was stronger than the PGK1 promoter (515 b) by 1.2-fold in terms of FVIII expression activity. Since the β-actin minimum promoter was stronger than the PGK1 promoter and was short enough to construct 5.1-kb AAV vectors carrying the BDD FVIII cDNA, we used the β-actin minimum promoter to produce AAV vectors carrying the BDD FVIII gene.

Expression of Lac Z gene by the ß-actin minimum promoter in vivo

To confirm that the β-actin minimum promoter can express a transgene in vivo, AAV vectors carrying the Lac Z gene located in the downstream of the β-actin minimum promoter (AAV1-β-actin-Lac Z, AAV8-β-actin-LacZ) were injected to wild-type mice and expression of the Lac Z gene was studied by X-gal staining. When AAV1-β-actin-Lac Z was injected to the skeletal muscles of lower extremities of wild-type mice, Lac Z gene expression was observed in muscle fibers as shown in Fig. 2A. No apparent Lac Z gene expression was observed in other organs in the AAV1-β-actin-Lac Z injected mice (not shown), suggesting that transgene expression in other organs was minimum. Lac Z gene expression of mice with intravenous injection of AAV8-β-actin-Lac Z mainly was observed in the liver as shown in Fig. 2B. Lac Z gene expression also was observed in other organs including the heart, lung, and skeletal muscles in accordance with the previous report [16]. The liver could be transduced with intravenously injected AAV8-β-actin-Lac Z almost as efficiently as intraportally injected vectors (not shown).

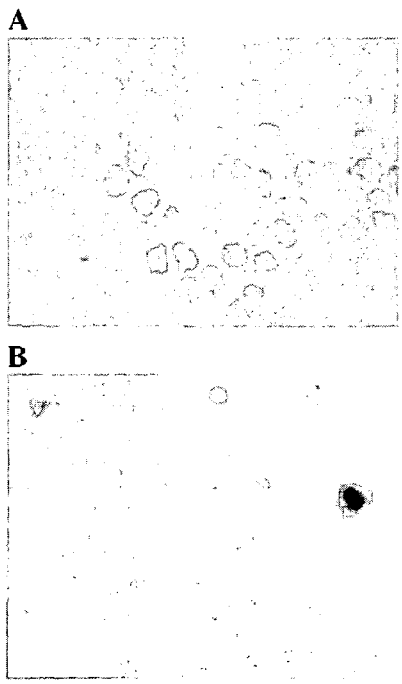

Figure 2 Expression of the Lac Z gene in mice transduced with AAV vectors carrying the Lac Z gene located downstream of the β-actin minimum promoter. X-gal staining of the skeletal muscles of mice with intramuscular injection of AAV1-β-actin-Lac Z (A) and of the liver of mice with intravenous injection of AAV8-β-actin-Lac Z (B) is shown.

Expression of FVIII by AAV vectors carrying the BDD cFVIII gene

AAV1-β-actin-FVIII vectors were injected into skeletal muscles of hemophilia A mice and AAV8-β-actin-FVIII vectors were intravenously injected into the cervical vein plexus of hemophilia A mice. FVIII clotting activities of citrated plasma drawn from mice were measured by the APTT method using FVIII-deficient human plasma.

FVIII clotting activities in mouse plasma increased on days 14 and 28 after AAV1 vector injection. The increase of FVIII clotting activities on day 28 after injection was dose-dependent. The FVIII activity levels in peripheral blood increased to $2.9 \pm 1.0\%$ in hemophilia A mice with the AAV1-β-actin-cFVIII dose of 1×10^{12} gc/body (Fig. 3), suggesting partial correction of the phenotype with AAV1-β-actin-cFVIII vectors. After these periods, FVIII activities decreased to the basal levels of mice before vector injection. FVIII antigen levels increased in parallel with levels of FVIII activity, confirming expression of cFVIII transgene in mice (not shown). Analyses for antibody against transgene products showed that neutralizing antibodies developed in 4 out of 6 tested mice by week 12 after vector injection, although the antibody titers were not high (Table 1). The RT-PCR analysis and the immunohistochemistry study suggested the presence of the transgene transcripts and products in the vector-injected muscles, suggesting that decrease of FVIII levels may be accounted for by the presence of neutralizing antibody to cFVIII.

FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently on day 28, achieving therapeutic FVIII levels (5—90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1-3 \times 10^{11}$ gc/body and supernormal FVIII levels (180~670%) were achieved in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body (Fig. 4). These data on AAV8 vector-transduced FVIII expression were almost comparable with the results of the previous study using the single AAV8 vector carrying the BDD cFVIII gene [6], suggesting that β-actin minimum promoter almost

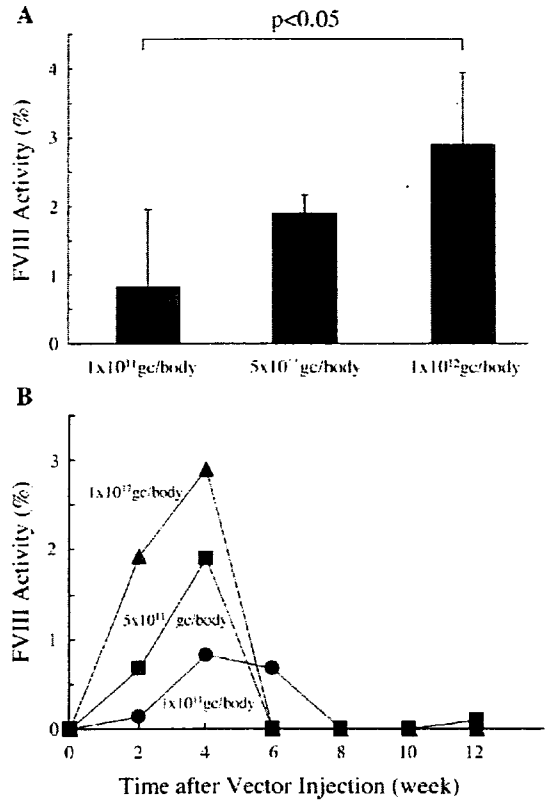

Figure 3 FVIII levels in plasma of hemophilia A mice after intramuscular injection of AAV1-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$) on day 28 after intramuscular injection of AAV1-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice with injection of AAV1-β-actin-cFVIII (circles, 1×10^{11} gc/body: squares, 5×10^{11} gc/body: triangles, 1×10^{12} gc/body) are shown in panel B.

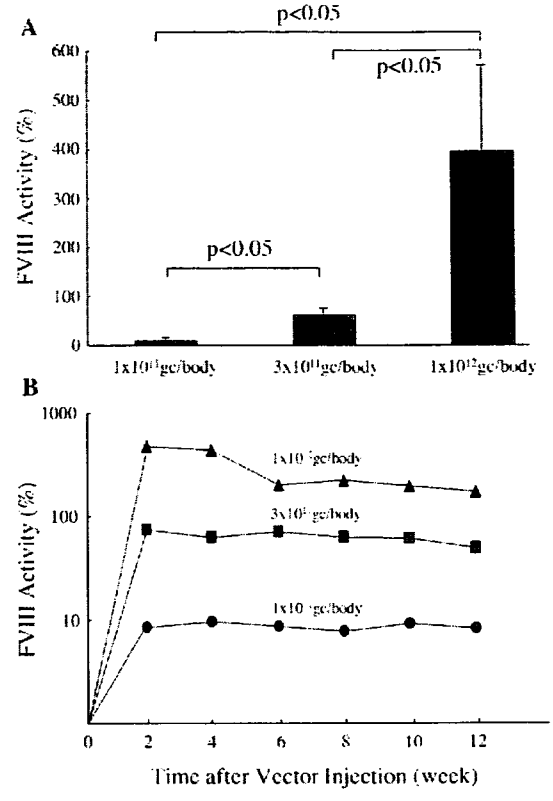

Figure 4 FVIII levels in plasma of hemophilia A mice after intravenous injection of AAV8-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$, each group) on day 28 after intravenous injection of AAV8-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice ($n=4$, each group) with injection of AAV8-β-actin-cFVIII (circles, 1×10^{11} gc/body; squares, 3×10^{11} gc/body; triangles, 1×10^{12} gc/body) are shown in panel B.

worked as efficiently as the chimeric IGBP promoter complexes. High-level expression of FVIII in the vector-injected hemophilia A mice was sustained for more than 12 weeks. No apparent neutralizing antibody developed during the 12-week period after vector injection (Table 1). FVIII antigen levels also increased in parallel with FVIII activity levels, confirming expression of the cFVIII transgene in mice (not shown). The antigen levels of cFVIII determined by the ELISA for human FVIII were approximately 1/5 of the FVIII activity levels determined by the APTT method. Analyses for cFVIII transcripts suggested that the cFVIII gene mainly was expressed in the liver (Fig. 5) together

Table 1 Neutralizing antibodies against cFVIII developed in hemophilia A mice

	Inhibitor positive mouse	Bethesda units/mL
AAV1cFVIII	4/6 (66.7%)	9.4 ±9.5[a]
AAV8cFVIII	0/9 (0%)	Not detected

[a] Neutralizing antibodies detected by week 12 after vector injection.

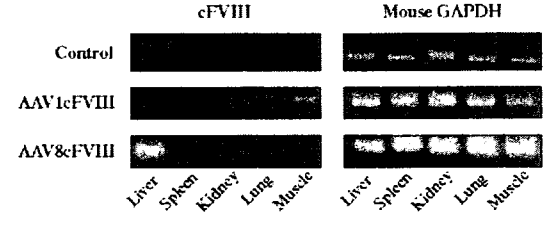

Figure 5 Analysis for cFVIII transcripts in mice. The RT-PCR analyses for the transcripts derived from the cFVIII gene (cFVIII) of RNA isolated from hemophilia A mouse organs without (control) or with intramuscular injection of AAV1-β-actin-cFVIII vectors (AAV1cFVIII) or intravenous injection of AAV8-β-actin-cFVIII vectors (AAV8cFVIII) are shown. For the control, the RT-PCR analysis for mouse GAPDH (Mouse GAPDH) of RNA isolated from hemophilia A mice with or without injection of AAV-β-actin-cFVIII vectors was performed simultaneously.

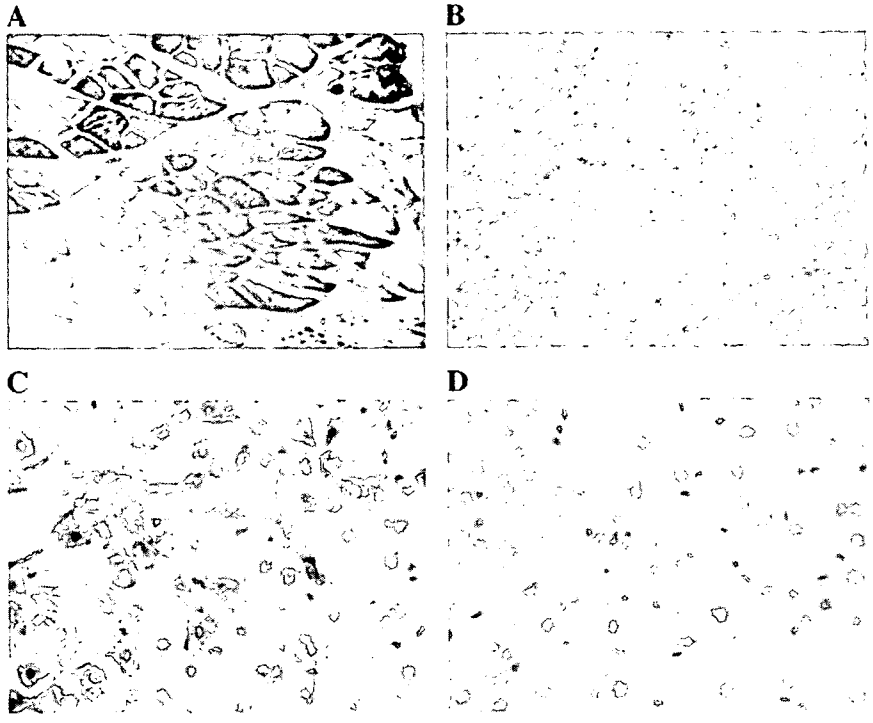

Figure 6 Immunohistochemical Analysis for cFVIII transgene products. Immunohistochemistry for cFVIII of the skeletal muscles of hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII vectors (A) and the liver of hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII vectors (C) is shown (positive stain: brown). For the control, sections of the skeletal muscles (B) and the liver (D) obtained from hemophilia A mice without vector injection were processed simultaneously with anti-FVIII antibodies.

with the traceable expression in the heart, lung, and spleen (not shown). In accordance with the data on cFVIII transcripts, cFVIII molecules were immunohistochemically detected in the skeletal muscles of AAV1-β-actin-cFVIII injected mice and in the liver of mice with intravenous injection of AAV8-β-actin-cFVIII (Fig. 6).

Discussion

Because of the size and nature of the FVIII gene (cDNA), there were difficulties in hemophilia A gene therapy compared with gene therapy for hemophilia B. These difficulties were solved by efforts of many investigators that allowed use of a modified FVIII gene such as BDD FVIII cDNA, improved vector systems, and new strategies. Based upon these studies, a few clinical trials of hemophilia A gene therapy were conducted [17–19]. Increase of FVIII activities in the circulation and clinical improvements were observed in patients who received vector injection or transplantation of genetically modified cells. However, long-term expression of FVIII from the transgenes was not achieved in these studies. Thus, reexamination of the vector systems, the target organs for transduction, and the promoters may be required.

The recombinant AAV vectors are thought to be one of the better vectors in terms of its capability to transduce non-dividing cells and long-term transgene expression, although delivery of the FVIII gene using AAV vectors were limited by its small packaging capacity [4]. The dual AAV vector system utilizing separate AAV2 vectors independently carrying the FVIII heavy chain gene and the FVIII light chain gene could express functionally active FVIII [5]. However, there was an imbalance in the expression levels of the FVIII heavy chain and FVIII light chain, suggesting that over-expressed free FVIII light chain molecules might be more immunogenic than the native molecules. The BDD FVIII gene could be packaged in AAV2 or AAV8 vectors in the previous studies and these vectors could efficiently transduce the liver with intraportal injection of the vectors [6]. Transduction of the liver with peripheral vein injection of AAV8 vectors was as efficient as portal vein injection of vectors, although that of AAV2 vectors was not [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ, so FVIII

synthesis in hepatocytes and its subsequent secretion into the circulation may be warranted. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs. AAV1 vector-based transduction of the skeletal muscles has beneficial characteristics of removing the transgenes. This is the first report of sufficient expression of FVIII in the skeletal muscles transduced with AAV vectors and suggests that skeletal muscle-directed FVIII expression has a potential for hemophilia A gene therapy.

Compared with synthesis and secretion of FVIII into the circulation from the liver, transport of sufficient FVIII into the circulation from the skeletal muscle fibers is not assured. Based upon our data, it is apparent that transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal muscles with AAV1-β-actin-cFVIII regarding FVIII production. The difference between FVIII levels in the peripheral blood of these vector-injected mice may be due to how the transduced cells secrete FVIII molecules into the circulation. Hepatocytes actively secrete a variety of molecules including FVIII into the circulation. Since recombinant cFVIII is in a BDD form, its expression in and secretion from hepatocytes is expected to be better than native FVIII [20], accounting for the high cFVIII expression in mice with intravenous injection of AAV8 vectors carrying the cFVIII gene though cFVIII expressing hepatocytes were not abundant. Although muscle fibers are surrounded by capillaries, transport of recombinant FVIII molecules from muscle fibers to capillaries would not be as efficient as that from hepatocytes.

In terms of the immune reaction to transgene products, muscle stem cells have been shown to function as antigen-presenting cells, suggesting that expression of the transgene by the ubiquitous promoter in the skeletal muscles might lead to development of antibodies against the transgene products if there is no immune tolerance to the transgene products [21]. This was confirmed by Wang et al. [22]. Neutralizing antibody formation was observed in 66.7% of mice with AAV1cFVIII injection even with administration of immunosuppressant, while it was not observed in mice with AAV8-β-actin cFVIII injection by week 12 after vector injection, supporting the potential advantage of AAV8 vector-based transduction of the liver over the muscle-directed transduction by AAV1 vectors.

Each vector system has advantages and disadvantages in these respects. We may need to confirm the results obtained in hemophilia mice using dogs and non-human primates that genetically are more close to humans because there may be differences in transduction efficiency of various serotypes between mice and humans [23]. Taken together, we may need to perform a comparative study using another animal models such as hemophilic dogs and non-human primates that are more genetically close to humans than mice to address these questions. Additionally, use of tissue-specific promoters to minimize neutralizing antibody formation may be a better strategy for expressing transgenes in a tissue- and organ-specific manner. These experiments will be performed in future studies.

In conclusion, our data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have the potential for hemophilia A gene therapy. Our present studies have provided important insight about selecting the appropriate target for delivery of the therapeutic genes and the vector system for the hemophilia A gene therapy.

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References

[1] Hoyer LW. Hemophilia A. *N Engl J Med* 1994;330:38-47.
[2] Kay MA, High K. Gene therapy for the hemophilias. *Proc Natl Acad Sci U S A* 1999;96:9973-5.
[3] High KA. Clinical gene transfer studies for hemophilia B. *Semin Thromb Hemost* 2004;30:257-67.
[4] Lu Y. Recombinant adeno-associated virus as delivery vector for gene therapy—a review. *Stem Cells Dev* 2004; 13:133-45.

[5] Scallan CD, Liu T, Parker AE, Patarroyo-White SL, Chen H, Jiang H, et al. Phenotypic correction of a mouse model of hemophilia A using AAV2 vectors encoding the heavy and light chains of FVIII. *Blood* 2003;**102**:3919-26.

[6] Sarkar R, Tetreault R, Gao G, Wang L, Bell P, Chandler R, et al. Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood* 2004;**103**:1253-60.

[7] Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003;**101**:2963-72.

[8] High KA, Manno CS, Sabatino DE, Hutchison S, Dake M, Razavi M, et al. Immune responses to AAV and to factor IX in a phase I study of AAV-mediated, liver-directed gene transfer for hemophilia B. *Blood*(suppl. 102):154a.

[9] Mochizuki S, Mizukami H, Kume A, Muramatsu S, Takeuchi K, Matsushita T, et al. Adeno-associated virus (AAV) vector-mediated liver- and muscle-directed transgene expression using various kinds of promoters and serotypes. *Gene Ther Mol Biol* 2004;**8**:9-18.

[10] Ogata K, Mimuro J, Kikuchi J, Tabata T, Ueda Y, Naito M, et al. Expression of human coagulation factor VIII in adipocytes transduced with the simian immunodeficiency virus agmTYO1-based vector for hemophilia A gene therapy. *Gene Ther* 2004;**11**:253-9.

[11] Kikuchi J, Mimuro J, Ogata K, Tabata T, Ueda Y, Ishiwata A, et al. Sustained transgene expression by human cord blood-derived $CD34^+$ cells transduced with simian immunodeficiency virus agmTYO1-based vectors carrying the human coagulation factor VIII gene in NOD/SCID mice. *J Gene Med* 2004;**6**:1049-60.

[12] Niwa H, Yamamura K, Miyazaki J. Efficient selection for high level expression transfectants with a novel eukaryotic vector. *Gene* 1991;**108**:193-200.

[13] Mimuro J, Muramatsu S, Hakamada Y, Mori K, Kikuchi J, Urabe M, et al. Recombinant adeno-associated virus vector-transduced vascular endothelial cells express the thrombomodulin transgene under the regulation of enhanced plasminogen activator inhibitor-1 promoter. *Gene Ther* 2001;**8**:1690-7.

[14] Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian Jr HH. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 1995;**10**:119-21.

[15] Madoiwa S, Yamauchi T, Hakamata Y, Kobayashi E, Arai M, Sugo T, et al. Induction of immune tolerance by neonatal intravenous injection of human factor VIII in murine hemophilia A. *J Thromb Haemost* 2004;**2**:754-62.

[16] Nakai H, Fuess S, Storm TA, Muramatsu S, Nara Y, Kay MA. Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J Virol* 2005;**79**:214-24.

[17] Roth DA, Tawa Jr NE, O'Brien JM, Treco DA, Selden R.F, The Factor VIII Transkaryotic Therapy Study Group. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med* 2001;**344**:1735-42.

[18] Powell JS, Ragni MV, White II GC, Lusher JM, Hillman-Wiseman C, Moon TE, et al. Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood* 2003;**102**:2038-45.

[19] Chuah MK, Collen D, VandenDriessche T. Clinical gene transfer studies for hemophilia A. *Semin Thromb Hemost* 2004;**30**:249-56.

[20] Miao HZ, Kucab PF, Pipe SW. Bioengineering of coagulation factor VIII for improved secretion. *Blood* 2004;**103**:3412-9.

[21] Cao B, Bruder J, Kovesdi I, Huard J. Muscle stem cells can act as antigen-presenting cells: implication for gene therapy. *Gene Ther* 2004;**11**:1321-30.

[22] Wang L, Dobrzynski E, Schlachterman A, Cao O, Herzog RW. Systemic protein delivery by muscle-gene transfer is limited by a local immune response. *Blood* 2005;**105**:4226-34.

[23] Wang L, Calcedo R, Nichols TC, Bellinger DA, Dillow A, Verma IM, et al. Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated, liver-directed gene therapy. *Blood* 2005;**105**:3079-86.

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RESEARCH ARTICLE

Utility of intraperitoneal administration as a route of AAV serotype 5 vector-mediated neonatal gene transfer

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Abstract

Background Gene transfer into a fetus or neonate can be a fundamental approach for treating genetic diseases, particularly disorders that have irreversible manifestations in adulthood. Although the potential utility of this technique has been suggested, the advantages of neonatal gene transfer have not been widely investigated. Here, we tested the usefulness of neonatal gene transfer using adeno-associated virus (AAV) vectors by comparing the administration routes and vector doses.

Methods To determine the optimal administration route, neonates were subjected to intravenous (*iv*) or intraperitoneal (*ip*) injections of AAV5-based vectors encoding the human coagulation factor IX (*hfIX*) gene, and the dose response was examined. To determine the distribution of transgene expression, vectors encoding *lacZ* or luciferase (*luc*) genes were used and assessed by X-gal staining and *in vivo* imaging, respectively. After the observation period, the vector distribution across tissues was quantified.

Results The factor IX concentration was higher in *ip*-injected mice than in *iv*-injected mice. All transgenes administered by *ip* injection were more efficiently expressed in neonates than in adults. The expression was confined to the peritoneal tissue. Interestingly, a sex-related difference was observed in transgene expression in adults, whereas this difference was not apparent in neonates.

Conclusions AAV vector administration to neonates using the *ip* route was clearly advantageous in obtaining robust transgene expression. Vector genomes and transgene expression were observed mainly in the peritoneal tissue. These findings indicate the advantages of neonatal gene therapy and would help in designing strategies for gene therapy using AAV vectors.

Keywords AAV vector; neonatal gene therapy; luciferase; coagulation factor IX

Introduction

Due to its unique properties, the adeno-associated virus (AAV) vector is one of the most promising vehicles for gene therapy. It can efficiently transduce a variety of tissues, and long-term transgene expression can be attained. Therefore, the AAV vector is suitable for supplemental gene therapy, particularly for hemophilia. However, despite the promising results obtained in animals [1–4], insignificant levels of human coagulation factor IX (hFIX)

were observed in humans after intramuscular (*im*) injection of the AAV vector [5,6]. The use of alternative serotypes may possibly improve the therapeutic outcome. To achieve therapeutic levels of hFIX expression, several reports have suggested the necessity of optimizing the serotypes of the AAV vector for each administration route [7–10].

It is also believed that neonatal or fetal gene therapy is potentially useful for improving the therapeutic outcome of genetic diseases. These methods are advantageous for preventing early manifestations of genetic diseases, for transducing organ systems that are not easily accessible in later life [11–13], and for providing robust transgene expression at relatively low vector doses. Moreover, since the neonatal and fetal immune systems are immature, gene transfer during this period may induce tolerance to transgene products [7,14,15].

With regard to the utility of the AAV serotypes for neonatal gene therapy, relatively little information is currently available. Limited utility of the AAV serotype 2 (AAV2) vector for *in utero* gene transfer was previously described [16]. It was reported that an intraperitoneal (*ip*) injection of AAV5-based vectors resulted in transgene expression that is at least 10 times higher than that obtained with an *ip* injection of the AAV2 vector [17]. In this study, based on these reports and our previous observations that demonstrated the advantages of AAV5 in gene transfer experiments [18,19], we compared the efficacy and distribution of transgene expression for evaluating the utility of AAV5-based vectors administered to neonates and adult mice either by an *ip* or intravenous (*iv*) injection.

Materials and methods

Plasmids and AAV vectors

Plasmids for AAV vector production were purchased from Stratagene (La Jolla, CA, USA). pAAV5-CMV-LacZ, a plasmid encoding LacZ, and 5RepCapA, a helper plasmid, were donated by Dr. J. A. Chiorini (National Institutes of Health, Bethesda, MD, USA). pAAV5-CMV-hFIX that contains the hFIX sequence was prepared as previously described [20,21], with the inverted terminal repeat (ITR) sequences changed to those of the AAV5 vector. pAAV5-CMV-Luc, which harbors the firefly luciferase gene, was originally purchased from Promega (Madison, WI, USA), and its ITR sequences were also changed to those of the AAV5 vector. Recombinant AAV vector stocks were prepared in accordance with an adenovirus-free triple-plasmid transfection protocol [22]. After harvest, vector solutions were purified twice on a cesium chloride (CsCl) gradient and quantified by DNA dot blot hybridization. The same vector stock was used in the same series of experiments in order to minimize the variability that could occur due to the potential differences in vector potency.

Animal procedures

All animal experiments were performed in accordance with the standards in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and the institutional guidelines. Pregnant female C57BL/6 mice were purchased from CLEA Japan, Inc. (Hamamatsu, Japan), and the neonates were subjected to vector injection within 24 h of birth. Isoflurane anesthesia was applied at the time of injection, and the injection volume was kept constant at 20 μl throughout the study. In order to determine a suitable route for administration in neonates, the AAV5-CMV-hFIX vector was injected either intravenously (*iv*, into the jugular vein) or intraperitoneally (*ip*). In order to validate the usefulness, *ip* injections of the AAV5-CMV-hFIX vector at higher doses were tested. In order to assess the tissue distribution of the vector and transgene expression, the AAV5-CMV-LacZ vector (n = 8) or the AAV5-CMV-Luc vector (n = 10) was injected into the peritoneal cavity. Along with the neonates, an adult group comprising 12-week-old mice were used as adults for *ip* injection, and the AAV5-CMV-hFIX vector (n = 8), AAV5-CMV-LacZ vector (n = 6), or AAV5-CMV-Luc vector (n = 10) was administered. All procedures were performed safely, and animal death was rarely observed following vector injection.

Determination of the plasma concentration of human factor IX

Whole blood was collected from the tail vein by using heparinized capillary tubes. Plasma concentrations of the hFIX protein were determined as described previously [21]. The detection limit of this assay was 1 ng/ml. Normal human plasma stock was used as the standard. This assay system did not react with murine factor IX [21].

Detection and quantitation of vector genomes

Organs were isolated from mice after 16 weeks of vector injection. Tissue samples were frozen in liquid nitrogen and stored at −70 °C. Total DNA was extracted from the tissue samples using the DNeasy tissue kit (Qiagen GmbH, Hilden, Germany). In order to analyze the vector distribution following *ip* administration, total DNA was extracted from various tissues and subjected to quantitative polymerase chain reaction (Q-PCR) using an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA), under conditions that were previously described [23]. The detection limit was 0.01 vector genome copies per diploid genome equivalent (g.c./d.g.e.).

Histochemistry

The mice were sacrificed, and each tissue was obtained at 8 or 10 weeks after the AAV5-CMV-LacZ injection. For microscopic evaluation, the tissues were washed, incubated with phosphate-buffered saline (PBS) containing sucrose (15–30%), frozen in OTC compound (Tissue Tek, Miles Inc., Elkhart, IN, USA) in dry ice/ethanol, attached to polylysine-coated glass slides, and analyzed by standard X-gal staining [24].

Bioluminescence studies

For *in vivo* bioluminescence imaging, the mice were anesthetized with isoflurane, and an aqueous solution of luciferin substrate (150 μg/10 μl/g body weight) was injected into the intraperitoneal cavity 12 min prior to imaging. The mice were placed in a light-tight chamber to maintain complete darkness. Photons transmitted through the tissues were then collected and analyzed using IVIS Imaging Systems and Living Image software (Xenogen Corp., Alameda, CA, USA). Imaging was performed with 5 s of the integration time. The range of the reference pseudocolor scale, representing the light intensity, was kept constant for all mice. For *ex vivo* luciferase analysis, in order to discontinue the follow up of the *in vivo* observation, the representative mice were chosen and sacrificed 10 min after *ip* injection of the luciferin substrate solution (150 μg/10 μl/g body weight), and the internal organs were then separated. Each organ was immediately placed into each well of a 24-well dish containing 1 : 50 dilutions of an aqueous solution of the luciferin substrate (final concentration, 300 μg/ml), and bioluminescence was measured using 60 s of the integration time. The light intensity was calculated based on the weight of the tissue.

Statistical analysis

All data are shown as means ± standard deviation (SD). To compare the means between the two groups, statistical analysis was performed by applying Student's *t* test after confirming the equality between the variances of the groups. If the variances were unequal, Mann-Whitney *U* tests were performed. Values of $p < 0.05$ were regarded to be significant.

Results

Comparison of delivery routes for neonatal injection

As shown in Figure 1A, the plasma levels of hFIX were higher in the *ip*-injected group than in the *iv*-injected group. The plasma concentration of hFIX at 8 weeks for the two groups was 21.8 ± 5.0 ng/ml and 10.2 ± 3.1 ng/ml, respectively, and the difference in the hFIX concentration was significant after 6 weeks ($p < 0.01$).

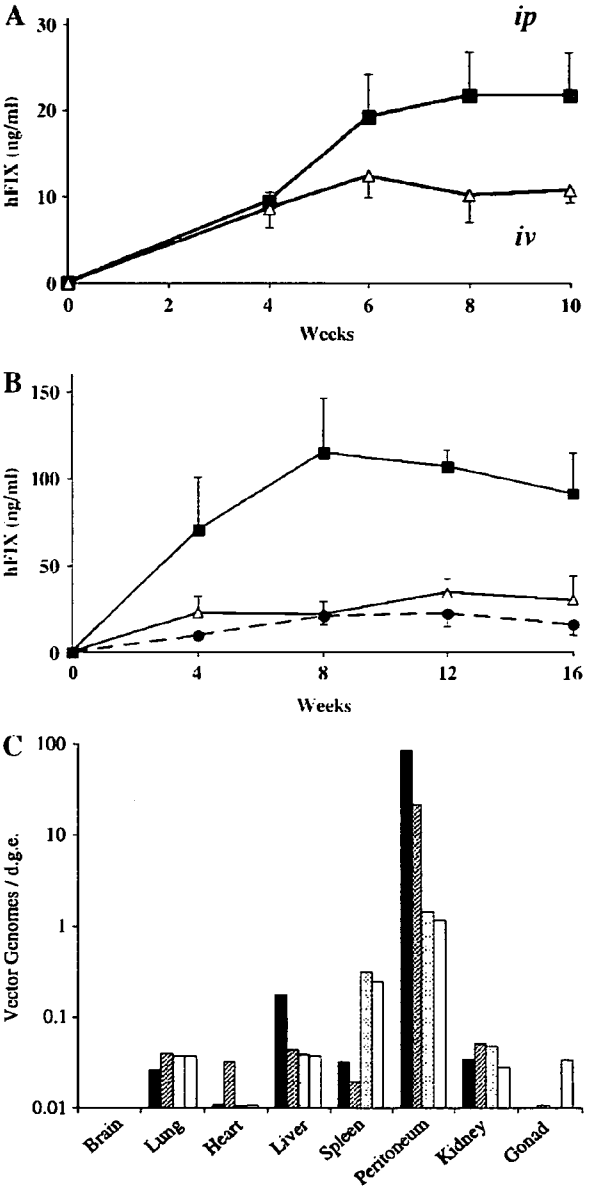

Figure 1. Analysis of C57BL/6 mice after intraperitoneal (*ip*) or intravenous (*iv*) injection of AAV vectors. (A) Plasma hFIX concentration after *ip* (n = 4, closed squares) and *iv* (n = 5, open triangles) administration of the AAV5-CMV-hFIX vector (1×10^{10} genome copies/body weight (g.c./g)) in the C57BL/6 neonatal mice. (B) Plasma hFIX concentration in neonatal mice after *ip* injections at different vector doses. The vector dose was 1×10^{10} g.c./g (closed circles), 3×10^{10} g.c./g (open triangles), or 3×10^{11} g.c./g (closed squares). (C) The number of vector genomes within the tissues at 10 weeks after *ip* injection into neonates. Total DNA (100 ng) was analyzed by Q-PCR, and the results were calculated as vector genomes per diploid genome equivalent (d.g.e.). Closed, hatched, dotted, and open columns indicate the results with neonatal males, neonatal females, adult males, and adult females, respectively

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Effect of the vector dose in *ip* administration

As *ip* administration appeared to be more promising than *iv*, we focused on the utility of *ip* in neonates. For this purpose, increasing doses of AAV5-CMV-hFIX vectors were tested. Higher hFIX concentrations were observed in animals with higher vector doses (Figure 1B). In the group with the highest vector dose (3×10^{11} genome copies/body weight (g.c./g)), the plasma hFIX concentrations were approximately 100 ng/ml, which is a therapeutically relevant level for severe hemophilia B, and these concentrations were sustained throughout the observation period.

Tissue distribution of the AAV vector genome

The tissue distribution of the vector genome after the *ip* injection into male mice was analyzed by real-time PCR. Substantial numbers of vector genomes were detected in the peritoneum and to a lesser extent in the liver and other tissues (Figure 1C). Note that the vector genomes are shown on a logarithmic scale.

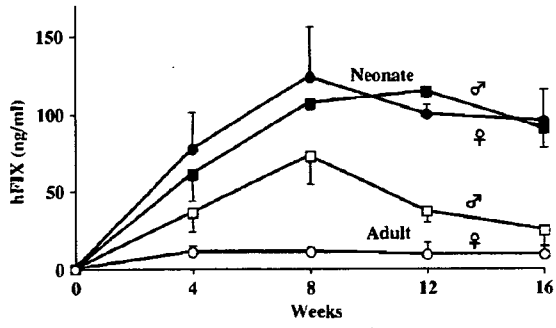

Figure 2. Plasma hFIX concentrations in mice after *ip* injections into different groups. The AAV5-CMV-hFIX vector at a dose of 3×10^{11} g.c./g was injected into C57BL/6 neonatal males (n = 6, closed squares), neonatal females (n = 4, closed circles), adult males (n = 4, open squares), and adult females (n = 4, open circles)

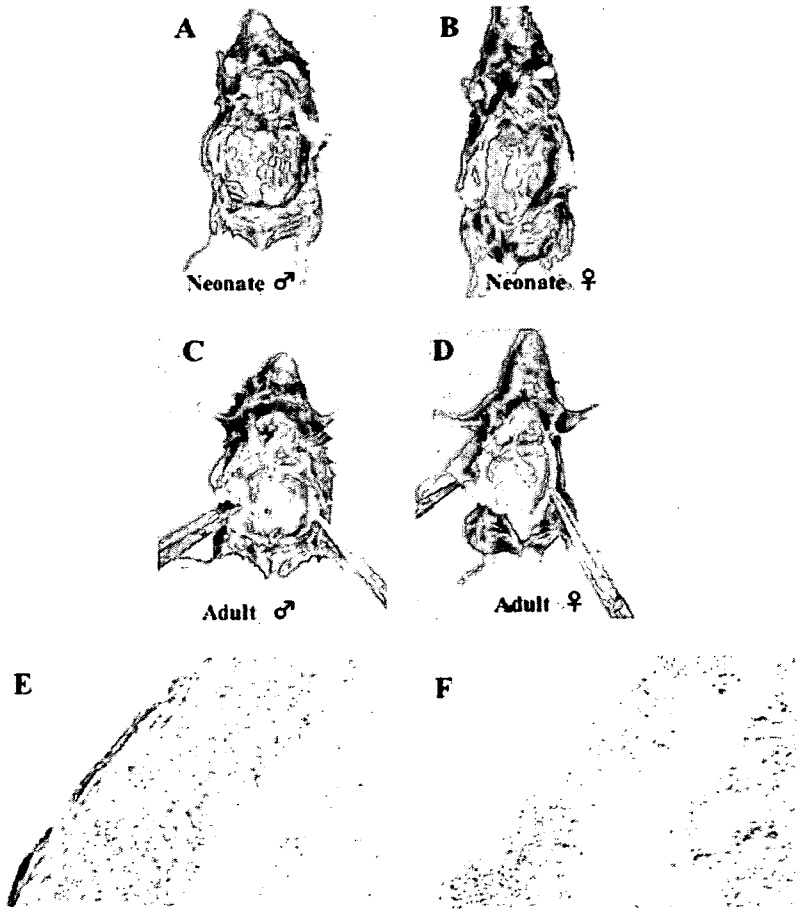

Figure 3. β-Galactosidase expression at 8 weeks after *ip* injection of the AAV5-CMV-LacZ vector at a dose of 1×10^{11} g.c./g in the C57BL/6 mice (A–D). X-gal staining was performed after removal of the intraperitoneal organs. Histochemistry with β-galactosidase performed on tissues from the neonatal male peritoneum after the injection stained the mesothelium (E) and the untransduced control (F) (final magnification x100)

Influence of sex and age of mice on transgene expression

In order to compare the efficiency with regard to the sex and age of mice during administration, the same dose of the AAV vector based on the body weight (3×10^{11} g.c./g) was administered by *ip* injection to both neonatal and adult mice. As summarized in Figure 2, the plasma levels of hFIX were significantly higher in males than in females when adults were used ($p < 0.05$). On the other hand, there were no sex-related differences in the hFIX concentration in neonates. Moreover, the hFIX levels were much higher in neonates (neonate vs. adult; $p < 0.05$ in males, $p < 0.01$ in females). After 8 weeks, a considerable reduction in the plasma hFIX concentration was observed in adult males.

Tissue distribution of transgene expression following *ip* injection

To evaluate the efficacy and location of transgene expression following *ip* vector administration, 1×10^{11} g.c./g of the AAV5-CMV-LacZ vector was injected into either neonatal or adult mice. After 8 weeks, the mice were sacrificed and their tissues were subjected to X-gal staining. As shown in Figures 3A–3D, β-galactosidase expression was observed in the peritoneum. Robust β-galactosidase expression was observed in both male and female mice in the neonatal group (Figures 3A and 3B). In contrast, in the injected adults, only weak β-galactosidase expression was observed in the male mice, and faint expression was detected in the female mice (Figures 3C and 3D). Other tissues were also analyzed by X-gal staining, and none of these, including liver and kidney, showed positive results (data not shown). Microscopic examination of the peritoneum of neonatally injected male mice revealed β-galactosidase expression in mesothelial cells, while the control mice did not show X-gal positivity (Figures 4E and 4F).

In vivo and *ex vivo* analysis using bioluminescence

To quantify the distribution of transgene expression, the AAV5-CMV-Luc vectors were administered *ip* to neonatal and adult mice at an equivalent vector dose based on the body weight (3×10^9 g.c./g). Luciferase expression was observed by *in vivo* bioluminescence imaging 10 weeks after the vector injection (Figures 4A–4D). Quantitative results of *in vivo* bioluminescence are shown in Figure 4E. In neonates, no sex-related difference was found in luciferase expression ($3.8 \times 10^9 \pm 1.2 \times 10^8$ photons/s and $2.9 \times 10^9 \pm 1.0 \times 10^9$ photons/s for the males and females, respectively, $p = 0.13$). In contrast, a significant difference in distribution and quantitation was observed in adults ($1.3 \times 10^9 \pm 7.2 \times 10^8$ photons/s and $5.3 \times 10^7 \pm 1.6 \times 10^7$ photons/s for males and

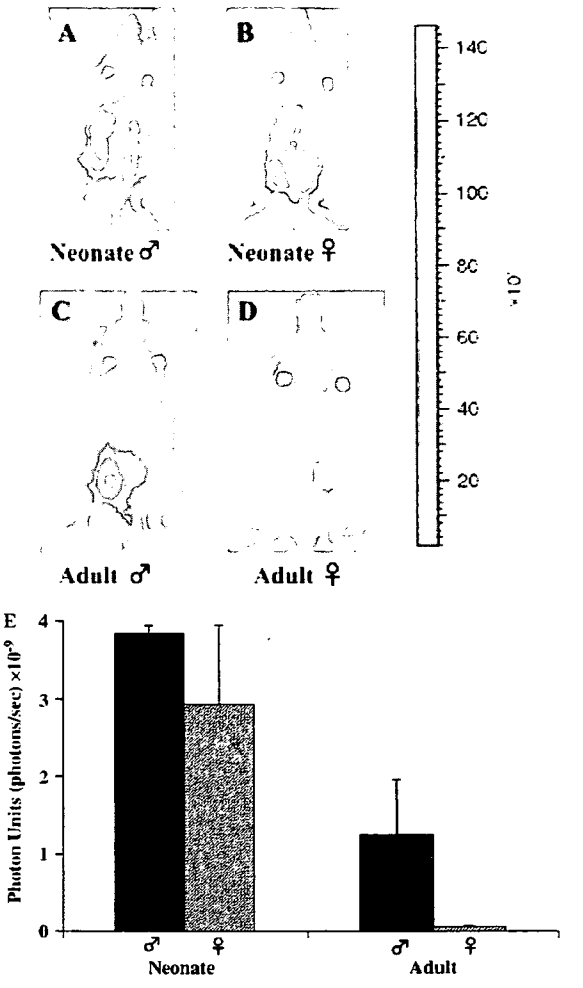

Figure 4. *In vivo* **bioluminescence imaging at 10 weeks after** *ip* **injection of the AAV5-CMV-Luc vector at a dose of 5×10^9 g.c./1.5 g in the C57BL/6 mice (A–D). Images were analyzed under the same condition, and the reference color bar, indicating the photon units (photons/s), is the same for all mice. (E) Quantitative results of** *in vivo* **bioluminescence imaging in neonatal males (n = 6, closed columns) and females (n = 4, hatched column), and adult males (n = 5, dotted column) and females (n = 5, open column), are shown. Mice were transduced with 5×10^9 g.c./1.5 g of the AAV5-CMV-Luc vector (2.5×10^8 g.c./μl). The ordinate indicates the photon units (photon/s)**

females, respectively, $p < 0.05$). In order to identify the tissues responsible for luciferase expression, an *ex vivo* bioluminescence analysis was performed at 10 weeks after the vector injection; this demonstrated that the luciferase expression was localized in the peritoneum (Figure 5A). As shown on the pseudocolor scale, the white color showed background of the assay and did not reflect luciferase expression. A luminometric analysis of individual tissues from representative animals revealed a difference in the expression in the peritoneum among the injected neonates and adults (3.1×10^8 and 1.6×10^8 photons/s/g for male and female neonates, respectively; 1.1×10^8 and 7.9×10^4 photons/s/g for male and female adults, respectively) (Figure 5B).

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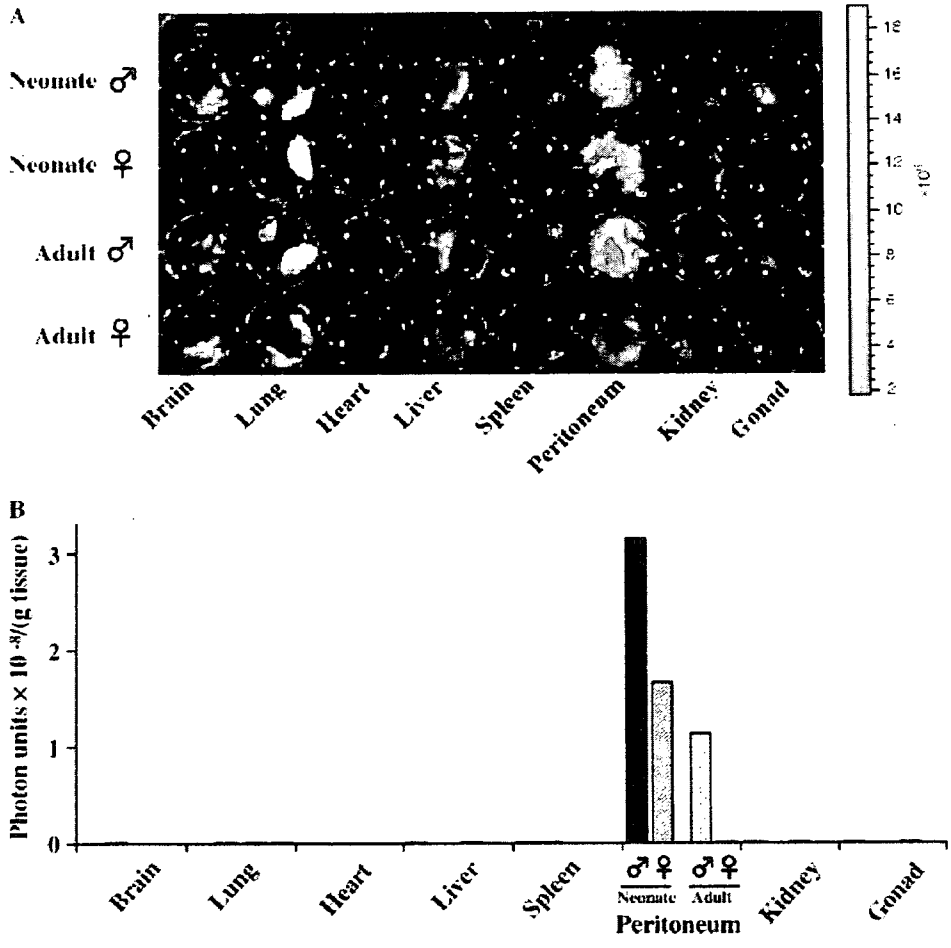

Figure 5. Analysis of tissue-specific expression after *ip* injection of the AAV5-CMV-Luc vector. (A) *Ex vivo* bioluminescence images of injected neonates and adults are shown. Mice were sacrificed at 10 weeks after vector injection and the major organs were extracted and placed into each well of a 24-well dish containing luciferin substrate solution in order to measure the individual bioluminescence. (B) Quantitative results of transgene expression are as indicated in (A). The ordinate shows the photon units (photons/s)

Discussion

In this study, we tested the utility of neonatal gene transfer by using AAV5-based vectors. All genes tested – *lacZ*, *hfIX*, and *luc* – demonstrated robust transgene expression after *ip* injection. The advantage of neonatal gene transfer was clearly demonstrated by the plasma hFIX levels after injecting both adult and neonatal mice with equivalent doses of the AAV-CMV-hFIX vector (3×10^{11} g.c./g). Throughout the observation period, a higher hFIX concentration was detected in neonates than in adults; therapeutic levels of hFIX were maintained even after maturation (Figure 2). Another comparison using vectors encoding luciferase at an equivalent vector dose also resulted in a higher transgene expression in neonates (Figure 4). These data support the advantages of neonatal gene transfer.

Neonatal gene delivery in mice is technically difficult due to their size. In this study, we demonstrated the usefulness of *ip* injections as a route of vector delivery. On the other hand, we did not include the *im* route in this series of experiments because the injection volume was strictly limited in neonates. However, this latter method is apparently an attractive route of administration in clinical applications. Therefore, the efficacy of *im* administration requires further analysis in larger animal models.

In this study, transgene expression was mostly confined to the peritoneum after *ip* injection into neonates. This was confirmed by different modes of detection. In addition, the vector genome distribution was mostly comparable to the level of transgene expression. However, in a previous report, transgene expression was also observed in tissues other than the peritoneum when fetuses were injected [17]. Since the vector system and the promoter were the same, the difference in tissue distribution may be related to the age at the time of injection, vector dose, technical details, or other unrecognized factors. At present, the mechanism responsible for tissue specificity is not clear. The abundance of receptor molecules, such as platelet-derived

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growth factor (PDGF) receptors [27], may contribute to this phenomenon. Using other vector systems may result in different tissue specificity. Recently, transgene expression in the whole peritoneal cavity was observed by *ip* administration of polyethylenimine (PEI)/DNA complexes [28]. Further, in neonates, a long-term expression was observed in factor IX concentration, whereas in adult males a sharp decrease was observed at 12 weeks and later (Figure 3). When the peritoneum was analyzed, only the surface epithelium of the peritoneal tissue was transduced (Figure 4E), and it appeared to be responsible for continuously supplying the transgene product at a therapeutic level. These cells contain an extremely high copy number of transgenes even after a prolonged period of time (Figure 2C). The copy number of the vector genome within the peritoneum appears to be underestimated thus far because the whole peritoneal tissue was used for DNA extraction prior to Q-PCR. The presence of an extremely high copy number of vector genomes within the peritoneum is possibly related to the robust and persistent transgene expression in neonatal gene transfer. The mechanism for the persistence of high copy number and transgene expression is interesting and may offer important insights into the biology of the AAV vector.

Interestingly, a sex-related difference in transgene expression within the peritoneal tissues was observed after *ip* injection into adult mice regardless of the transgene. In a previous study, a sex-related difference in transgene expression was demonstrated in the liver, and an androgen-dependent pathway appeared to be involved [25,26]. We have also demonstrated an overwhelming sex-related difference in liver transduction efficiency in a mouse model [19]. Based on our knowledge, this is the first report that demonstrates a sex-related difference in transgene expression in tissues other than the liver. At present, it is not clear whether the same mechanism is involved in the peritoneal tissue. The difference may be a drawback when an attempt is made to transfer genes into females. However, our results indicate that this problem can be circumvented if neonates are targeted for gene therapy.

Neonatal gene transfer is also advantageous from an immunological point of view. Due to the immaturity of the neonatal immune system, tolerance to an 'immunogenic' transgene product can be induced. Recently, neonatal and fetal gene transfer experiments using adenoviral and retroviral vectors demonstrated the induction of tolerance to transgene products [14,15]. In our series of experiments, it is difficult to prove this point because all transgenes were expressed for a long period even in adults. Nonetheless, divergent levels of transgene expression between adults and neonates may reflect a difference in immunology, and needs to be analyzed in the future.

In conclusion, our findings support the efficacy of neonatal gene therapy and would help to design strategies for neonatal gene therapy using AAV vectors.

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References

1. Chao H, Samulski R, Bellinger D, *et al.* Persistent expression of canine factor IX in hemophilia B canines. *Gene Ther* 1999; **6**: 1695–1704.
2. Herzog RW, Yang EY, Couto LB, *et al.* Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. *Nat Med* 1999; **5**: 56–63.
3. Mount JD, Herzog RW, Tillson DM, *et al.* Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. *Blood* 2002; **99**: 2670–2676.
4. Snyder RO, Miao C, Meuse L, *et al.* Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nat Med* 1999; **5**: 64–70.
5. Kay MA, Manno CS, Ragni MV, *et al.* Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; **24**: 257–261.
6. Manno CS, Chew AJ, Hutchison S, *et al.* AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* 2003; **101**: 2963–2972.
7. Arruda VR, Schuettrumpf J, Herzog RW, *et al.* Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. *Blood* 2004; **103**: 85–92.
8. Chao H, Liu Y, Rabinowitz J, *et al.* Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Mol Ther* 2000; **2**: 619–623.
9. Chao H, Monahan PE, Liu Y, Samulski RJ, Walsh CE. Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. *Mol Ther* 2001; **4**: 217–222.
10. Mingozzi F, Schuettrumpf J, Arruda VR, *et al.* Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. *J Virol* 2002; **76**: 10497–10502.
11. Coutelle C, Themis M, Waddington S, *et al.* The hopes and fears of in utero gene therapy for genetic disease–a review. *Placenta* 2003; **24**(Suppl B): S114–121.
12. Mitchell M, Jerebtsova M, Batshaw ML, Newman K, Ye X. Long-term gene transfer to mouse fetuses with recombinant adenovirus and adeno-associated virus (AAV) vectors. *Gene Ther* 2000; **7**: 1986–1992.
13. Themis M, Schneider H, Kiserud T, *et al.* Successful expression of beta-galactosidase and factor IX transgenes in fetal and neonatal sheep after ultrasound-guided percutaneous adenovirus vector administration into the umbilical vein. *Gene Ther* 1999; **6**: 1239–1248.
14. Waddington SN, Buckley SM, Nivsarkar M, *et al.* In utero gene transfer of human factor IX to fetal mice can induce postnatal tolerance of the exogenous clotting factor. *Blood* 2003; **101**: 1359–1366.
15. Zhang J, Xu L, Haskins ME, Parker Ponder K. Neonatal gene transfer with a retroviral vector results in tolerance to human factor IX in mice and dogs. *Blood* 2004; **103**: 143–151.
16. Schneider H, Muhle C, Douar AM, *et al.* Sustained delivery of therapeutic concentrations of human clotting factor IX–a comparison of adenoviral and AAV vectors administered in utero. *J Gene Med* 2002; **4**: 46–53.
17. Lipshutz GS, Titre D, Brindle M, *et al.* Comparison of gene expression after intraperitoneal delivery of AAV2 or AAV5 in utero. *Mol Ther* 2003; **8**: 90–98.

18. Mochizuki S, Mizukami H, Kume A, *et al.* Adeno-associated virus (AAV) vector-mediated liver- and muscle-directed transgene expression using various kinds of promoters and serotypes. *Gene Ther Mol Biol* 2004; **8**: 9–18.
19. Mochizuki S, Mizukami H, Ogura T, *et al.* Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice. *Gene Ther* 2004; **11**: 1081–1086.
20. Herzog RW, Hagstrom JN, Kung SH, *et al.* Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proc Natl Acad Sci U S A* 1997; **94**: 5804–5809.
21. Mimuro J, Mizukami H, Ono F, *et al.* Specific detection of human coagulation factor IX in cynomolgus macaques. *J Thromb Haemost* 2004; **2**: 275–280.
22. Matsushita T, Elliger S, Elliger C, *et al.* Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* 1998; **5**: 938–945.
23. Grimm D, Zhou S, Nakai H, *et al.* Preclinical in vivo evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Blood* 2003; **102**: 2412–2419.
24. Kanazawa T, Mizukami H, Okada T, *et al.* Suicide gene therapy using AAV-HSVtk/ganciclovir in combination with irradiation results in regression of human head and neck cancer xenografts in nude mice. *Gene Ther* 2003; **10**: 51–58.
25. Davidoff AM, Ng CY, Zhou J, Spence Y, Nathwani AC. Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. *Blood* 2003; **102**: 480–488.
26. Nathwani AC, Davidoff A, Hanawa H, *et al.* Factors influencing in vivo transduction by recombinant adeno- associated viral vectors expressing the human factor IX cDNA. *Blood* 2001; **97**: 1258–1265.
27. Di Pasquale G, Davidson BL, Stein CS, *et al.* Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med* 2003; **9**: 1306–1312.
28. Louis M-H, Dutoit S, Denoux Y, *et al.* Intraperitoneal linear polyethylenimine (L-PEI)-mediated gene delivery to ovarian carcinoma nodes in mice. *Cancer Gene Ther* 2006; **13**: 367–374.

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ORIGINAL ARTICLE

Aspirin resistance detected with aggregometry cannot be explained by cyclooxygenase activity: involvement of other signaling pathway(s) in cardiovascular events of aspirin-treated patients

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Summary. *Objectives:* Although the concept of aspirin resistance is extensively reported in medical literature, its precise mechanisms and clinical outcomes are largely unknown. In this study, we examined individual thromboxane biosynthesis and platelet aggregation in aspirin-treated patients, and whether the results of a platelet aggregation test influenced clinical outcomes. *Results:* Subjects taking 81 mg of aspirin ($n = 50$) and controls ($n = 38$) were evaluated for platelet aggregation and platelet cyclooxygenase-1 (COX-1) activity by measuring collagen-induced thromboxane B_2 production. For aggregometry, both light transmission (LT) and laser-light scattering methods were employed to quantitatively evaluate aggregate sizes and numbers. Aspirin treatment resulted in the inhibition of collagen-induced platelet aggregation, particularly the transition from small to large platelet aggregates. Although platelet COX-1 activity seemed to be uniformly inhibited in all patients, platelet aggregation studies showed great inter-individual differences; variation in platelet COX-1 activity only accounted for 6–20% of the individual aggregations. Factor analysis revealed the existence of a common factor (other than platelet COX-1) that explained 48.4% of the variations in platelet aggregation induced by collagen, adenosine diphosphate (ADP), and collagen-related peptide. We then prospectively enrolled 136 aspirin-treated patients in our study, and we found that being in the upper quartile level of LT, or with large aggregate formation induced by collagen, was an independent risk factor for developing cardiovascular events within 12 months [hazard ratio (HR) = 7.98, $P = 0.008$ for LT; HR = 7.76, $P = 0.007$ for large aggregates]. On the other hand, the existence of diabetes mellitus was an independent risk factor for overall outcomes (HR 1.30–11.9, $P = 0.015$–0.033). *Conclusions:* Aspirin resistance expressed as unsuppressed platelet COX-1 activity is a rare condition in an out-patient population. Other factor(s) affecting collagen-induced platelet aggregation may influence early outcomes in aspirin-treated patients.

Keywords: aspirin, cerebrovascular diseases, coronary heart diseases, cyclooxygenase, platelets.

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Introduction

Aspirin reduces the risk of cardiovascular events by approximately 25% in a broad category of patients with arterial vascular disease [1,2]. Aspirin exerts its anti-thrombotic effects through the inhibition of platelet cyclooxygenase-1 (COX-1) by the irreversible acetylation of a specific serine moiety, thereby blocking the formation of thromboxane (Tx) A_2 for the lifetime of the platelets [3–5]. The term 'aspirin resistance' has been used to describe the clinical inability of aspirin to protect individuals from arterial thrombotic events or when laboratory methods indicate the failure of aspirin to inhibit platelet activity [3,6]. Previous studies have estimated that between 8–45% of patients who suffered an ischemic stroke or cardiovascular disease are aspirin resistant [2,5,6]. Although the problem of aspirin resistance has been greatly emphasized in the medical literature, its precise definition and even its frequency are still unknown. Indeed, the term 'aspirin resistance' has been given different definitions by different researchers.

It was shown that those with a higher concentration of urinary 11-dehydro TxB_2, a stable marker of TxA_2 production,

had a 3.5-fold higher risk of cardiovascular death [7]. Furthermore, aspirin resistance defined by aggregation tests was associated with a greater than 3-fold increase in the risk of major adverse events [8]. These data indicate that screening for aspirin resistance may optimize the anti-platelet treatment for the prevention of cardiovascular events. Accordingly, we must clarify the mechanism(s) by which some patients' platelets are resistant and thereby establish a concise definition of aspirin resistance. In the present studies to investigate the mechanism(s) of aspirin resistance in aspirin-treated patients, we measured platelet function and COX activity by directly assaying collagen-induced TxB_2 production and determined the level of urinary 11-dehydroTxB_2. For platelet aggregometry, we used both the conventional light transmission (LT) method and a laser-light scattering method to quantitatively evaluate aggregate sizes and numbers. Finally, we prospectively enrolled 136 aspirin-treated patients to assess whether platelet aggregation is an attractive test for determining clinical outcome.

Methods

Patients and study protocol

The institutional review board of Minobusan Hospital (Yamanashi, Japan) approved the study protocols and informed consent was obtained from all participants. The first part of these series of investigations was a study called 'Mechanisms of Aspirin Resistance: the Relationship Between TxB_2 Production and Platelet Aggregation'; 51 aspirin-treated individuals (81 mg), who had all given informed consent, and 50 control individuals took part in this study. The baseline characteristics, including age; sex; additional medication use; plasma fibrinogen; the prevalence of hypertension (HT), diabetes mellitus (DM), and hyperlipidemia (HL); and additional treatment such as antihypertensive drugs were not significantly different between the two groups (data not shown). Collagen-induced TxB_2 production, platelet aggregation induced by 0.3, 1, and 3 $\mu g\ mL^{-1}$ collagen; 2 $\mu mol\ L^{-1}$ adenosine diphosphate (ADP); and 0.03 and 0.1 $\mu g\ mL^{-1}$ collagen-related peptide (CRP) were measured in all patients. Urinary 11-dehydroTxB_2 was measured in urine samples from 40 aspirin-treated patients that had given informed consent. One patient who did not comply with taking aspirin was excluded from the analysis.

The next study, entitled 'The Prospective Determination of the History of Aspirin Sensitivity Measured by Aggregometry', was performed in April, 2002 to determine whether inter-individual aggregation differences influenced the clinical outcomes. Finally, we prospectively enrolled 140 stable, aspirin-treated out-patients (81 mg) with a previous history of cerebral infarction or ischemic heart diseases in December 2003. At the time of enrollment, platelet aggregation induced by 0.3 and 1 $\mu g\ mL^{-1}$ collagen and 2 $\mu mol\ L^{-1}$ ADP was examined. Exclusion criteria included the following: ingestion of ticlopidine, dipyridamole, anti-inflammatory drugs, or other drugs affecting platelet function; platelet count $< 10 \times 10^7\ mL^{-1}$ or $40 \times 10^7\ mL^{-1}$; myeloproliferative disorders; atrial fibrillation. Compliance on aspirin was determined by patient interview both at study enrollment and follow-up. The primary endpoint was the composite of myocardial infarction (MI), cerebrovascular infarction, or death from cardiovascular events. Follow-up was performed by telephone interview and from medical records. Persons performing follow-up were unaware of the aspirin sensitivity status.

Platelet aggregation

There are many pre-analytical and analytical variables that affect the results of platelet aggregation [3]. In addition, it is difficult to compare the results obtained in one laboratory to those of another because of the lack of standardization. Therefore, we carefully standardized the conditions of blood collecting and time to measurement, and only two experienced laboratory staff members who were kept unaware of the patient information performed assays to increase the precision.

Fasting venous blood was carefully collected using a 21-guage needle into a syringe containing 1/10 sodium citrate. Blood collection was carried out at 08.30–10.00 hours to minimize the change of platelet activation with circadian variation. Platelet-rich plasma (PRP) was obtained by centrifuging the whole blood at $200 \times g$ for 12 min. The platelet count of the PRP was measured and then adjusted to $20 \times 10^7\ mL^{-1}$ with platelet-poor plasma. Time to measurement was standardized to 1–1.5 h from blood collection. The aggregation response was measured simultaneously using two methods: the conventional method, which is based on changes in LT [9], and light scattering intensities with a PA-20 platelet aggregation analyzer (Kowa Co., Ltd., Tokyo, Japan) [10]. This device is particularly sensitive for detecting the size of small platelet aggregates and can subdivide platelet aggregates according to size into small, medium, or large aggregates [10,11]. Platelet aggregation was performed with collagen (Hormon-Chemie, Munich, Germany), ADP (MC Medical Co., Tokyo, Japan) or CRP (collagen-related peptide), a specific agonist for platelet glycoprotein (GP) VI [12], at 37°C under continuous stirring at 1000 rpm (0.82 dyn/cm^2) for 5 min. CRP (GCP*$[GPP]_{10}$ GCP*G, where P* represents hydroxyproline) was kindly provided by Toray Co. Ltd. (Tokyo, Japan), and was cross-linked as described previously [13].

Measurement of thromboxane metabolites

Although serum TxB_2 concentration is widely used in the assessment of COX activity in aspirin-treated patients, we wanted to examine the correlation between Tx biosynthesis and platelet aggregation under the same condition, and to minimize the influence of TxB_2 derived from other hematopoietic cells. Accordingly, we measured the TxB_2 concentrations and the supernatant from collagen-stimulated PRP in patients. When serum TxB_2 concentration and collagen-stimulated TxB_2 were simultaneously measured, both measurement values correlated with each other [$R = 0.97$, $P < 0.0001$ ($n = 20$)].

The PRP (20×10^7 mL^{-1}) was stimulated with 3 μg mL^{-1} collagen for 5 min and then centrifuged at 2000 × *g* for 15 min to remove the platelets. Supernatants were immediately stored at −30°C. Samples were shipped to the laboratory of BML, Inc. (Tokyo, Japan) in dry ice, and the concentration of TxB_2 (stable metabolite of TxA_2) was measured by radioimmunoassay [14]. Urinary 11-dehydroTxB_2 was measured using enzyme-linked immunosorbent assay (ELISA) (Neogen Co., Lexington, KY, USA) according to the manufacturer's instructions. All investigators were kept unaware of the sample information. To reduce the possibility of systematic bias of the control and aspirin-treated subjects, the samples were assayed in random order.

Statistics

All data analyses were performed with StatView 4.5 (SAS Institute Inc., Cary, NC, USA) for the Macintosh computer. Normally distributed variables were presented as mean ± SD and compared with Student's *t*-test or one-way analysis of variance (ANOVA). Non-normally distributed variables were analyzed with the Mann–Whitney *U*-test. The correlation coefficient was obtained by simple regression analysis. Factor analysis consisting of (i) extraction of the initial components by use of principal-component analysis and (ii) interpretation of factors with loadings > 0.1 ($P < 0.05$) was used to assess the relationship between several inter-correlated variables. Kaplan–Meier product limits were computed for the freedom from endpoint, and the Breslow–Gehan–Wilcoxon test was used for screening univariable group results regarding the outcomes. Multivariate Cox regression models were used to investigate the association of cardiovascular risk factors (age, HT, DM, HL, and platelet aggregation status) with the incidence of endpoints.

Results

Effects of aspirin treatment on platelet aggregation

We first examined the effect of aspirin treatment on platelet aggregation patterns with a newly-developed aggregometry that simultaneously measures both LT and light scattering. In patients treated with aspirin, the platelet aggregation assessed by LT was significantly decreased (Fig. 1). The aspirin treatment was more effective against collagen-induced aggregation than ADP-induced aggregation. Aspirin efficiently

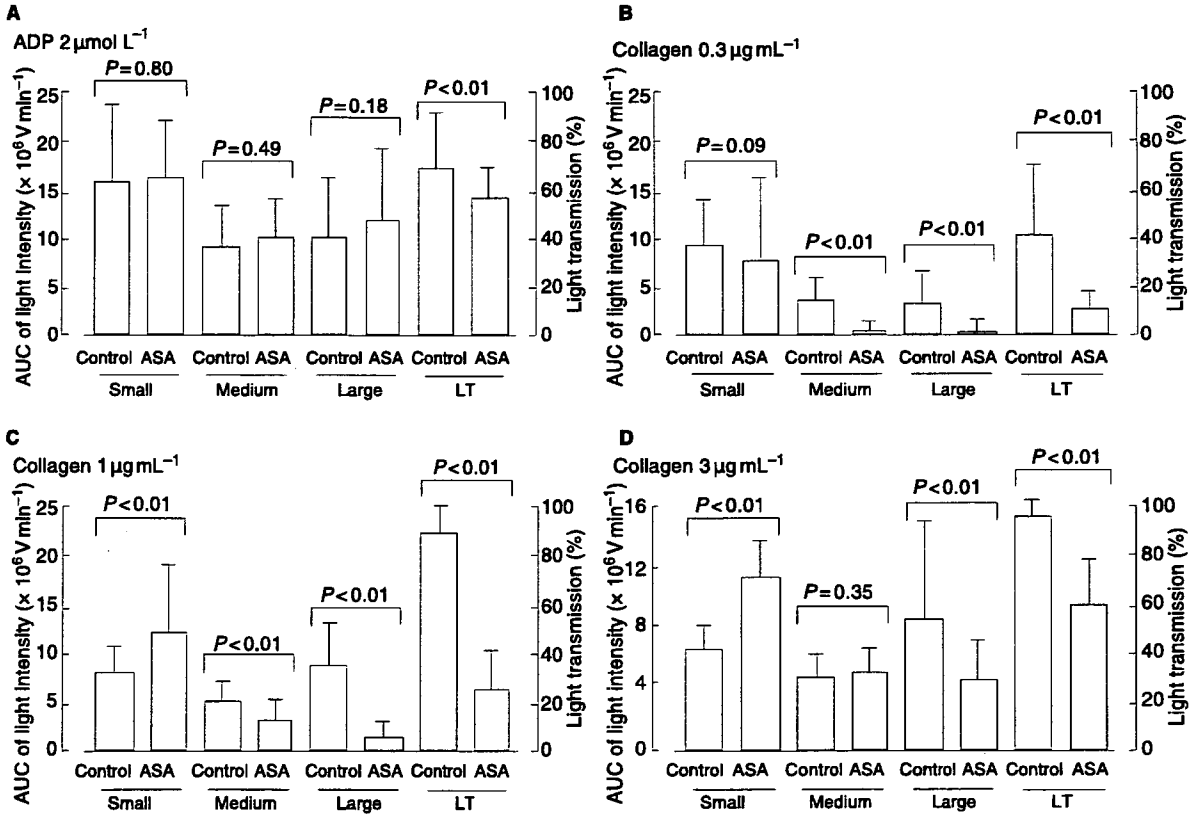

Fig. 1. The effects of aspirin intake on platelet aggregation assessed by light transmission and light scattering methods. Platelets in platelet-rich plasma (PRP) obtained from the control (Control) or aspirin-treated patients (ASA) were stimulated with 2 μmol L^{-1} adenosine diphosphate (ADP) (A), 0.3 μg mL^{-1} (B), 1 μg mL^{-1} (C), or 3 μg mL^{-1} collagen (D) for 5 min. Changes in the maximum light transmission (LT) were monitored using conventional methods. Light scattering intensities were measured simultaneously to detect small, medium, and large aggregates. Data represent the mean ± SD (n = 38 for control and n = 50 for aspirin-treated patients).

inhibited the formation of large aggregates; however, the number of small aggregates increased, suggesting that aspirin treatment prevented small aggregates from developing into large aggregates (Fig. 1). These data indicate that the screening of aspirin sensitivity using this type of aggregometry should be defined by the variation of the LT or the large aggregate formation induced by collagen.

Involvement of platelet COX-1 activity in platelet aggregation and urinary 11-dehydroTxB$_2$

In a preliminary study, we attempted to define 'aspirin resistance' as the failure of aspirin to inhibit platelet COX-1 activity by measuring arachidonic acid-induced platelet aggregation. However, none of the aspirin-treated patients (n = 20) responded to 10 μmol L^{-1} arachidonic acid (data not shown), suggesting that platelet COX-1 activity is sufficiently suppressed in all patients. Accordingly, we directly measured the production of TxB$_2$ in collagen-activated platelets in this study to prove this hypothesis. As shown in Fig. 2A, collagen-stimulated TxB$_2$ formation was almost completely inhibited in all aspirin-treated patients, compared with the control patients. These data suggest that oral low-dose aspirin therapy is sufficient to inhibit platelet COX-1 activity in the Japanese population. On the other hand, the results of platelet aggregation tests induced by collagen had great inter-individual differences (Fig. 2B).

We next performed regression analysis to evaluate whether TxB$_2$ production was associated with the differences of collagen-stimulated platelet aggregation (Table 1). Variables that were found to be associated with platelet TxB$_2$ production were (i) small and medium aggregates (0.3 μg mL^{-1} collagen), (ii) medium and large aggregates (1 μg mL^{-1} collagen), and (iii) large aggregates and LT (3 μg mL^{-1} collagen) (Table 1). However, the TxB$_2$ concentration was able to predict only 6–20% of the variation in collagen-stimulated platelet aggregations (Table 1). These data indicate the existence of other variable(s) that account for the inter-individual variation of collagen-induced platelet activation.

Urinary 11-dehydroTxB$_2$ reflects *in vivo* platelet activation and may be useful for monitoring platelet activity when testing for aspirin resistance [7,15]. Although urinary 11-dehydroTxB$_2$ excretion in aspirin-treated patients was significantly lower

Table 1 Correlation coefficients between platelet TxB$_2$ production (independent variable) and different measurements of platelet aggregation among 50 aspirin-treated patients

Dependent variable	R	R^2	P
Collagen (0.3 μg mL^{-1})			
Small aggregates	0.29	0.084	0.041
Medium aggregates	0.32	0.10	0.023
Large aggregates	0.031	0.00098	0.82
Light transmission	0.15	0.023	0.29
Collagen (1 μg mL^{-1})			
Small aggregates	0.18	0.035	0.19
Medium aggregates	0.37	0.13	0.0080
Large aggregates	0.44	0.20	0.0011
Light transmission	0.22	0.050	0.11
Collagen (3 μg mL^{-1})			
Small aggregates	0.02	0.00044	0.88
Medium aggregates	0.21	0.045	0.14
Large aggregates	0.32	0.10	0.025
Light transmission	0.25	0.06	0.076
Urinary 11-dehydro TxB$_2$/creatinine	0.10	0.01	0.57

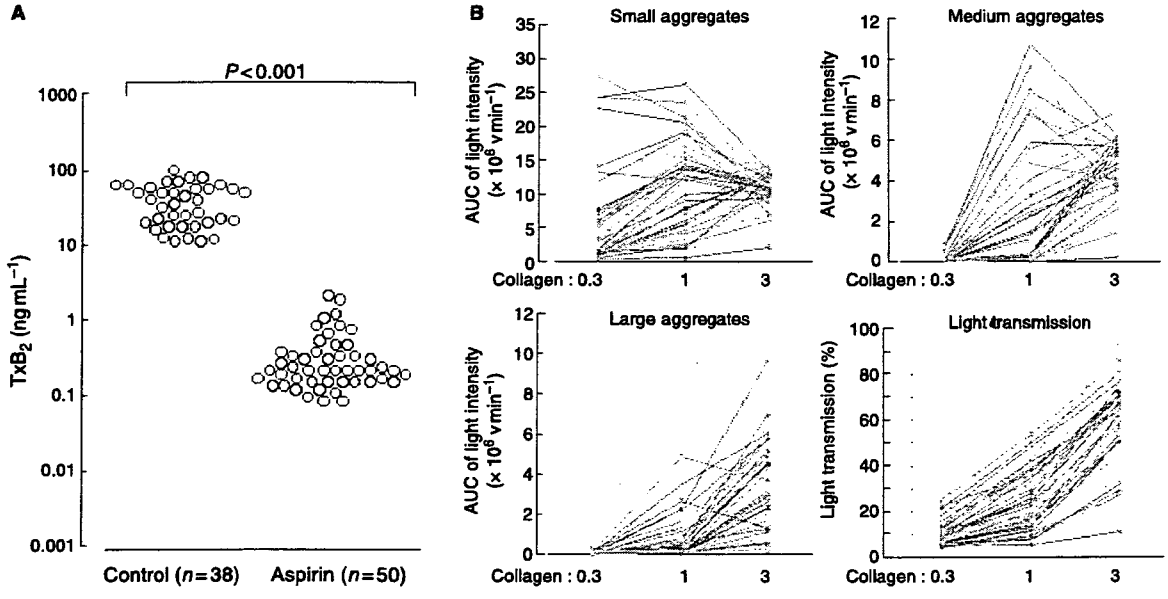

Fig. 2. Platelet TxB$_2$ production and aggregation induced by collagen. In (A), platelets in PRP obtained from control (Control) or aspirin-treated patients (Aspirin) were stimulated with 3 μg mL^{-1} of collagen for 5 min. The TxB$_2$ concentration of the supernatant after stimulation was measured by radioimmunoassay. In (B), individual platelet aggregations induced by collagen in aspirin-treated patient are shown (n = 50).

than that in the control patients (12.0 ± 10.9 vs. 20.6 ± 14.8 ng mg^{-1} creatinine, $P = 0.03$), urinary 11-dehydroTxB_2 excretion was not associated with platelet COX-1 activity (Table 1). As platelet COX-1 activity was inhibited in all patients enrolled, it is possible that the measurement of urinary 11-dehydroTxB_2 instead reflected the Tx biosynthesis of other cells such as endothelial cells and macrophages in aspirin-treated patients.

Determination of a factor that influences collagen-induced platelet aggregation

Aspirin resistance was reportedly caused by an increased sensitivity of platelets to collagen [16]. However, not only the platelet aggregation induced by CRP, but also that induced by ADP, was significantly higher among the patients who had increased responses to collagen (data not shown), suggesting that differences in collagen-induced aggregation in aspirin-treated patients could not be solely explained by alterations in collagen-specific signaling pathways. To explore the confounding factor(s) in platelet aggregation, we next performed factor analysis, including the platelet aggregation induced by collagen, CRP, and ADP, in addition to TxB_2. This model resulted in three separate factors: factor 1, aggregation of CRP, ADP, and collagen-induced platelet aggregation (other than factor 2); factor 2, small and medium aggregates induced by 3 μg mL^{-1} collagen; and factor 3, TxB_2 (Table 2). Factors 1, 2, and 3 explained 48.4%, 17.7%, and 9.9% of the total variance, respectively. These data suggest that platelet aggregations elicited not only by collagen but also those by CRP and ADP were greatly influenced by a factor other than TxB_2 in aspirin-treated patients.

Table 2 Factor analysis using different measurements of platelet aggregation and TxB_2 in 50 aspirin-treated patients

	Factor 1	Factor 2	Factor 3
Collagen (1 μg mL^{-1})			
Small aggregates	0.784	0.251	0.229
Medium aggregates	0.826	−0.093	0.352
Large aggregates	0.769	−0.412	0.035
Light transmission	0.776	−0.059	0.011
Collagen (3 μg mL^{-1})			
Small aggregates	0.076	0.861	−0.090
Medium aggregates	0.574	0.758	0.027
Large aggregates	0.822	0.277	0.034
Light transmission	0.856	0.190	−0.149
CRP (0.03 μg mL^{-1})			
Light transmission	0.711	−0.463	−0.196
CRP (0.1 μg mL^{-1})			
Light transmission	0.764	−0.169	−0.215
ADP (2 μmol L^{-1})			
Light transmission	0.617	0.109	−0.579
TxB_2	0.325	−0.083	0.751
Variance expected (%)	48.4	17.7	9.9

CRP, collagen-related peptide; ADP, adenosine diphosphate.

Involvement of collagen-induced platelet aggregation in cardiovascular events

Finally, we prospectively enrolled 140 patients who took 81 mg of aspirin for the secondary prevention of cardiovascular or cerebrovascular disease to determine whether the variation of platelet aggregation during aspirin treatment influences clinical outcomes. Of the 140 patients enrolled, three patients that developed atrial fibrillation and one patient who did not take aspirin were excluded from the analysis. The mean follow-up duration was 721 days. The baseline characteristics of the patients were as follows: age, 75.4 ± 9.4 years; HT, 105 (77.2%); DM, 15 (11.0%); HL, 22 (16.2%); current cigarette smoking, seven (5.1%). Major events occurred in 21 (15.4%) of the 136 patients (non-fatal MI in two, stroke in 15, and cardiovascular death in four). The patient characteristics of the study according to platelet aggregation status [1 μg mL^{-1} collagen (LT)] are described in Table 3. The previous history of stroke and cardiovascular diseases is 63.2% and 36.8%, respectively. The use of statins and anti-hypertensive drugs was not different among the groups (Table 3).

Figure 3 depicts the Kaplan–Meier time-to-event curves for event-free survival based on platelet aggregation induced by 1 μg mL^{-1} collagen. The composite outcome increased in the upper quartile of large platelet aggregation and light transmission, but not in small or medium aggregates (Fig. 3). ADP sensitivity did not appear to predict the outcomes (data not shown). Total events of the upper quintile of light transmission, but not other indicators, were significantly higher than the lowest quintile ($P = 0.029$) or other groups ($P = 0.045$). In addition, the upper quartile of the large aggregates and light transmission were more likely to experience major clinical events within 12 months (Fig. 3). To evaluate whether the increased baseline collagen-induced platelet aggregations were associated with early rather than late cardiovascular events, we performed separate analyses in patients who had an event within 12 months of study enrollment and those whose events occurred at > 12 months after study entry. As expected with Kaplan–Meier analysis, the adjusted odds for the primary endpoint within 12 months were significantly higher in the highest quartile of the large aggregates and light transmission in multivariable analysis (Table 4). On the other hand, the existence of DM seemed to be an independent risk factor for overall outcomes (HR 1.30–11.9, $P = 0.015$–0.033).

Discussion

Aspirin is the most prescribed antiplatelet drug and its effectiveness in preventing cardiovascular events is well established [1–3]. As low-dose aspirin has been believed to be potent enough to inhibit platelet COX-1 activity, the HOPE study, showing that the inability of aspirin to inhibit Tx biosynthesis resulted in poor clinical outcomes [7], makes us re-consider whether aspirin efficiently inhibits platelet COX activity in all patients. Several mechanisms can be proposed to account for the incomplete suppression of platelet COX-1 by aspirin [2,3]:

Table 3 Patients' characteristics according to quintiles of platelet aggregation induced by 1 μg mL^{-1} collagen (light transmission) in a prospective study

	Total (n = 136)	Q1 (n = 34)	Q2 (n = 34)	Q3 (n = 34)	Q4 (n = 34)
Age	75.4 ± 9.4	74.2 ± 11.2	74.4 ± 8.8	76.7 ± 8.0	76.3 ± 9.4
Gender (female) (%)	73 (53.7)	12 (35.2)	18 (52.9)	22 (64.7)	21 (61.7)
Hypertension (%)	105 (77.2)	24 (70.6)	27 (79.4)	27 (79.4)	27 (79.4)
Diabetes mellitus (%)	15 (11.0)	3 (8.82)	4 (11.8)	7 (20.6)	1 (2.9)
Hyperlipidemia (%)	22 (16.2)	4 (11.8)	6 (17.6)	6 (17.6)	6 (17.6)
Previous history (%)					
Cardiovascular disease	50 (36.8)	14 (41.2)	10 (29.4)	14 (41.2)	12 (35.3)
Stroke	86 (63.2)	20 (58.8)	24 (70.6)	20 (58.8)	22 (64.7)
Drug use (%)					
Ca^{2+} channel blocker	62 (45.6)	10 (29.4)	15 (44.1)	18 (52.9)	19 (55.9)
ACEI/ARB	47 (34.6)	10 (29.4)	11 (32.4)	14 (41.2)	12 (35.3)
Π	21 (15.4)	4 (11.8)	3 (8.8)	8 (23.5)	6 (17.6)
Other anti-HT	30 (22.1)	13 (38.2)	6 (17.6)	3 (8.8)	8 (23.5)
Statin	25 (18.4)	5 (14.7)	5 (14.7)	6 (17.6)	9 (26.4)

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor II blocker; HT, hypertension.

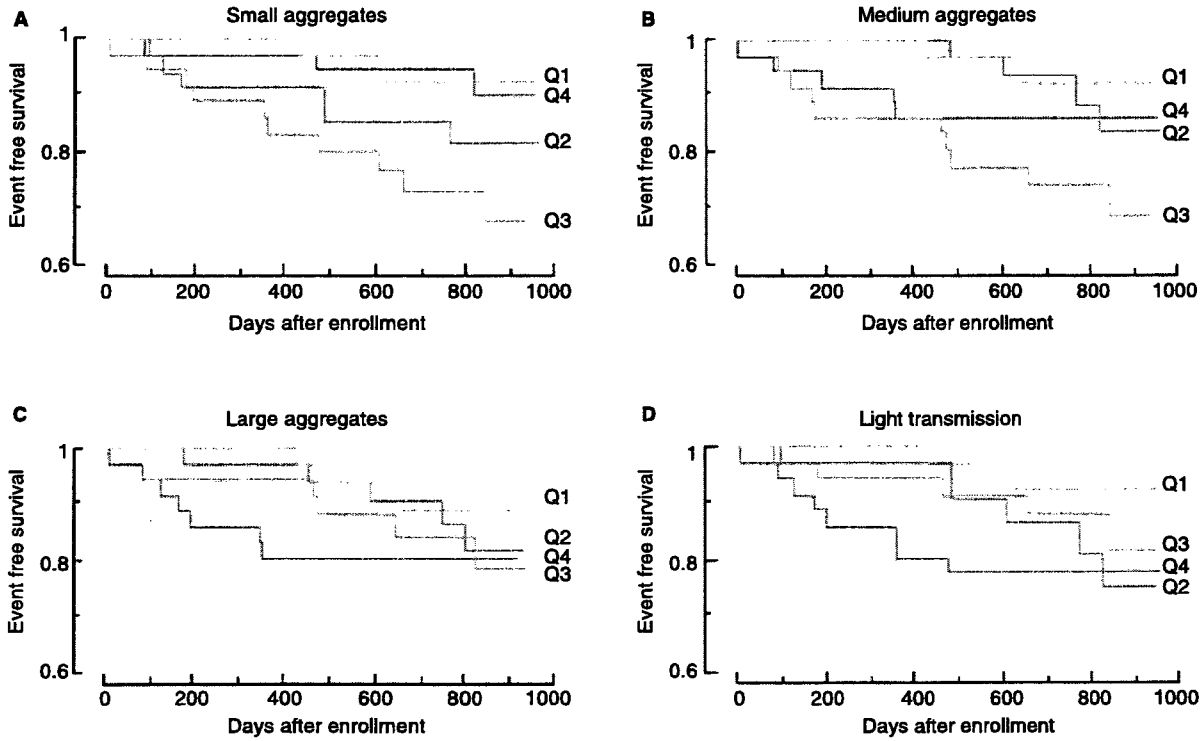

Fig. 3. Kaplan–Meier plot relating quartiles of small (A), medium (B), large aggregates (C), and light transmission (D) (1 μg mL^{-1} of collagen) to the composite risk of myocardial infarction, stroke, and cardiovascular death (Q1 = lowest quartile < Q2 < Q3 < Q4 = highest quartile).

TxA_2 production by the induction of aspirin-insensitive COX in platelets [17,18] and COX-1 polymorphism [19]. Aspirin is approximately 50- to 100-fold more potent in inhibiting COX-1 than COX-2 [2], it is ideally suited to act on a nucleate platelet, inducing a permanent defect in Tx-dependent platelet function. Thus the transient expression of COX-2 in newly formed platelets in a clinical setting of enhanced platelet turn-over [17,20], is a potentially important mechanism that deserves further investigation. Indeed, the incomplete inhibition of COX has been reported in specific clinical settings, such as severe unstable angina [21], coronary artery bypass surgery [18], and endarterectomy [22]. On the other hand, low-dose aspirin treatment invariably inhibited platelet COX-1 activity in our study, suggesting that 'aspirin resistance', which is unsuppressed platelet COX-1 activity, is likely to be rare, at least in an out-patient population which has normal platelet turn-over.

Recent studies assessed the response to aspirin by measuring the urinary level of 11-dehydroTxB_2 [3,7,21] and cardiovascular death associated with aspirin resistance as documented by the urinary TxB_2 concentration [7]. A major portion of this

Table 4 Multivariate analyses of time-to-event among patients according to aspirin sensitivity assessed by 1 μg mL^{-1} collagen (n = 136)

	Early events (< 12 months)		Total events	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Small aggregates				
Upper quartile	0.27 (0.032–2.21)	0.22	0.34 (0.10–1.21)	0.10
Medium aggregates				
Upper quartile	1.98 (0.51–7.62)	0.32	0.73 (0.26–2.01)	0.54
Large aggregates				
Upper quartile	7.98 (1.78–35.7)	0.0066	1.93 (0.72–5.01)	0.18
Light transmission				
Upper quartile	7.76 (1.72–35.3)	0.0077	2.32 (0.89–6.01)	0.084

CI, confidence internal; HR, hazard ratio.

metabolite is believed to come from platelets, but there are also additional cellular sources including monocytes and endothelial cells [2,21,23]. Our results suggest that urinary 11-dehydroTxB_2 variations reflect systemic COX activity other than that from platelets in aspirin-treated patients. Recently, the polymorphism of the COX-2 gene was reportedly associated with a decreased risk of a MI and stroke [24]. It is necessary to clarify whether patients with high 11-dehydroTxB_2 levels, despite the inhibition of platelet COX-1, should be treated with a higher dose of aspirin, and whether the COX-2 polymorphism is involved in differences in systemic Tx production.

Although aspirin resistance was also defined by *in vitro* platelet function in previous studies, there appears to be widespread misunderstanding that *in vitro* platelet function directly represents the COX-1 activity with the results of platelet aggregation tests. In reality, the inter-individual differences of platelet aggregation under aspirin-treatment were caused by a factor(s) other than COX-1. There are several explanations for the factor-influenced platelet activation. First, the threshold of platelet aggregation might decrease in some patients. It is supposed that the decreased basal levels of (or decreased sensitivity to) c-AMP or c-GMP [25,26] and the increases in the level of myosin light chain phosphorylation in platelets are possible factors causing hyperaggregability of platelets in high-risk patients such as diabetics [27]. Furthermore, ADP signaling may be involved in the inter-individual differences [28]. A strategy targeting the ADP signaling pathway is attractive because recent trials have demonstrated the superior clinical benefit of the combination of clopidogrel with aspirin, compared with aspirin alone [29,30]. The elucidation of the factor(s) leading to the inter-individual differences would lead to a new therapeutic approach, and the specific antagonists of the factor may supplement the therapeutic effect of aspirin in some aspirin-treated patients.

We found that the upper quartile of LT or large aggregate formation induced by collagen was an independent risk factor for developing cardiovascular events within 12 months in multivariable analyses, suggesting that the necessity of inhibiting platelet function to an optimized level, even if aspirin effectively abolishes platelet COX-1 activity. Unfortunately, the presented data appears to show no advantage of measuring small and medium aggregates in aspirin-treated patients. Taking into consideration that aspirin inhibits the transition from small aggregates to large aggregates, it is likely that the conventional aggregometry which preferentially detects large aggregates has proved useful in monitoring the effects of aspirin on platelet aggregation. An association between suboptimal platelet function inhibition during aspirin treatment and the heightened incidence of cardiovascular events has been also described [8]. However, this study used inadequate techniques to measure the response to aspirin: ADP at 10 μM induces full platelet aggregation, and a high concentration of arachidonic acid induces platelet lysis [3] and activates platelets through unknown signaling pathways [31]. As collagen-induced platelet aggregation is more sensitive to aspirin therapy than the aggregation elicited by ADP or other soluble agonists and reflects clinical outcomes in our study, we recommend collagen as a standard platelet agonist for assessment of aspirin treatment. Considering the small number of individuals studied, our current findings should be interpreted with some caution, and larger-scale studies are awaited to draw more conclusive answers to this issue. Furthermore, the study to evaluate additional treatment and elucidate the precise biological effects of aspirin should be performed.

Acknowledgements

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References

1 Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy-I: prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *BMJ* 1994; **308**: 81–106.
2 Patrono C, Coller B, FitzGerald GA, Hirsh J, Roth G. Platelet-active drugs: the relationships among dose, effectiveness, and side effects: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. *Chest* 2004; **126**: 234S–64S.
3 Cattaneo M. Aspirin and clopidogrel: efficacy, safety, and the issue of drug resistance. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1980–7.
4 Awtry EH, Loscalzo J. Aspirin. *Circulation* 2000; **101**: 1206–18.
5 Mason PJ, Jacobs AK, Freedman JE. Aspirin resistance and atherothrombotic disease. *J Am Coll Cardiol* 2005; **46**: 986–93.
6 McKee SA, Sane DC, Deliargyris EN. Aspirin resistance in cardiovascular disease: a review of prevalence, mechanisms, and clinical significance. *Thromb Haemost* 2002; **88**: 711–5.
7 Eikelboom JW, Hirsh J, Weitz JI, Johnston M, Yi Q, Yusuf S. Aspirin-resistant thromboxane biosynthesis and the risk of myocardial infarction, stroke, or cardiovascular death in patients at high risk for cardiovascular events. *Circulation* 2002; **105**: 1650–5.