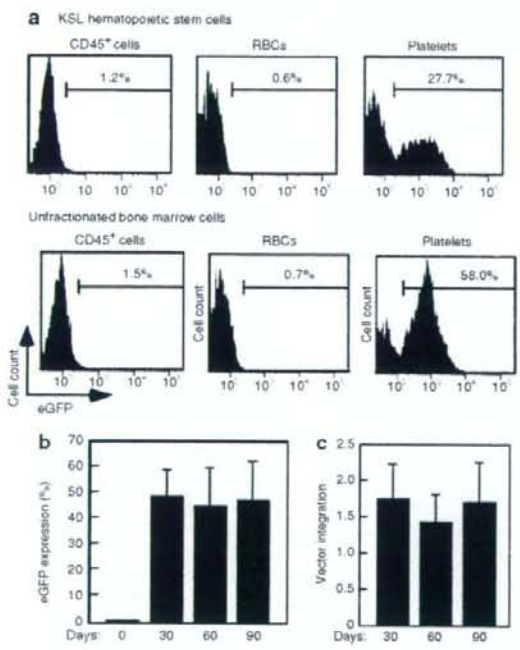

Figure 1 Effects of different stem cell sources on enhanced green fluorescent protein (eGFP) expression in platelets *in vivo*. KSL cells or whole bone marrow cells obtained from Ly5.1 mice were transduced with SIV-GPIbα-eGFP at a multiplicity of infection of 30. Irradiated Ly5.2 mice received either transduced KSL cells (1×10^4) together with competitor cells (2×10^5), or transduced unfractionated bone marrow cells (2×10^6). (**a**) Representative flow cytometry analysis of eGFP-positive cells among CD45$^+$ lymphocytes and granulocytes, red blood cells (RBCs), and platelets in peripheral blood 30 days after transplantation. (**b**) Percentages of eGFP-positive platelets at 30, 60, and 90 days after transplantation. Columns and error bars represent the mean ± SD ($n = 7$). (**c**) Proviral integration into the genomic DNA of bone marrow cells was quantified at 30, 60, and 90 days after transplantation by real-time quantitative PCR. Columns and error bars represent the mean ± SD ($n = 7$). SIV, simian immunodeficiency virus.

hFVII-2RKR expression in platelets induced by platelet-directed gene transduction

The rhFVIIa product currently in clinical use is produced in a single-chain form and activated to the two-chain form during protein purification.[7] Approximately 1% of circulating hFVII in healthy individuals is in the activated form, and the amount of hFVIIa required for bypassing is much larger than the physiological concentration.[7] In order to secrete the activated form of hFVII from transduced cells, we inserted into the factor X activation–cleavage site two arginine/lysine/arginine (RKR) sequences recognized by an intracellular paired basic amino-acid cleaving enzyme/furin type protease, resulting in the secretion of the two-chain molecule with a structure similar to hFVIIa (hFVII-2RKR; Figure 2a).[9]

We first examined whether functional FVIIa was produced in megakaryocytes. After transduction with SIV vector containing *hFVII-2RKR* driven by cytomegalovirus promoter, hFVII antigen in the supernatant from the megakaryoblastic cell line UT-7/TPO was detected and found to have activity similar to that from HEP-G2 cells (Figure 3a and c). The FVII activity of FVII-2RKR was much higher than that of plasma-derived hFVII (Figure 3c), thereby suggesting that FVII-2RKR could be cleaved into two chains. In addition, mRNA expression of γ-glutamyl carboxylase, which post-translationally modifies glutamyl residues into γ-carboxyglutamyl residues of vitamin K–dependent coagulation

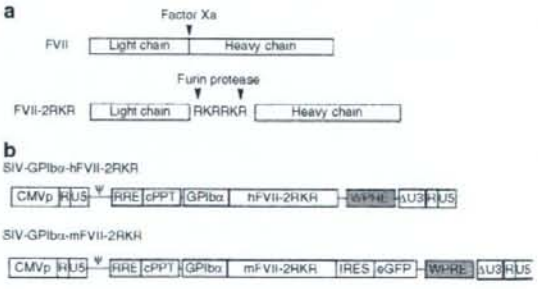

Figure 2 Schematic representation of factor VII (FVII) and simian immunodeficiency virus (SIV) lentiviral vector. (**a**) FVII and engineered activated FVII (FVII-2RKR) construct showing the light and heavy chains. Arrows indicate the recognition sites of physiological factor X activation (FXa) and the intracellular paired basic amino-acid cleaving enzyme/furin type protease. (**b**) The SIV lentiviral vector for platelet-specific gene expression consisted of a cytomegalovirus (CMV)/long-terminal repeat (LTR) chimeric promoter followed by a packaging signal (ψ), a rev-binding element (RRE) for cytoplasmic export of the RNA, the transgene expression region consisting of an internal promoter (*GPIbα*) and the transgene (*hFVII-2RKR* or *mFVII-2RKR-IRES-eGFP*), woodchuck hepatitis virus post regulatory element (WPRE), and a 3′-self-inactivating LTR. cPPT, central polypurine tract; eGFP, enhanced green fluorescent protein; hFVII, human FVII; IRES, internal ribosomal entry site; mFVII, murine FVII.

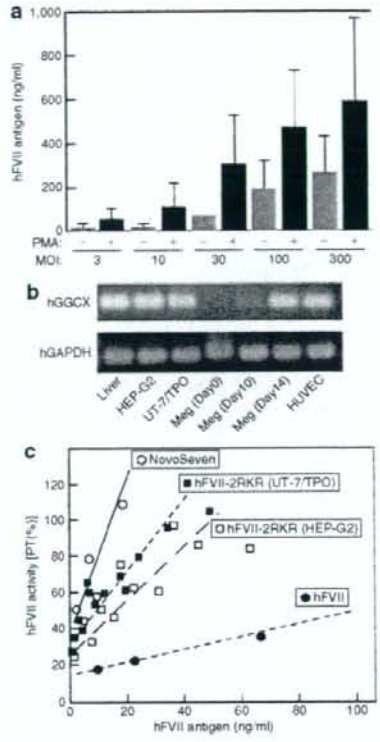

Figure 3 Production of functional hFVII-2RKR by UT-7/TPO cells transduced with simian immunodeficiency virus (SIV) vector. (**a**) UT-7/TPO cells were transduced with SIV-CMV-hFVII-2RKR at the multiplicity of infection (MOI) indicated. hFVII antigen in the supernatant from the cells treated without or with 5 μmol/l phorbol 12-myristate 13-acetate (PMA) for 24 hours were measured using enzyme-linked immunosorbent assay. Columns and error bars represent the mean ± SD (n = 3). (**b**) mRNA expression of γ-glutamyl carboxylase (GGCX) was determined using reverse transcriptase-PCR (RT-PCR). As a control, RT-PCR analysis for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript was performed simultaneously. (**c**) Comparison between activity and antigen levels of hFVII-2RKR produced by UT-7/TPO and HEP-G2 cells, NovoSeven, and plasma-derived full-length hFVII. CMV, cytomegalovirus; hFVII, human factor VII; HUVEC, human umbilical vein endothelial cells; Meg, CD34$^+$-derived megakaryocytes at days 0, 10, and 14 after the start of differentiation with 10 ng/ml of interleukin-3 and 50 ng/ml of thrombopoietin.

factor, was confirmed in megakaryocytes (Figure 3b). These data suggest that platelet precursor megakaryocytes efficiently produce functional FVIIa after transduction.

We next examined whether transduction of HSCs enabled FVIIa expression in platelets. For this purpose, hFVII was used to discriminate transduced FVII from endogenous mFVII. An SIV vector equipped with *hFVII-2RKR* driven by *GPIbα* promoter was created (Figure 2b) and used for infecting unfractionated bone marrow cells. After transplantation of the transduced cells into lethally irradiated FVIII-deficient mice, hFVII antigen levels were detected in platelet lysates for at least 90 days (Figure 4a). Importantly, hFVII antigen was not detected in the plasma even after stimulation of the

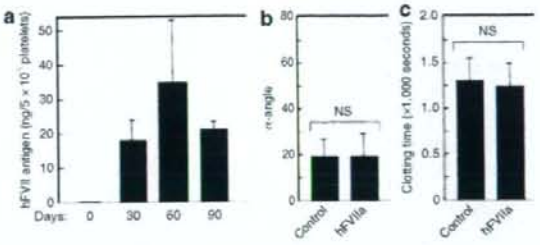

Figure 4 Human factor VII (hFVII) expression in platelets after transplantation of transduced stem cells. FVIII-deficient mice were given transplants of unfractionated bone marrow cells transduced without (control) or with SIV-GPIbα-hFVII-2RKR (hFVIIa). (**a**) Peripheral blood was drawn from the transplant-recipient mice at the indicated times, and the hFVII antigen levels in platelet lysates were measured using enzyme-linked immunosorbent assay. Columns and error bars represent the mean ± SD (n = 4 per group). (**b** and **c**) Whole-blood coagulation was assessed using thromboelastography. Quantitative data of panel **b** α-angle and panel **c** clotting time are shown (n = 7 for control; n = 8 for hFVIIa). Columns and error bars represent the mean ± SEM. Differences between the two groups were analyzed statistically using Student's t-test. NS, not significant. SIV, simian immunodeficiency virus.

platelets with collagen and phorbol 12-myristate 13-acetate (data not shown); therefore, we did not assay for FVII activity in plasma.

We next examined the improvement of whole-blood coagulation in the transplant-recipient mice. The conventional activated partial thromboplastin time, a useful marker for gene therapy in mouse models of hemophilia, did not seem to reflect the phenotypic correction directly, as seen from the fact that platelet-derived hFVII antigen could not be detected in plasma. Therefore we employed thromboelastography (TEG) to record the continuous profile of whole-blood coagulation.[10] TEG can be used for evaluating the effects of hemostatic agents such as rhFVIIa,[11] and to assess the effects of different pharmacological interventions on various factors (coagulation, platelet activation, and platelet–fibrin interaction) involved in clot formation. As shown in Figure 4b and c, whole-blood coagulation in FVIII-deficient mice, as assessed by TEG, did not improve after transplantation. The mortality rate after tail clipping was not altered by transplantation (data not shown).

In order to investigate why phenotypic correction was not observed after transplantation, we validated the expression and localization of hFVII by immunogold electron microscopy. We confirmed that hFVII antigen was abundant in cytoplasm of platelets obtained from transplant-recipient mice (Figure 5a). When platelets were stimulated with phorbol 12-myristate 13-acetate, most of the hFVII antigen was redistributed to the sub-membrane zone, and was partly expressed on the surface (Figure 5b). These data confirmed that hFVII is efficiently stored in platelets after transplantation of transduced bone marrow cells, and is expressed on the cell surface after platelet activation.

Phenotype correction of FVIII-deficient mice by expression of mFVII-2RKR in platelets

We hypothesized that the failure of hFVII-2RKR expression in platelets may be because of inefficient interaction of hFVII-2RKR with murine TF.[12,13] Indeed, higher concentrations of rhFVIIa (NovoSeven: ≥20 μg/ml) were required to restore coagulation in

FVIII-deficient mice (data not shown). We therefore generated an mFVII construct in an analogous fashion (mFVII-2RKR) to further prove its efficacy in FVIII-deficient mice (Figure 2b). Because we could not create an enzyme-linked immunosorbent assay for mFVII antigen, an *eGFP* gene driven by the internal ribosomal entry site was inserted just after the *mFVII-2RKR* gene to give indirect confirmation of mFVII-2RKR expression in platelets (Figure 2b). Although eGFP expression in platelets after transplantation was limited to 3–12% of the platelets, the pattern of eGFP expression under the control of the internal ribosomal entry site sequence was much weaker than that driven directly by the upstream promoter (data not shown). It appears, therefore, that the levels of ectopically expressed mFVII-2RKR in platelets are much higher than would be expected from the results of eGFP expression. This reasoning was supported by the results of proviral integration into the genome (1.15 ± 2.42; $n = 13$), which were similar to those obtained from mice expressing eGFP in 28.6–74.3% of platelets by transduction with SIV-GPIbα-eGFP (see Figure 1c).

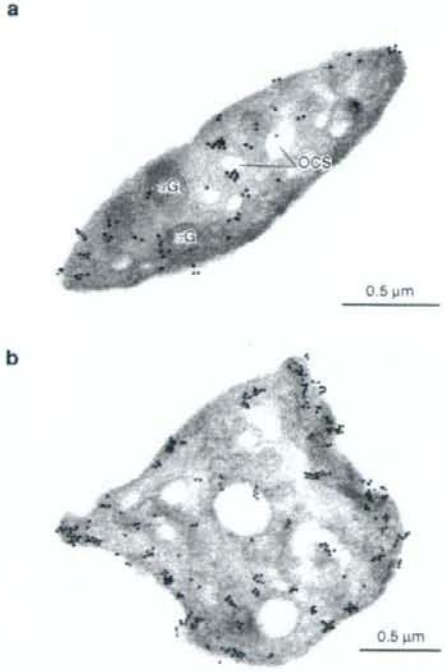

Figure 5 Immunoelectron microscopic localization of ectopically expressed hFVII-2RKR in platelets. FVIII-deficient mice were given transplants of unfractionated bone marrow cells transduced with SIV-GPIbα-hFVII-2RKR. Isolated platelets from the transplant-recipient mice were stimulated (**a**) without or (**b**) with 100 nmol/l phorbol 12-myristate 13-acetate for 15 minutes. Cells were incubated with a biotin-labeled rabbit anti-hFVII antibody. Bound antibodies were detected using a colloidal gold–conjugated goat anti-biotin secondary antibody. hFVII expression in platelets was examined by electron microscopy. aG, α-granule; hFVII, human factor VII; OCS, open canalicular system; SIV, simian immunodeficiency virus.

In comparison with the results obtained from hFVII-2RKR, whole-blood coagulation, as assessed by TEG, was significantly improved in mice that had received the transplant (Figure 6a). The α-angle, which represents the rate of clot formation, was enhanced (Figure 6b), and the clotting time was significantly shortened

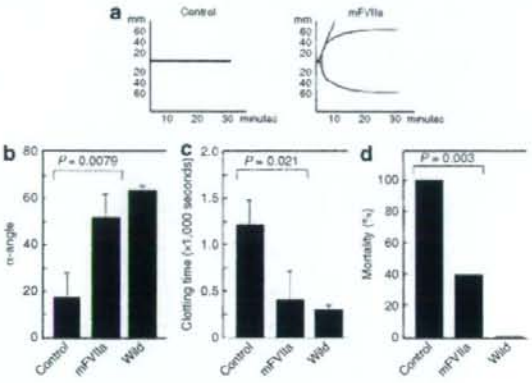

Figure 6 Phenotype correction of factor VIII (FVIII)-deficient mice by ectopic expression of mFVII-2RKR in platelets. FVIII-deficient mice were given transplants of unfractionated bone marrow cells transduced without (control) or with SIV-GPIbα-mFVII-2RKR (mFVIIa). (**a**) Representative thromboelastography data obtained from control and mFVII-2RKR-transfected mice. (**b** and **c**) Quantitative data of panel **b** α-angle and panel **c** clotting time are shown ($n = 8$ for control; $n = 8$ for mFVIIa). The data obtained from wild-type mice are also shown ($n = 6$). Columns and error bars represent the mean ± SEM. Differences between the two groups were analyzed statistically using Student's *t*-test. (**d**) Mortality rates within 24 hours of tail clipping in wild-type mice ($n = 6$) or FVIII-deficient mice given transplants of control or SIV-GPIbα-mFVII-2RKR-transduced bone marrow cells ($n = 10$ for control; $n = 10$ for mFVIIa). The mortality rate was analyzed statistically using the χ^2-test. mFVIIa, murine activated factor VII; SIV, simian immunodeficiency virus.

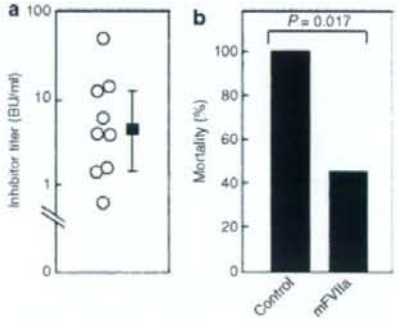

Figure 7 Effects of mFVII-2RKR expression in platelets on phenotypic correction in factor VIII (FVIII)-deficient mice in the presence of anti-FVIII inhibitors. (**a**) Circulating inhibitors were detected in 9 of the 13 FVIII-deficient mice after peritoneal injection of recombinant hFVIII. (**b**) In the presence of circulating inhibitors, mortality ratio at 24 hours after tail clipping was examined in FVIII-deficient mice given transplants of control or SIV-GPIbα-mFVII-2RKR-transduced bone marrow cells [$n = 10$ for control; $n = 9$ for mFVII-2RKR (mFVIIa)]. The mortality rate was analyzed statistically using the χ^2-test. mFVIIa, murine activated factor VII; SIV, simian immunodeficiency virus.

(Figure 6c). The mortality rate after tail clipping was significantly reduced in mice with transplants (Figure 6d). In addition, five of the nine FVIII-deficient mice that received bone marrow cells transduced with SIV-GPIbα-mFVII-2RKR survived after tail clipping despite the presence of circulating inhibitors against hFVIII (Figure 7a and b). Blood coagulation, as assessed by TEG, was similarly corrected in FVIII-deficient mice having FVIII-neutralizing inhibitors, by treating them with SIV-GPIbα-mFVII-2RKR (data not shown). Taking these results together, platelet-specific mFVII-2RKR expression results in efficient bypass therapy to activate factor X activation, thereby resulting in thrombin generation on platelet surfaces in FVIII-deficient mice.

DISCUSSION

Hemophilia is considered to be a suitable condition for gene therapy because it is caused by a single gene abnormality, and therapeutic coagulation factor levels may vary across a broad range (5–100%).[14] Although sustained therapeutic expression of FVIII has been achieved in preclinical studies using a wide range of gene transfer technologies targeted at different tissues, the emergence of neutralizing antibodies often limits the clinical applications.[15] Blood platelets have been receiving much attention as novel target cells for hemophilia gene therapy, because platelet-specific expression of FVIII abolishes the emergence of neutralizing antibodies, and platelet-derived FVIII supports hemostasis in the presence of high titers of FVIII-neutralizing antibodies.[2–5] Here, we extended the application of platelet-directed gene therapy to demonstrate that FVII-2RKR expression in platelets improved the bleeding phenotype of FVIII-deficient mice, even in the presence of FVIII-neutralizing antibodies. Given that rhFVIIa has proven efficacy in the treatment of hemophilia patients with the inhibitors,[8] platelets expressing FVII-2RKR may be a potential alternative to bolus administration of rhFVIIa in such patients.

It is possible that platelets store ectopically expressed FVII-2RKR in the cytoplasm, and that this is specifically expressed on the cell surface after activation. Although hFVII antigen levels achieved here seemed to be much lower than the therapeutic level, whole-blood clotting, as assessed by TEG, and mortality rate after tail clipping were significantly improved when mFVII-2RKR was expressed. These data suggest that FVII-2RKR in platelets can locally generate a thrombin burst at the site of vascular injury. Although an important drawback of rhFVIIa administration is its short half-life (2.6–2.8 hours), which necessitates frequent bolus injections to stop bleeding,[7,16] platelets may be able to store stable FVII-2RKR in the circulation. The importance of coagulation factor stored in platelets is supported by the role of platelet factor V in hemostasis. Platelet-derived platelet factor V appears to support hemostasis even in patients with an acquired platelet factor V inhibitor, thereby suggesting that platelets can deliver coagulation factors and protect them from being degraded by platelet factor V inhibitors.[17] Recently, erythroid-specific factor IX expression, driven by the *β-globin* promoter, has been shown to result in phenotypic correction of hemophilia B in mice.[18] However, given the context of the specific release or expression of a target protein at the site of thrombus formation, platelet-directed gene therapy has an advantage as a therapy for inherited coagulation factor deficiencies.

Contrary to our initial expectations, the sub-localization of ectopically expressed hFVII-2RKR in platelets is quite different from that described in previous reports of FVIII expression.[3,19] Retroviral transduction of FVIII in human $CD34^+$ HSCs enables FVIII-transduced megakaryocytes to store human FVIII with von Willebrand factor, a natural carrier protein of FVIII, within α-granules.[19] In this study, hFVII-2RKR was found to be localized in the cytoplasm, but not in α-granules, suggesting that storage of this ectopically expressed protein in α-granules requires specific binding with an endogenous protein, as is the case for the FVIII–von Willebrand factor interaction.[20] Despite the failure to localize in α-granules, cytoplasmic hFVII-2RKR translocated to a sub-membrane fraction and was expressed on the cell surface after platelet activation. It is possible that activated platelets can express FVII-2RKR through a mechanism of protein secretion other than granule release. Phosphatidylserine flip-flop is one candidate that may be responsible for the surface expression of FVII-2RKR; however, we do not yet have a clear explanation for this. Recently, it has been reported that platelets supply their own TF for thrombin generation in a temporally and spatially circumscribed process.[21,22] This would suggest that TF expression in platelets is involved in the hemostatic function of ectopically expressed FVII-2RKR, even in the absence of soluble TF. The failure of hFVII-2RKR expression in platelets to correct the bleeding phenotype, in contrast to mFVII-2RKR, further demonstrates the TF-dependence of coagulation mediated by platelet-derived FVIIa; murine TF appears to be more species-specific and interacts poorly with hFVIIa.[12,13]

Before proceeding to further clinical application, we should weigh the clinical benefits and risks of FVII-2RKR expression in platelets. The most important drawback of FVIIa expression is a potential risk for thromboembolic events. In this study, the clinical effect of FVII-2RKR expression seemed to be less than that of FVIII expression in platelets. Although a correction of as little as 1% of FVIII in platelets was reportedly enough to cure FVIII-null mice,[3–5] our strategy resulted only in a partial cure in the current model. One explanation of the lower efficacy is that much higher expression of FVII-2RKR is needed to generate sufficient thrombin in FVIII-deficient mice. We will need to further improve the transduction efficiency and modify the enzymatic activity of FVIIa. We believe that megakaryocyte- and platelet-specific expression of FVIIa in targeting HSCs is important for safety, given that expression of FVIIa in neutrophils or monocytes may alter the coagulation properties of blood cells, leading to an unexpected thrombosis similar to disseminated intravascular coagulation. Once the approach involving the targeting of platelets has been optimized for greater efficacy, it is possible that the risk of unexpected thrombosis will become a major concern. Further, we must consider the risk of insertional mutagenesis of the integrating vector. While lentiviral vectors offer a means to correct genetic diseases by integration into chromosomal DNA permanently, all the integrating gene transfer vectors in current use carry a risk of insertional mutagenesis.[23] In view of the fact that the target diseases for platelet-directed gene therapy, including hemophilia, are not generally lethal disorders, we have to continue investigating the safety of platelet-directed gene therapy using integrating vectors to the maximum. Observations should be carried on for extended durations in order

to substantiate long-term *in vivo* gene expression and vector safety. Our observations were limited to 3 months.

In this study, we demonstrated that FVII-2RKR expression in platelets by SIV vectors could be an important strategy for treating hemophilia A. Given that platelets play a central role and provide the scaffolding for the coagulation cascade, this would be a reasonable approach, similar to the proven therapeutic effects of rhFVIIa infusion, for treating a number of hemorrhagic diseases, such as hemophilia, Glanzmann's thrombasthenia, and FVII deficiency. Further evaluations utilizing larger animals such as Cynomolgus monkeys or dogs will be necessary to determine efficient and safe protocols for platelet-directed gene therapy.

MATERIALS AND METHODS

The materials and methods for cell culture, reverse transcriptase-PCR, proviral integration, mouse blood preparation, enzyme-linked immunosorbent assay for hFVII antigen, determination of hFVII activity, and flow cytometry are described in the **Supplementary Materials and Methods**.

Plasmid constructs and production of SIV lentiviruses. The methods for cDNA cloning and plasmid construction are described in detail in **Supplementary Materials and Methods**. A self-inactivating SIV vector plasmid was generated as described earlier (Figure 1b).[24] SIV lentiviral vectors were produced as described earlier.[4] Briefly, the SIV vector and each packaging vector (*gag/pol*, *rev*, and *VSV-G*) were co-transfected into HEK293T cells using the Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA). The supernatants were collected 48 hours after transfection and filtered through a 0.4-μm filter. The transduction units of the lentiviral vector and proviral integration into the genomic DNA were measured as described earlier.[4]

Isolation of KSL cells, viral transduction, and stem cell transplantation. Mice with FVIII-deficient hemophilia A with targeted destruction of exon 16 of the *FVIII* gene were kindly provided by Dr H.H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA). C57BL/6 (Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6 mice congenic for the Ly5 locus (Ly5.1) were purchased from Sankyo-Lab Service (Tokyo, Japan). All animal procedures were approved by the Institutional Animal Care and Concern Committee at Jichi Medical University (Tochigi, Japan), and animal care was in accordance with the committee's guidelines.

KSL HSCs were isolated as described earlier.[4] KSL cells or unfractionated bone marrow cells were precultured for 24 hours before viral transduction in Stem Pro Medium (Invitrogen, Carlsbad, CA) supplemented with 100 ng/ml stem cell factor, 10 ng/ml thrombopoietin, 100 ng/ml interleukin-6, 100 ng/ml Flt-3 ligand, and 400 ng/ml soluble interleukin-6 receptor. The cells were transduced with SIV vectors at a multiplicity of infection of 30 in the presence of the same cytokine combination, and incubated at 37 °C for 12 hours. The recipient mice (Ly5.2 mice or FVIII-deficient mice at 8–12 weeks of age) were irradiated with a single lethal dose of 9.5 Gy (Gamma Cell; Norton International, Ontario, Canada), and then administered either transduced KSL cells (1×10^4) and Ly5.2 competitor cells (2×10^5), or transduced whole bone marrow cells (2×10^6). The methods for transduction of UT-7/TPO and HEP-G2 cells by SIV vector are described in **Supplementary Materials and Methods**.

Immunogold electron microscopy. Washed murine platelets were obtained as described earlier.[25] The resting and phorbol 12-myristate 13-acetate-stimulated platelets were fixed in 0.1% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) at 4 °C for 1 hour. The fixed platelets were transferred into Eppendorf tubes, centrifuged at 3,000 rpm for 3 minutes, and rinsed with phosphate-buffered saline five times at 4 °C. The specimens were sequentially immersed in 1 mol/l sucrose in phosphate-buffered saline for 1 hour, and then in 1.84 mol/l sucrose containing 20% polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline overnight at 4 °C.[26] The specimens were frozen in liquid nitrogen and ultra-thin frozen sections were prepared, incubated with biotin-labeled rabbit anti-hFVII antibody, washed with phosphate-buffered saline, and then incubated with goat anti-biotin antibody coupled to 15 colloidal gold (BBI International, Cardiff, UK). After being stained with uranyl acetate, the sections were examined using a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

TEG and tail clipping. The principle of TEG is based on measurement of the physical viscoelastic characteristics of blood clots. Whole blood for TEG was drawn at 30–60 days after transplantation. Clot formation was monitored in whole blood at 37 °C, in an oscillating plastic cylindrical cuvette having a coaxially suspended stationary piston with 1-mm clearance between the surfaces, using a computerized TEG (ROTEG; Pentapharm, Munich, Germany). A sample (270 μl) of whole blood was carefully drawn from the superior vena cava of anesthetized mice using a syringe containing 30 μl sodium citrate. Whole-blood coagulation was initiated with the addition of 20 μl of 200 mmol/l $CaCl_2$, and TEG was used to assess the coagulation by measuring various parameters, such as the latency for clotting time, and the kinetics of clot development, as determined by the α-angle.[10] When blood coagulation was not observed within 30 minutes, the clotting time and α-angle were defined as 1,800 seconds and 0°, respectively. Phenotypic correction was tested in some of the transplant-recipient mice by anesthetizing them with diethyl ether and clipping 1.0 cm off the ends of their tails. The mice were then observed for 24 hours to determine the rate of mortality.

Circulating FVIII inhibitors. Antibodies against hFVIII were produced by administering weekly peritoneal injections of 0.05 U/g body weight of rhFVIII (Kogenate FS; Bayer AG, Wuppertal, Germany). In view of the possibility that lethal irradiation could cancel the circulating anti-FVIII antibodies (data not shown), we started immunization with hFVIII 1 month after transplantating bone marrow cells transduced with SIV-GPIbα-mFVII-2RKR. Analysis of neutralizing antibodies against hFVIII was performed using the Bethesda method described earlier.[27] After five immunizations, we could detect circulating inhibitors against hFVIII, as assessed in Bethesda units (Figure 7a).

SUPPLEMENTARY MATERIAL

Materials and Methods.

ACKNOWLEDGMENTS

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RESEARCH ARTICLE

Adeno-associated virus vector-mediated systemic interleukin-10 expression ameliorates hypertensive organ damage in Dahl salt-sensitive rats

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Abstract

Background Inflammation plays an important role in the pathogenesis of hypertension and hypertensive organ damage. Interleukin (IL)-10, a pleiotropic anti-inflammatory cytokine, exerts vasculoprotective effects in many animal models. In the present study, we examined the preventive effects of adeno-associated virus (AAV) vector-mediated sustained IL-10 expression against hypertensive heart disease and renal dysfunction in Dahl salt-sensitive rats.

Methods We injected the rats intramuscularly with an AAV type 1-based vector encoding rat IL-10 or enhanced green fluorescent protein (EGFP) at 5 weeks of age; subsequently, the rats were fed a high-sodium diet from 6 weeks of age.

Results Sustained IL-10 expression significantly improved survival rate of Dahl salt-sensitive rats compared with EGFP expression (62.5% versus 0%, $p < 0.001$); it also caused 26.0% reduction in systolic blood pressure at 15 weeks ($p < 0.0001$). Echocardiography exhibited a 22.0% reduction in hypertrophy ($p < 0.0001$) and a 26.3% improvement in fractional shortening ($p < 0.0001$) of the rat left ventricle in the IL-10 group compared to the EGFP group. IL-10 expression also caused a 21.7% decrease in the heart weight/body weight index and cardiac atrial natriuretic peptide levels. Histopathological studies revealed that IL-10 decreased inflammatory cell infiltration, fibrosis, and transforming growth factor-β_1 levels in the failing heart. Furthermore, IL-10 expression significantly reduced urine protein excretion with increased glomerular filtration rates.

Conclusions This is the first study to demonstrate that the anti-inflammatory cytokine IL-10 has a significant anti-hypertensive effect. AAV vector-mediated IL-10 expression potentially prevents the progression of refractory hypertension and hypertensive organ damage in humans. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords AAV vector; gene therapy; hypertension; inflammation; interleukin-10

Introduction

Inflammation plays an important role in the pathogenesis of hypertension and hypertensive organ damage. Congestive heart failure (CHF) is a crucial life-threatening sequalae of hypertensive organ damage, and

its severity is closely related with the serum tumor necrosis factor (TNF) levels [1,2]. Recent studies have demonstrated the marked anti-hypertensive and renoprotective effects of an immunosuppressant *in vivo* [3,4]. Although these observations suggest a therapeutic potential of anti-inflammatory molecules, anti-TNF antibody treatments (e.g. infliximab and etanercept) have failed to improve the survival of CHF patients partly because of their cytokine-inducing effects and cytotoxicity [5,6].

Interleukin (IL)-10 is a pleiotropic cytokine produced by monocytes/macrophages and type 2 helper T cells. It regulates inflammatory and immune reactions by inhibiting macrophage activation, T-cell proliferation, and the production of proinflammatory cytokines such as TNF-α [7]. IL-10 also enhances endothelial nitric oxide synthase expression [8] and inhibits vascular smooth muscle cell proliferation [9,10]. Previous studies have demonstrated the therapeutic effects of IL-10 on CHF models resulting from acute viral or autoimmune myocarditis [11,12]. However, no studies have examined the effects of IL-10 on chronic CHF resulting from hypertensive heart disease that occurs far more frequently than acute myocarditis. In the present study, we examined the effects of IL-10 using Dahl salt-sensitive (DS) rats that present with severe hypertension and chronic CHF when fed a salt-rich diet [13].

We employed an adeno-associated virus (AAV) type 1-based vector in order to sustain serum levels of IL-10 because it has a short biological half-life. AAV vectors permit long-term transgene expression with minimal inflammatory and immune responses [14]. If the intramuscular injection of the AAV serotype 1 vector carrying the IL-10 gene (AAV1-IL-10) produces sufficient amount of IL-10 in skeletal myocytes, then IL-10 should be secreted into the systemic circulation [10]. We examined the preventive effects of IL-10 on chronic CHF progression in DS rats, focusing on its effects on survival, hypertension, pathological cardiac remodelling and renal function.

Materials and methods

AAV vector production

Rat IL-10 was cloned from rat splenocyte cDNA by the polymerase chain reaction (PCR) using the primers: 5′-GCACGAGAGCCACAACGCA-3′ (upstream) and 5′-GATTTGAGTACGATCCATTTATTCAAAACGAGGAT-3′ (downstream) [10]. To achieve efficient transduction of the skeletal muscles, we developed a recombinant AAV type 1-based vector encoding rat IL-10 (AAV1-IL-10) or enhanced green fluorescence protein (EGFP, AAV1-EGFP) controlled by the modified chicken β-actin promoter with the cytomegalovirus immediate-early enhancer and by the woodchuck hepatitis virus post-transcriptional regulatory element [pBS II SK (+) WPRE-B11, provided by Dr Thomas Hope, University of Illinois, Chicago, IL, USA]. The AAV vectors were prepared by the previously described three-plasmid transfection adenovirus-free protocol modified by the use of the active gassing system [15,16]. Briefly, 60% confluent human embryonic kidney 293 cells were co-transfected with the proviral transgene plasmid, the AAV-1 chimeric helper plasmid p1RepCap (provided by Dr James M. Wilson, University of Pennsylvania, Philadelphia, PA, USA), and the adenoviral helper plasmid pAdeno (provided by Avigen, Inc., Alamada, CA, USA). The crude viral lysate was purified by two rounds of two-tier CsCl centrifugation [14]. The viral stock titer was determined by dot blot hybridization with plasmid standards.

Animal experiment protocols

All animal studies were performed in accordance with the guidelines issued by the committee on animal research and approved by the ethics committee of Jichi Medical University. For histopathological and physiological studies (Protocol 1; Figure 1), we divided the male DS rats (Japan SLC, Shizuoka, Japan) into the following three groups:

<Study groups>
(1) Normal (no vector, 0.3% NaCl)
(2) AAV1-EGFP (8% NaCl)
(3) AAV1-IL-10 (8% NaCl)

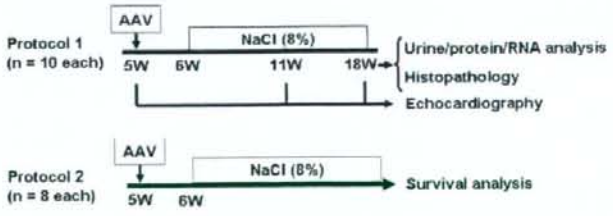

Figure 1. Study protocols. The male DS rats were divided into the three groups: (1) normal group, (2) EGFP group, and (3) IL-10 group. The rats without normal group were injected with AAV1 vectors at 5 weeks of age. The rats in the normal group were fed a low-sodium diet (containing 0.3% NaCl), whereas those in the EGFP or IL-10 group were fed a high-sodium diet (containing 8% NaCl) from 6 weeks of age

IL-10, EGFP and normal ($n = 10$, respectively). AAV1-IL-10 or AAV1-EGFP [1×10^{12} genome copies (g.c.)/body] was injected bilaterally into the anterior tibial muscles of the 5-week-old rats in the IL-10 or EGFP groups, respectively. From 6 weeks onwards, these rats were fed a high-sodium diet (containing 8% NaCl). DS rats in the normal group were fed a low-sodium diet (containing 0.3% NaCl). Systolic blood pressure (SBP) was measured every 2 weeks by the tail-cuff method using a manometer tachometer (MK-1030; Muromachi Kikai Co., Ltd, Tokyo, Japan). During the acclimatization period (3–5 weeks), training for blood pressure measurements was performed three times a week. The mean of the three measurements following a 10-min rest at 37 °C was used in the calculations. Blood was collected from the tail vein at 5, 11 and 18 weeks; the sera and plasma were stored at −80 °C. At 18 weeks, the rats were sacrificed by administering an overdose of isoflurane, and their hearts and lungs were harvested and weighed. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C to obtain proteins and RNA for the subsequent analysis. For survival analysis (Protocol 2; Figure 1), the rats were randomly divided into three groups ($n = 8$ each). Those in the IL-10 or EGFP group were injected at 5 weeks of age with the AAV1-IL-10 or AAV1-EGFP (1×10^{11} g.c./body), respectively, and this was followed by a high-sodium diet from 6 weeks of age. By contrast, those in the normal group were fed a low-sodium diet.

Echocardiography

Transthoracic two-dimensional echocardiography was performed at 5, 11 and 18 weeks of age using a 13- MHz transducer (ProSound SSD-α5; Aloka Co., Ltd, Tokyo, Japan). The internal diameter in end-diastole or end-systole of the left ventricle (LVDd or LVDs, respectively) or the posterior wall thickness (PWT) of the left ventricle (LV) in end-diastole was measured by M-mode tracing at the papillary muscle level. The relative wall thickness (RWT) or the percentage fractional shortening (%FS) of LV was calculated according to the formula: RWT = 2 × PWT/LVDd, %FS = (LVDd − LVDs)/LVDd × 100 (%).

Cytokine measurements

At 18 weeks, protein samples were prepared by homogenizing the frozen heart tissues in a lysis buffer [10 mmol/l Tris-HCl (pH 8.0), 0.2% NP40, 1 mmol/l ethylenediaminetetraacetic acid] containing the protease inhibitor cocktail Complete Mini (Roche Diagnostics, Mannheim, Germany). After centrifugation of the homogenates or serum samples, the supernatants were used for measurement. The serum IL-10 and the tissue transforming growth factor (TGF)-β_1 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (Amersham PharmaciaBiotech, Bucks, UK; BioSource International, Inc., Camarillo, CA, USA; R&D Systems Inc., Minneapolis, MN, USA). The tissue cytokine levels were standardized using the total protein concentrations estimated by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Quantitative reverse transcriptase (RT)-PCR

At 18 weeks, total RNA was extracted from the heart by using RNAzol B (Tel-Test, Inc., Friendswood, TX, USA) and reverse-transcribed into double-stranded cDNA by using the Superscript Preamplification System (Invitrogen, Carlsbad, CA, USA) with the T7-dT primer (5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGA-GGCGGTTTTTTTTTTTTTTTTTTTTTTT-3′). To estimate the atrial natriuretic protein (ANP) mRNA levels, quantitative PCR analysis was conducted using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The GAPDH mRNA was quantified for normalization. The oligonucleotide primers used were: for GADPH, 5′-CAGCAATGCAT CCTGCAC-3′ (upstream) and 5′-GAGTTGCTGTTGAAGTCACAGG-3′ (downstream) [17]; for ANP, 5′-GGTAGGATTGACAGGATTGGAGCC-3′ (upstream) and 5′-ACATCGATCGTGATAGATGAAGAC-3′ (downstream) [18]. Quantitative values were obtained from the threshold cycle (C_t) number that indicates exponential amplification of a PCR product.

Histopathology

At 18 weeks of age, the anesthetized rats were perfused with 50 ml of saline, followed by 100 ml of cold 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4). The hearts were fixed in the same fixative and finally embedded in paraffin. For evaluation of light microscopic findings, we stained sections (3 μm thick) with hematoxylin and eosin (H&E) or the Azan–Mallory stain using the standard methods.

Statistical analysis

The data were assessed using the StatView, version 5.0 (Statview, Abacus Concepts, Berkeley, CA, USA). Differences in the values at specific stages between the groups were assessed by one-way analysis of variance combined with Fisher's test. $p < 0.05$ was considered statistically significant. Survival curves were analysed by the Kaplan–Meier method and compared using log-rank tests.

Results

Pro-survival effect of systemic IL-10 in DS rats

Compared to the control EGFP transduction, IL-10 transduction significantly improved survival rates in DS

rats fed a high-sodium diet ($p < 0.001$, Figure 2). After 13 weeks of the gene delivery, serum IL-10 concentrations significantly increased in the IL-10-transduced rats compared to the normal untreated rats or control EGFP-transduced rats (986.6 ± 278.5 pg/ml versus <3 or 20.8 ± 18.1 pg/ml, $p < 0.001$, respectively; Figure 3). At this time point, the EGFP transduction generated a slight but significant increase of endogenous IL-10 levels compared to control ($p < 0.01$).

Anti-hypertensive effects of IL-10

SBP gradually increased in the EGFP group, resulting in levels of 184 ± 7 mmHg at 15 weeks of age (Figure 4). At 9 weeks (i.e. after 4 weeks of the vector injection), SBP in the IL-10 group (151 ± 7 mmHg) was significantly lower

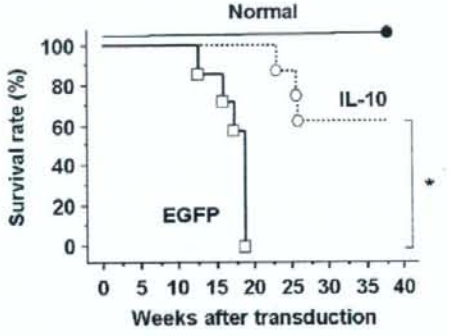

Figure 2. The pro-survival effects of IL-10 in DS rats. The 5-week-old rats were intramuscularly injected with AAV1-IL-10 or AAV1-EGFP at 1×10^{11} g.c./body. Kaplan–Meier survival analysis was performed. Closed circle, normal group; open circles, IL-10 group; open squares, EGFP group ($n = 8$ each). $^{*}p < 0.001$ versus EGFP group

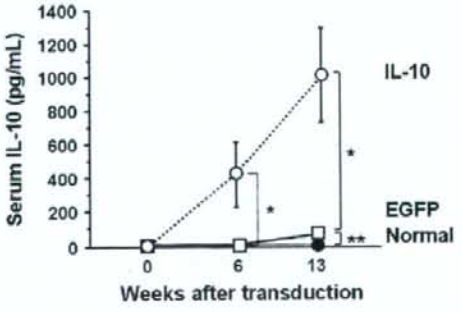

Figure 3. AAV vector-mediated systemic IL-10 expression in DS rats. AAV1-EGFP or AAV1-IL-10, at 1×10^{12} g.c./body, respectively, was injected bilaterally into the anterior tibial muscles of the 5-week-old rats. Serum IL-10 levels were determined periodically by ELISA. The normal group includes DS rats fed a low-sodium diet and not administered the vector injection. The results are presented as means ± SD ($n = 10$ each). $^{*}p < 0.001$, $^{**}p < 0.01$

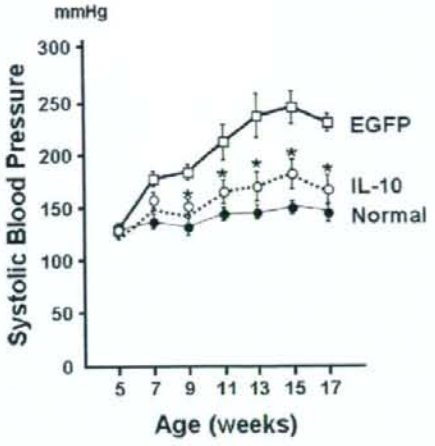

Figure 4. The anti-hypertensive effect of IL-10. Longitudinal tracing of systolic blood pressure evaluated by the tail-cuff method after injecting the AAV vectors in 5-week-old DS rats. Open squares, EGFP group; open circles, IL-10 group; closed circles, normal group ($n = 10$ each). The results are presented as means ± SD. $^{*}p < 0.001$ versus EGFP group

Table 1. Effects of IL-10 on left ventricular hypertrophy and function

	RWT (mm)		%FS (%)	
Age (weeks)	5	11	5	18
Normal	0.46 ± 0.03	0.48 ± 0.02	58.7 ± 3.7	57.2 ± 3.9
EGFP	0.45 ± 0.03	0.63 ± 0.04*	59.8 ± 1.9	32.9 ± 4.4*
IL-10	0.45 ± 0.04	0.49 ± 0.02**	59.4 ± 2.6	59.2 ± 4.6**

M-mode echocardiograms of the LV at the papillary muscle level were traced for analysis. RWT of the LV as an index of LV hypertrophy and %FS as an index of systolic LV function were calculated as described in the Materials and methods. The results are presented as means ± SD ($n = 10$ each). $^{*}p < 0.0001$ versus Normal group, $^{**}p < 0.0001$ versus EGFP group at the same time-point, respectively.

than that in the EGFP group ($p < 0.0001$). The anti-hypertensive effect of IL-10 persisted until the animals were sacrificed at 18 weeks of age.

Effects of IL-10 on left ventricular hypertrophy, function and CHF

Echocardiography exhibited a 22.0% reduction in the RWT of the LV posterior wall at 11 weeks of age ($p < 0.0001$) and a 26.3% improvement in %FS of the LV wall at 18 weeks of age ($p < 0.0001$) in the IL-10 group compared to the EGFP group (Table 1). As compared to EGFP expression, IL-10 expression caused a 21.7% or 52.7% decrease in the heart or lung weight/body weight index, respectively (all $p < 0.05$; Figures 5a and 5b). Similarly, the cardiac ANP mRNA level significantly increased in the EGFP group compared to the control (46.5 ± 23.8-fold); whereas, IL-10 transduction significantly suppressed this increase (9.28 ± 5.2-fold) compared to control (Figure 5c).

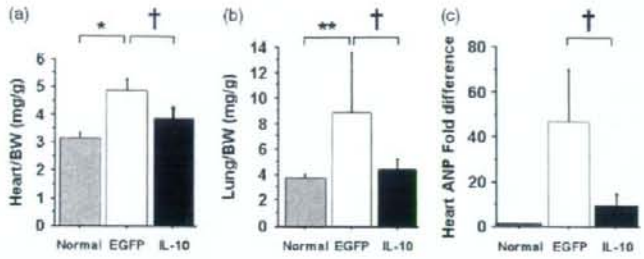

Figure 5. Effects of IL-10 on congestive heart failure. The hearts (a) and lungs (b) of DS rats were harvested and weighed at 18 weeks of age. Data were expressed after normalization using body weight. The cardiac ANP mRNA levels determined by real-time RT-PCR (c). The total RNA was extracted from the heart at 18 weeks of age. The mRNA levels were corrected by using the GAPDH mRNA level of each animal and then normalized to the mean value of the normal group. The results are presented as means ± SD ($n = 10$ each). $^{*}p < 0.01$ versus Normal group, $^{**}p < 0.05$ versus Normal group, $\dagger p < 0.05$ versus EGFP

Effects of IL-10 on pathological cardiac remodelling

H&E staining demonstrated increased interstitial and perivascular cell infiltration in the failing heart of the EGFP-transduced rats (Figure 6a). Azan–Mallory staining demonstrated that interstitial and perivascular fibrosis increased in the EGFP group (Figure 6b). IL-10 transduction inhibited fibrosis and significantly decreased the cardiac TGF-β_1 levels in DS rats compared to the EGFP transduction (64.5 ± 45.3 pg/mg protein versus 197.1 ± 91.9 pg/mg protein, $p < 0.05$; Figure 6c).

Effects of IL-10 on renal function

Compared to control rats, DS rats fed a high-sodium diet exhibited a 68.0% increase in serum creatinine, a 243.0% increase in urine protein levels, and a 49.9% decrease in glomerular filtration rate (all $p < 0.05$; Figure 7). Sustained IL-10 expression reduced these changes by 88.2%, 100% and 45.8%, respectively (all $p < 0.05$).

Discussion

The present study demonstrates that systemic IL-10 expression via the AAV serotype 1 vector prevented the progression of hypertension, CHF and renal dysfunction in DS rats. A single intramuscular injection of AAV1-IL-10 achieved long-term systemic IL-10 expression, leading to the prolonged survival of the rats. The IL-10 transduction not only preserved systolic LV function, but also reduced fibrosis of the LV at the heart failure phase. The anti-hypertensive effect of IL-10 occurred prior to the

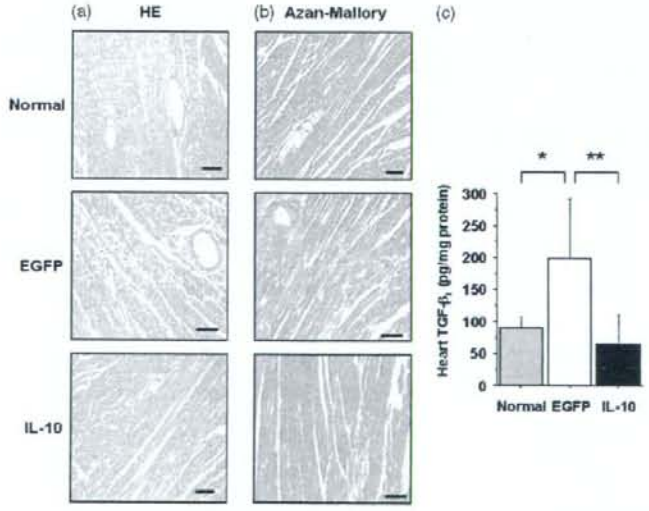

Figure 6. Histopathology and cardiac TGF-β_1 levels of the 18-week-old DS rats. (a) Representative micrographs of the H&E staining. (b) Representative micrographs of Azan–Mallory staining. Magnification, ×200; scale bar = 100 μm. (c) TGF-β_1 concentrations in the heart homogenates determined by ELISA. The results are presented as means ± SD ($n = 10$ each). $^{*}p < 0.05$ versus Normal group, $^{**}p < 0.05$ versus EGFP group

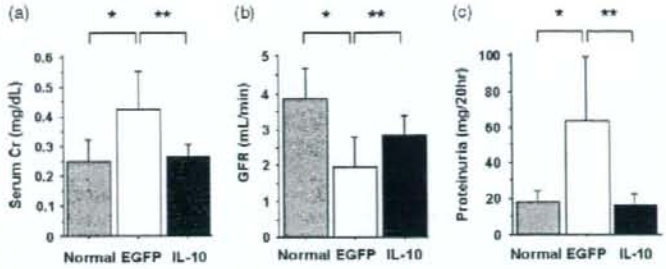

Figure 7. Effects of IL-10 on renal function in DS rats. (a) Serum creatinine (Cr), (b) glomerular filtration rate (GFR) and (c) urine protein levels were determined at 18 weeks of age. The results are presented as means ± SD ($n = 10$ each). $^{*}p < 0.001$ versus Normal group, $^{**}p < 0.05$ versus EGFP group

development of CHF and LVH, suggesting that this effect may largely contribute to amelioration of sodium-induced hypertensive organ damage.

Many studies have suggested the therapeutic potentials of IL-10 for CHF. Serum IL-10 levels decrease in CHF patients [19], and exogenous IL-10 administration retards progression of the disease in many cardiovascular disease models [20]. However, these studies used CHF models in which CHF was a result of acute viral or autoimmune myocarditis, and they examined the short-term IL-10 effects against initial inflammatory responses [11,12]. In the present study, we demonstrated the effects of long-term IL-10 expression against chronic CHF progression, hypertension and inflammatory changes of the cardiac tissue.

We detected a slight but significant increase of endogenous IL-10 levels in the heart failure phase in control DS rats. However, this increase was insufficient to cause beneficial effects. On the other hand, conventional IL-10 therapies based on recombinant drugs or plasmids require frequent administration for sufficient and sustained IL-10 expression. Thus, we used AAV vectors that permit long-term transgene expression *in vivo* [14]. Previously, we demonstrated that a single intramuscular injection of the AAV5-based vector caused systemic IL-10 expression for 1 year [21]. Since AAV1 is more efficient for muscle transduction than AAV2 or AAV5 [22], we used AAV1 as the vector in the present study [23].

A clinical trial using infliximab, a chimeric monoclonal antibody to TNF-α, failed to prolong the survival of CHF patients over the long term [5]. We speculate that the failure might be in part based on an insufficient regulation of the cytokine network, which may be involved in the progression of CHF and other related diseases such as hypertension and renal failure. Recent studies have shown the marked anti-hypertensive effects of an immunosuppressant mycophenolate mofetil (MMF) in DS rats [3,4]. MMF administration also ameliorates renal dysfunction via anti-inflammatory effects. Interestingly, an intramuscular injection of AAV1-IL-10 successfully ameliorated renal function in a rat model after nephrectomy [24]. We also observed that systemic IL-10 expression significantly attenuated hypertension and renal dysfunction, along with a decrease of inflammatory cell infiltration, in the kidney of stroke-prone spontaneously hypertensive rats (T. Nomoto *et al.*, unpublished data). In the present study, we demonstrate that IL-10 gene therapy successfully ameliorated heart failure and renal dysfunction along with a suppression of severe hypertension in DS rats. These observations suggest that anti-inflammatory action of IL-10 may attenuate the target organ damage related to high blood pressure. However, precise mechanism underlying the anti-hypertensive effect of IL-10 require further investigation.

The synthesis of ANP, a cardioprotective hormone predominantly produced by the ventricle, as well as its circulating levels, increases in accordance with the severity of CHF [25,26]. Administration of exogenous ANP ameliorates CHF in clinical settings via its diuretic and vasodilatory effects. In the present study, the cardiac ANP mRNA level significantly decreased in the IL-10 group. These observations suggest that IL-10 ameliorated CHF independently of direct ANP production but inhibited the adaptive increase in ANP levels.

The present study demonstrates that IL-10 expression attenuated pathological cardiac remodelling with reduced expression of TGF-β_1, a hallmark of cardiac fibrosis in DS rats [27]. Expression of monocyte chemoattractant protein (MCP)-1 in the endothelium of intramyocardial arterioles triggers perivascular macrophage accumulation [28]. Macrophage infiltration induces TGF-β_1 production, leading to fibroblast proliferation and extracellular matrix production [29]. Interestingly, a neutralizing antibody against TGF-β inhibits fibroblast activation, resulting in reduced collagen production and subsequent myocardial fibrosis [30]. Previously, we reported that systemic IL-10 expression significantly decreased serum MCP-1 levels, perivascular macrophage infiltration, and pulmonary tissue TGF-β_1 levels *in vivo* [10,21]. These observations suggest that the reduced macrophage-derived TGF-β_1 expression following MCP-1 suppression might be responsible for the anti-remodelling effects of IL-10. However, the direct effects of IL-10 on TGF-β_1 in the pathogenesis of CHF remain unclear.

Epidemiological studies have demonstrated that the increased pro-inflammatory cytokine expression is related to the incidence of pre-hypertension [31]. These results

suggest a possible link between the inflammatory response and the development of hypertension. This is the first study to demonstrate the anti-hypertensive effects of IL-10, which might be a key molecule to explain this relationship. Exploring the mechanisms underlying the effects of IL-10 would provide new molecular targets for refractory hypertension and its sequalae.

In conclusion, the sustained IL-10 expression achieved by the single AAV-IL-10 injection ameliorated CHF and prolonged survival in DS rats. IL-10 expression attenuated salt-sensitive hypertension, LV remodelling and renal dysfunction. These results suggest that our IL-10-based strategy potentially prevents the progression of refractory hypertensive organ damage in humans.

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Protection Against Aminoglycoside-induced Ototoxicity by Regulated AAV Vector–mediated GDNF Gene Transfer Into the Cochlea

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Since standard aminoglycoside treatment progressively causes hearing disturbance with hair cell degeneration, systemic use of the drugs is limited. Adeno-associated virus (AAV)-based vectors have been of great interest because they mediate stable transgene expression in a variety of postmitotic cells with minimal toxicity. In this study, we investigated the effects of regulated AAV1-mediated glial cell line–derived neurotrophic factor (GDNF) expression in the cochlea on aminoglycoside-induced damage. AAV1-based vectors encoding GDNF or vectors encoding GDNF with an rtTA2s-S2 Tet-on regulation system were directly microinjected into the rat cochleae through the round window at 5×10^{10} genome copies/body. Seven days after the virus injection, a dose of 333 mg/kg of kanamycin was subcutaneously given twice daily for 12 consecutive days. GDNF expression in the cochlea was confirmed and successfully modulated by the Tet-on system. Monitoring of the auditory brain stem response revealed an improvement of cochlear function after GDNF transduction over the frequencies tested. Damaged spiral ganglion cells and hair cells were significantly reduced by GDNF expression. Our results suggest that AAV1-mediated expression of GDNF using a regulated expression system in the cochlea is a promising strategy to protect the cochlea from aminoglycoside-induced damage.

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INTRODUCTION

Aminoglycoside antibiotics are frequently used in empiric therapy for serious infections, such as septicemia, complicated intra-abdominal infections, complicated urinary tract infections, and nosocomial respiratory tract infections. However, it is well known that aminoglycosides are associated with severe side effects, such as ototoxicity and nephrotoxicity, which attack the cochlea or vestibule and destroys the auditory and vestibular hair cells that pass information to the auditory nerve.[1] In addition, aminoglycosides predominantly destroy the outer hair cells by ototoxicity. Although the exact mechanism of damage is not well established,[2] aminoglycoside-induced hair cell loss results in a permanent hearing deficit[3] that can progressively occur 6 months to a year after exposure to these drugs. Therefore, the development of a strategy to prevent aminoglycoside-associated ototoxicity before adverse events occur is a critical issue in clinical settings.

The expression of a transgene using viral vectors is a potential approach to introduce neurotrophic factors into the cochlea to prevent and treat aminoglycoside-induced hearing loss. However, most of the currently used vectors, such as adenovirus vectors or herpes simplex virus vectors, have an associated vector-related cytotoxicity.[4,5] Hence, adeno-associated virus (AAV) vectors may be good candidates for gene transfer into the cochlear cells because of their efficient transduction and their safety and potential in long-term expression.[6] We have previously demonstrated that an AAV1-based vector efficiently transduced the inner hair cells, the spiral ganglion cells, and many other types of cells.[7] Therefore, an AAV1-based vector should successfully introduce secretory proteins, such as glial cell line–derived neurotrophic factor (GDNF), into the cochlea to prevent aminoglycoside-induced ototoxicity.

GDNF, a member of the transforming growth factor β family, was initially identified as a survival factor for mid-brain dopaminergic neurons and for a wide range of neuronal populations in the central and peripheral nervous systems.[8–10] Although it is still unclear whether GDNF protects against ototoxicity, sustained infusion of recombinant GDNF protected the cochlear structure and function from noise- and drug-induced damage and stress,[11–16] although its half-life is very short. However, an overdose of GDNF was shown to enhance the sensitivity of the cochleae to insult and

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