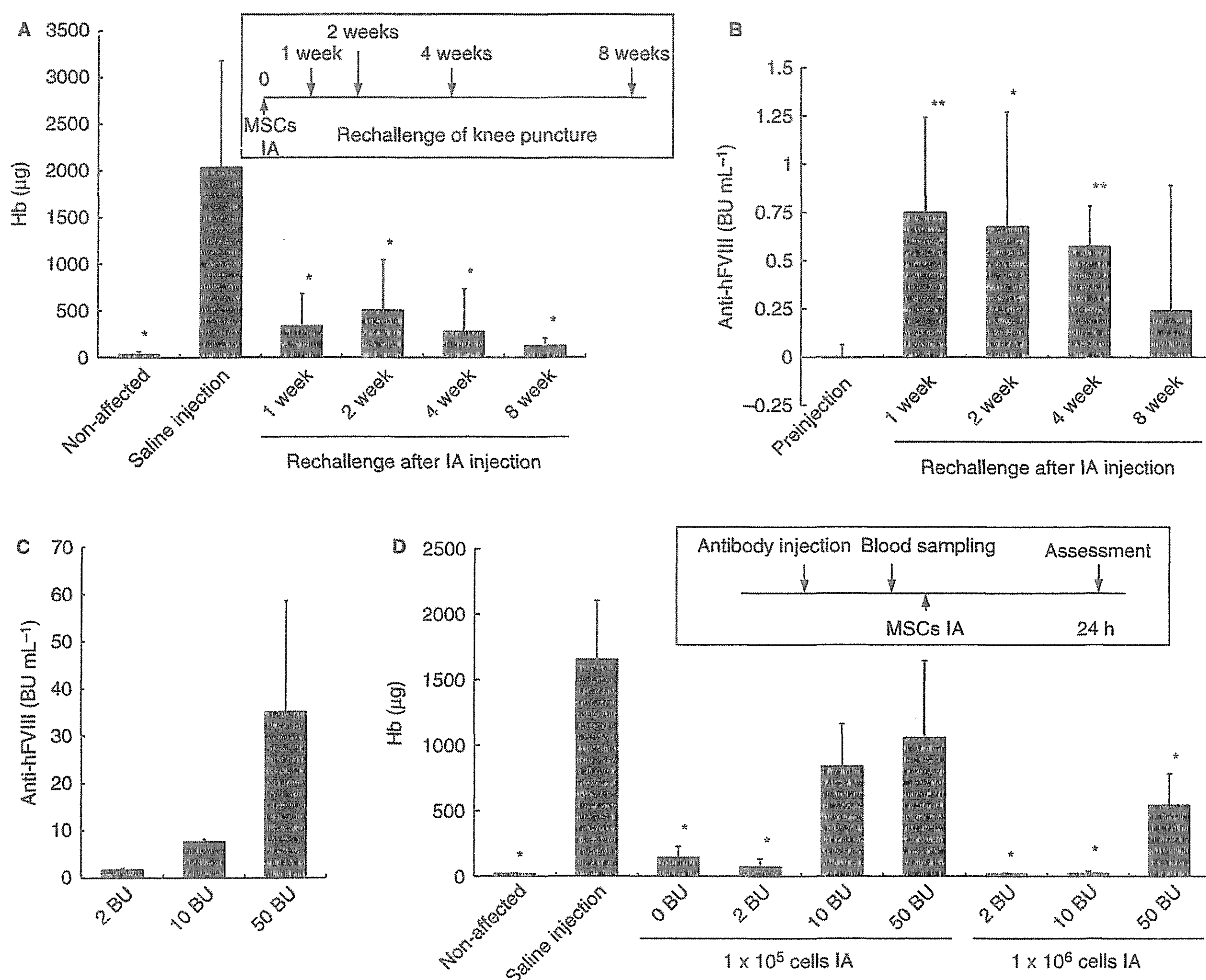

Fig. 7. Intra-articular (IA) injection of transduced mesenchymal stem cells (MSCs) expressing human FVIII (hFVIII) persistently inhibits hemarthrosis, and protects against hemarthrosis in the presence of low titers of neutralizing antibodies against hFVIII. (A) FVIII-deficient mice received an IA injection of transduced MSCs expressing hFVIII (1×10^5 cells), and this was followed by joint capsular needle puncture at the indicated times after IA injection. A schematic diagram of the procedure is shown in the box. Hemarthrosis at 24 h after knee puncture was quantified as the hemoglobin (Hb) concentration. Values are means ± standard deviations (SDs) (n = 4–9). *$P < 0.05$ as compared with the saline-injected control group (two-tailed Student's t-test). (B) Circulating plasma inhibitors were assessed as Bethesda Units (BU) mL^{-1} at the indicated times after IA injection. Values are means ± SDs (n = 4–10). (C) Neutralizing antibodies against hFVIII (2, 10 or 50 BU per mouse) were intravenously administrated into FVIII-deficient mice. Circulating plasma inhibitor concentrations were assessed as BU mL^{-1} (n = 6). (D) FVIII-deficient mice received an IA injection of transduced MSCs expressing hFVIII (1×10^5 cells or 1×10^6 cells), and this was followed by joint capsular needle puncture. Hemarthrosis at 24 h after knee puncture was quantified as the Hb concentration. A schematic diagram of the procedure is shown in the box. Values are means ± SDs (n = 3). *$P < 0.05$ as compared with the saline-injected control group (two-tailed Student's t-test).

conditions further enhances coagulation factor expression to ameliorate hemarthrosis.

Several studies have focused on cell-based therapy with MSCs expressing coagulation factor to treat hemophilia by increasing the plasma levels of coagulation factor [14,38–40]. However, long-term protein production from MSCs was not achieved *in vivo* after transplantation, because of the loss of cell viability and/or the emergence of inhibitory antibodies [14]. Recently, Coutu *et al.* [39] successfully achieved long-term expression of FIX by implanting a three-dimensional porous scaffold containing gene-modified MSCs to increase graft survival. They used the murine R333Q model of hemophilia B, which avoids the development of inhibitory antibodies [39]. In addition, Porada *et al.* [40] described the interesting treatment effect of MSCs in a sheep model of severe hemophilia A. They intraperitoneally transplanted MSCs expressing porcine FVIII into sheep with hemophilia A [40]. An increase in plasma FVIII activity could not be detected, and titers of the inhibitory antibody against hFVIII and porcine FVIII dramatically increased [40]. Nevertheless, transplantation of MSCs expressing coagulation factor resolved hemarthrosis and improved joint function [40]. The authors also observed the migration of

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transduced MSCs into a number of organs, including the synovium [40]. In our study, the level of circulating inhibitors of hFVIII induced by intra-articular injection of transduced MSCs was much lower than that following subcutaneous transplantation of MSCs, although a low titer of BUs was observed. Our results also suggest that implanting engineered MSCs expressing coagulation factor into the synovial joint space ameliorates hemarthrosis, even in the presence of inhibitory antibodies. Furthermore, a small number of transduced cells might be sufficient to achieve therapeutic effects, as compared with systemic transplantation of transduced cells. Accordingly, we believe that intra-articular injection of transduced MSCs represents a more realistic approach to ameliorate hemarthrosis and arthropathy, because of several advantages, including minimally invasive surgical procedures, the need for a small number of transduced cells, and a lower titer of inhibitory antibodies following treatment.

One of the main barriers to implementing clinical trials of gene and cell-based therapy is concern over the safety of viral vectors. We used the third generation of the SIV lentiviral vector to express coagulation factor in MSCs, because it has a better safety profile than gamma retroviral vectors (γRVs) [41]. As compared with γRVs, lentiviral vectors preferentially integrate within active transcription units without an obvious bias for proliferation-associated genes or transcriptional start sites, suggesting that lentiviral vectors are less likely to trigger oncogenic events [42]. Self-inactivating vector systems, in which the promoter activity in the U3 region of the viral long-terminal repeat (LTR) is deleted, have been used in many studies because the promoter activity of the viral LTR is associated with transcriptional activation of oncogenes in γRVs [43,44]. It is possible that the use of a physiologic promoter, such as the PAI-1 promoter in a self-inactivating vector, may be safer than using a ubiquitous viral promoter. We did not observe any tumorigenesis in the transplanted sites or abnormal proliferation of the transduced MSCs during the observation period. We believe that the safety of cell-based therapies could be further enhanced by several approaches. First, we can investigate the proviral integration sites of the transduced cells before using cell-based therapy, but not after direct injection of a viral vector. Second, we can improve the safety of cell-based therapy by blocking the cell cycle of transduced MSCs before transplantation by irradiation or pretreatment with a cytotoxic agent such as mitomycin C, if repeated injections of transduced MSCs are possible.

Some limitations of this study merit discussion before the clinical application of this procedure. The main limitation of our work is the relatively modest improvement in prevention of hemarthrosis. Although the local concentration of hFVIII achieved by intra-articular injection of the transduced cells should be higher than that reaching the joint following intravenous infusion, needle puncture-induced hemarthrosis was not completely abolished (Fig. 5). Second, our procedure induced a low neutralizing antibody titer, suggesting the possibility that our procedure would enhance the immune responses to hFVIII in patients expressing the inhibitor, particularly those with high responder inhibitor levels. Although intra-articular injection might be effective in the presence of low circulating inhibitor titers, our procedure may be more appropriate for adults who have already undergone replacement therapy several times, and might not develop inhibitory antibodies after intra-articular injection of the transduced MSCs. Furthermore, we could not fully assess the duration of transgene expression required to inhibit hemarthrosis or the fate of the transplanted MSCs. As we could recover very little RNA from around the knee joint from mice, we could not detect transgene mRNA in the joint space (data not shown). Although we believe that the therapeutic range of FVIII expression would be maintained for at least for 8 weeks, on the basis of the results shown in Fig. 7, it is important to confirm the long-term therapeutic effect and safety of this procedure. In addition, it is important to assess transgene expression and cell fate in larger animals to determine how frequently this procedure should be conducted.

In conclusion, we have proposed a new treatment strategy for hemophilic arthropathy in which MSCs expressing coagulation factor are directly injected into the target tissue. Considering that intra-articular injection is a minimally invasive procedure and that the MSCs can facilitate repair of the damaged joint structure, the procedure described here may become an attractive approach to prevent and/or treat blood-induced joint disease in hemophilic patients. Further evaluations of cell-based therapy in larger animals (e.g. cynomolgus monkey) and of the long-term safety of lentivirally transduced cells after transplantation are necessary before these procedures can be tested in clinical trials.

Addendum

Y. Kashiwakura and T. Ohmori: designed and performed the experiments, analyzed the data, and wrote the manuscript; J. Mimuro: performed experiments, analyzed the data, and revised the manuscript; A. Yasumoto, A. Sakata, and A. Ishiwata: performed experiments; M. Inoue and M. Hasegawa: provided vital reagents and critically reviewed the manuscript; S. Madoiwa, K. Ozawa, and Y. Sakata: analyzed data and revised the manuscript.

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Disclosure of Conflict of Interests

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Activity and antigen levels of hFVIII produced from MSCs, MEFs and HepG2 cells transduced with the SIV vector.
Figure S2. Association between hFVIII activity and proviral integration into the genome in MSCs transduced with the SIV vector.
Figure S3. Increases in plasma FVIII antigen after direct injection of supernatant from transduced MSCs or subcutaneous implantation of transduced MSCs.
Figure S4. Immunohistochemical staining of luciferase.
Data S1. Supplemental methods.

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Cell Stem Cell
Brief Report

Il2rg Gene-Targeted Severe Combined Immunodeficiency Pigs

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SUMMARY

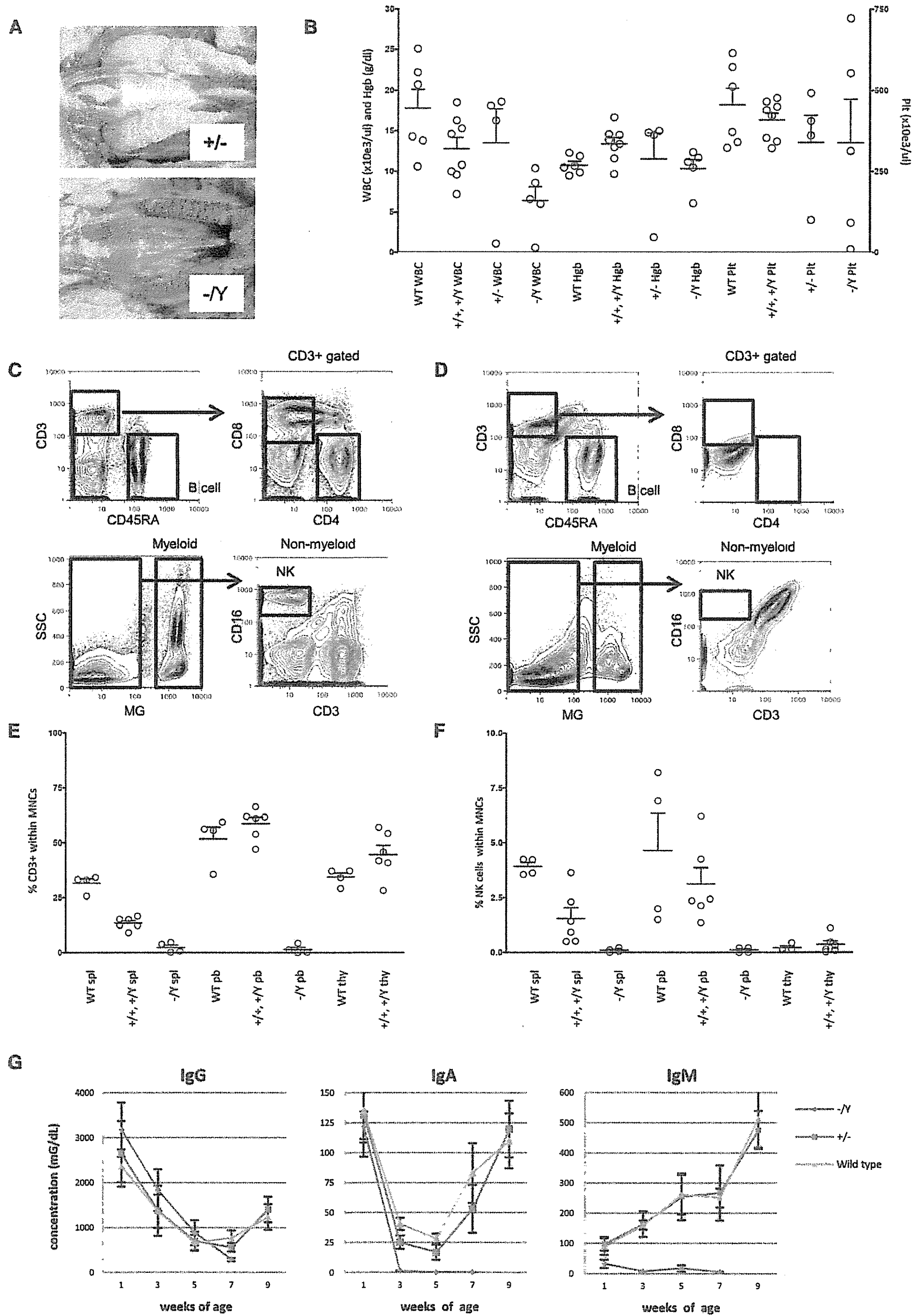
A porcine model of severe combined immunodeficiency (SCID) promises to facilitate human cancer studies, the humanization of tissue for xenotransplantation, and the evaluation of stem cells for clinical therapy, but SCID pigs have not been described. We report here the generation and preliminary evaluation of a porcine SCID model. Fibroblasts containing a targeted disruption of the X-linked interleukin-2 receptor gamma chain gene, *Il2rg*, were used as donors to generate cloned pigs by serial nuclear transfer. Germline transmission of the *Il2rg* deletion produced healthy $Il2rg^{+/-}$ females, while $Il2rg^{-/Y}$ males were athymic and exhibited markedly impaired immunoglobulin and T and NK cell production, robustly recapitulating human SCID. Following allogeneic bone marrow transplantation, donor cells stably integrated in $Il2rg^{-/Y}$ heterozygotes and reconstituted the $Il2rg^{-/Y}$ lymphoid lineage. The SCID pigs described here represent a step toward the comprehensive evaluation of preclinical cellular regenerative strategies.

The common gamma chain, IL2RG, is an IL-2 receptor subunit (Takeshita et al., 1992) shared by IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Kondo et al., 1993; Noguchi et al., 1993a; Russell et al., 1993; Giri et al., 1994; Kimura et al., 1995; Asao et al., 2001). IL2RG is present in T, NK, NKT, and dendritic cells (Ishii et al., 1994) and plays an essential role in lymphoid development by activating, through its cytoplasmic domain, Janus kinase 3 (Nakamura et al., 1994; Nelson et al., 1994, 1997).

Mammalian *IL2RG* orthologs are typically located on the X chromosome; in humans, *IL2RG* mutations result in X-linked severe combined immunodeficiency (XSCID) in which T and NK cells are absent or profoundly reduced in number, while B cells are numerically normal (or increased) but functionally impaired (Noguchi et al., 1993b; Leonard, 1996; Fischer et al., 1997). Gene-targeted mice lacking *Il2rg* also exhibit immunological defects (Cao et al., 1995; Ohbo et al., 1996) including the ablation of NK cell activity. NOD/SCID/$Il2rg^{null}$, NOG, and $Rag2^{null}/Il2rg^{null}$ mice permit the functional reconstitution of human hematopoietic and immune systems following the injection of purified human hematopoietic stem cells (Traggiai et al., 2004; Ishikawa et al., 2005; Shultz et al., 2005). Unfortunately, phenotypic differences exist between XSCID humans and *Il2rg* null mice, including a pronounced numerical B cell reduction in the latter. Two dog breeds develop SCID caused by *Il2rg* mutations (Felsburg et al., 1999; Perryman, 2004), but dogs are poorly characterized research models.

In contrast, the pig more closely resembles humans regarding anatomy, hematology, physiology, size, and longevity. By enabling long-term follow-up, pig models will permit the evaluation of human cancer and stem cell transplantation over clinically relevant time frames. We here describe disruption of the porcine *Il2rg* gene to generate SCID pigs, their phenotypic characterization, and proof-of-principle transplantation studies.

A conventional positive-negative selection *Il2rg* gene targeting vector (TV) (Figure S1A) enabled the functional inactivation of porcine *Il2rg* by removing exon 6 (Kanai et al., 1999). Following fetal fibroblast transfection, selection, and PCR screening, 1 of 3 TV-targeted cell lines was expanded for nuclear transfer (Table S1). Even after screening, PCR-positive colonies often contain a substantial proportion of nontargeted cells (not shown) that would result in a corresponding proportion of nontargeted cloned pigs following nuclear transfer. To ensure that all clones harbored a targeted *Il2rg* locus, we adopted a serial cloning strategy. Nine embryonic day 35 (E35) or E39 nuclear transfer embryos were collected and screened by PCR and Southern blotting. PCR (not shown) and Southern blotting (Figure S1B) revealed that six contained the genomic configuration predicted for a single-targeted *Il2rg* allele. Fibroblasts were cultured from one targeted embryo and used in secondary nuclear transfer to


A
+/-
-/Y
B
WBC (x10e3/ul) and Hgb (g/dl)
Plt (x10e3/ul)
WT WBC
+/+, +/Y WBC
+/- WBC
-/Y WBC
WT Hgb
+/+, +/Y Hgb
+/- Hgb
-/Y Hgb
WT Plt
+/+, +/Y Plt
+/- Plt
-/Y Plt
C
CD3+ gated
CD3
CD45RA
B cell
CD8
CD4
Myeloid
Non-myeloid
NK
SSC
MG
CD16
CD3
D
CD3+ gated
CD3
CD45RA
B cell
CD8
CD4
Myeloid
Non-myeloid
NK
SSC
MG
CD16
CD3
E
% CD3+ within MNCs
WT spl
+/+, +/Y spl
-/Y spl
WT pb
+/+, +/Y pb
-/Y pb
WT thy
+/+, +/Y thy
F
% NK cells within MNCs
WT spl
+/+, +/Y spl
-/Y spl
WT pb
+/+, +/Y pb
-/Y pb
WT thy
+/+, +/Y thy
G
IgG
IgA
IgM
concentration (mG/dL)
weeks of age
-/Y
+/-
Wild type

produce 31 cloned F_0 piglets; all were females heterozygously targeted at their *Il2rg* genes as judged by PCR (not shown) and Southern blotting (Figure S1C).

Fourteen of the 31 were stillborn and 3 of the 17 live-born died neonatally of unknown cause(s) (Table S1). Ten survivors died from pneumonia and severe arthritis (five were euthanized) between postnatal day 7 (P7) and P70. The remaining four (#5, 9, 15 and 20) survived for >1 year.

Stillborn and neonatal fatalities often had spleens with hypoplastic lymphoid aggregations (Figure S1D). Most (24/31, 77%) had undetectable or severely hypoplastic thymi (Figure S1E and Table S2). F_0 clones that died within 10 weeks also lacked detectable thymi and had few, if any, T cells either in their spleens or circulating (Figures S1F and S1G). In contrast, levels of $CD4^+$ and $CD8^+$ T cells in four long-lived F_0 lines were comparable to those of WT controls. Analysis of peripheral blood (PB) mononuclear cell (PBMC) RNA corroborated this: *Il2rg*, *CD4*, and *CD8* transcript levels were reduced in athymic *Il2rg*$^{+/-}$ clones that perished relative to respective levels in long-lived clones and WT controls (Figure S1H).

Thus, most *Il2rg*$^{+/-}$ clones exhibited SCID-like phenotypes, albeit that they had one WT allele. We attributed this high proportion to aberrant X-inactivation, a previously observed epigenetic cloning phenotype (Senda et al., 2004; Nolen et al., 2005; Jiang et al., 2008). However, epigenetic cloning phenotypes are corrected by germline transmission (Shimozawa et al., 2002). To confirm this and isolate the *Il2rg*$^{+/-}$ phenotype, we analyzed progeny derived by fertilization from *Il2rg*$^{+/-}$ cloned female #9.

Female *Il2rg*$^{+/-}$ #9 inseminated with WT sperm produced 19 F_1 (12 m, 7 f) offspring, and of these F_1 offspring, two *Il2rg*$^{+/-}$ females produced 21 F_2 (13 m, 8 f) when inseminated with WT sperm. Autopsies of representative F_1 and F_2 progeny revealed that, as expected, all *Il2rg*$^{-/Y}$ males had undetectable thymi, whereas *Il2rg*$^{+/-}$ females had thymi of normal size (Figure 1A and Table S3).

Hematological parameters in PB exhibited a significantly ($p = 0.0041$) reduced white blood cell (WBC) count in F_1 *Il2rg*$^{-/Y}$ males ($6.4 \pm 1.6 \times 10^3/\mu l$, n = 5) compared to WT controls ($17.8 \pm 2.3 \times 10^3/\mu l$, n = 6), while hemoglobin levels and platelet counts were unaffected (Figure 1B). F_1 *Il2rg*$^{+/-}$ females and WT littermates yielded comparable PB T, NK, and B cell numbers, indicative of intact acquired and innate immunity (Figures 1C, 1E, and 1F). In contrast, *Il2rg*$^{-/Y}$ males harbored significantly reduced PB T cells (*Il2rg*$^{-/Y}$ males, 1.5% ± 1.0%; *Il2rg*$^{+/Y}$ males, 57.3% ± 4.3%; n = 4 each, $p < 0.0001$) and NK cells (*Il2rg*$^{-/Y}$ males, 0.1% ± 0.1%, *Il2rg*$^{+/Y}$ males, 3.6% ± 1.1%; n = 4 each, $p = 0.0162$) (Figures 1D, 1E, and 1F). In proportion to the PB reductions, *Il2rg*$^{-/Y}$ spleens exhibited significant numerical reductions of T cells (*Il2rg*$^{-/Y}$ males, 2.3% ± 1.1%; *Il2rg*$^{+/Y}$ males, 13.0% ± 1.4%; n = 4 each, $p = 0.0011$) and NK cells (*Il2rg*$^{-/Y}$ males, 0.1% ± 0.0%; *Il2rg*$^{+/Y}$ males, 0.8% ± 0.2%; n = 4 each, $p = 0.0162$) (Figures 1E and 1F). B cells and myeloid cells accounted for the majority of $CD45^+$ leukocytes in *Il2rg*$^{-/Y}$ males, indicating that their immune deficiency was limited to T and NK cell lineages. The presence in *Il2rg*$^{-/Y}$ males of $CD33^+$ myeloid cells with both mononuclear and polynuclear properties suggests the differentiation of both granulocyte lineages and antigen-presenting cells, including monocytes and dendritic cells (Figures 1C and 1D).

We next evaluated humoral immune status in *Il2rg*-targeted pigs (Figure 1G). Serum IgG and IgA levels were high at 1 week (P7) and decreased gradually from 3 to 5 weeks in *Il2rg*$^{-/Y}$ (males), WT littermates, and *Il2rg*$^{+/-}$ female controls. After 7 weeks, IgG and IgA levels re-elevated in WT controls, while levels of both remained low in *Il2rg*$^{-/Y}$ males. Serum IgM was low at 1 week and increased gradually in controls but remained low in *Il2rg*$^{-/Y}$ males. Because both IgG and IgA at 1 week of age are entirely transferred via the colostrum in pigs, these results indicate that there had been no de novo Ig production in *Il2rg*$^{-/Y}$ males after weaning at 4 weeks. Impaired antibody production by *Il2rg*$^{-/Y}$ B cells is likely due to the absence of critical $CD4^+$ T helper cells. Consistent with their impaired immunity, all F_1 *Il2rg*$^{-/Y}$ males became systemically ill in the conventional housing conditions used, while F_1 *Il2rg*$^{+/-}$ females appeared healthy.

Collectively, this shows that when produced by conventional breeding, *Il2rg*$^{-/Y}$ F_1 males, but not *Il2rg*$^{+/-}$ females, present SCID phenotypes. SCID-like phenotypes observed in many *Il2rg*$^{+/-}$ female clones are attributable to aberrant, nonrandom X-inactivation during somatic cell cloning. Following germline transmission, *Il2rg*-targeted phenotypes resembled those of X-linked SCID in other species, with greatly reduced T and NK cell development and function (Cao et al., 1995; Puck et al., 1987). *Il2rg*-targeted pigs harbor B cells and thereby recapitulate human XSCID more closely than do *Il2rg*-targeted mice.

We next performed proof-of-principle allogeneic transplantation experiments using *Il2rg*-targeted pigs as recipients. Preliminary conditioning with orally administered fludarabine and busulfan produced significantly decreased WBC and platelet counts (not shown), which might promote the engraftment of transplanted cells. However, 2 of 6 *Il2rg*$^{-/Y}$ males died within 2 weeks postadministration, suggesting that the regimen was lethal for some piglets. Bone marrow (BM) cells from WT siblings were intravenously transplanted to four P11-12 *Il2rg*$^{-/Y}$ males with (#113, #115) or without (#605, #610) conditioning. Ubiquitously GFP-expressing (#184) BM cells (Watanabe et al., 2005)

Figure 1. Phenotypes of F_1 and F_2 Progeny Derived from *Il2rg*$^{+/-}$ Clone #9 by Germline Transmission
(A) Thymic phenotype in an *Il2rg*$^{+/-}$ female at 10 weeks and an *Il2rg*$^{-/Y}$ male at 9 weeks.
(B) Peripheral blood (PB) white blood cell (WBC), hemoglobin (Hgb), and platelet count (Plt) at 2 months of age in WT controls, *Il2rg*$^{+/+}$ female littermates, *Il2rg*$^{+/-}$ female littermates, and *IL2rg*$^{-/Y}$ males.
(C) Identification of acquired and innate immune subsets in an *Il2rg*$^{+/+}$ female by surface phenotype of $CD45RA^+CD3^-$ B cells, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and $CD3^-CD16^+$ NK cells among the nonmyeloid fraction and myeloid cells.
(D) Analysis as for (C), but of an *IL2rg*$^{-/Y}$ male.
(E) Proportion of $CD3^+$ T cells in the spleen (spl), PB, and thymus (thy) in control *Il2rg*$^{+/+}$ females and male *Il2rg*$^{+/Y}$ littermates and nonlittermates (WT).
(F) Analysis as for (E), except showing the proportion of $CD3^-CD16^+$ NK cells.
(G) Changes with time postpartum in serum IgG (left), IgA (middle), and IgM levels. All error bars indicate SEM. See also Figure S1 and Tables S1–S3.

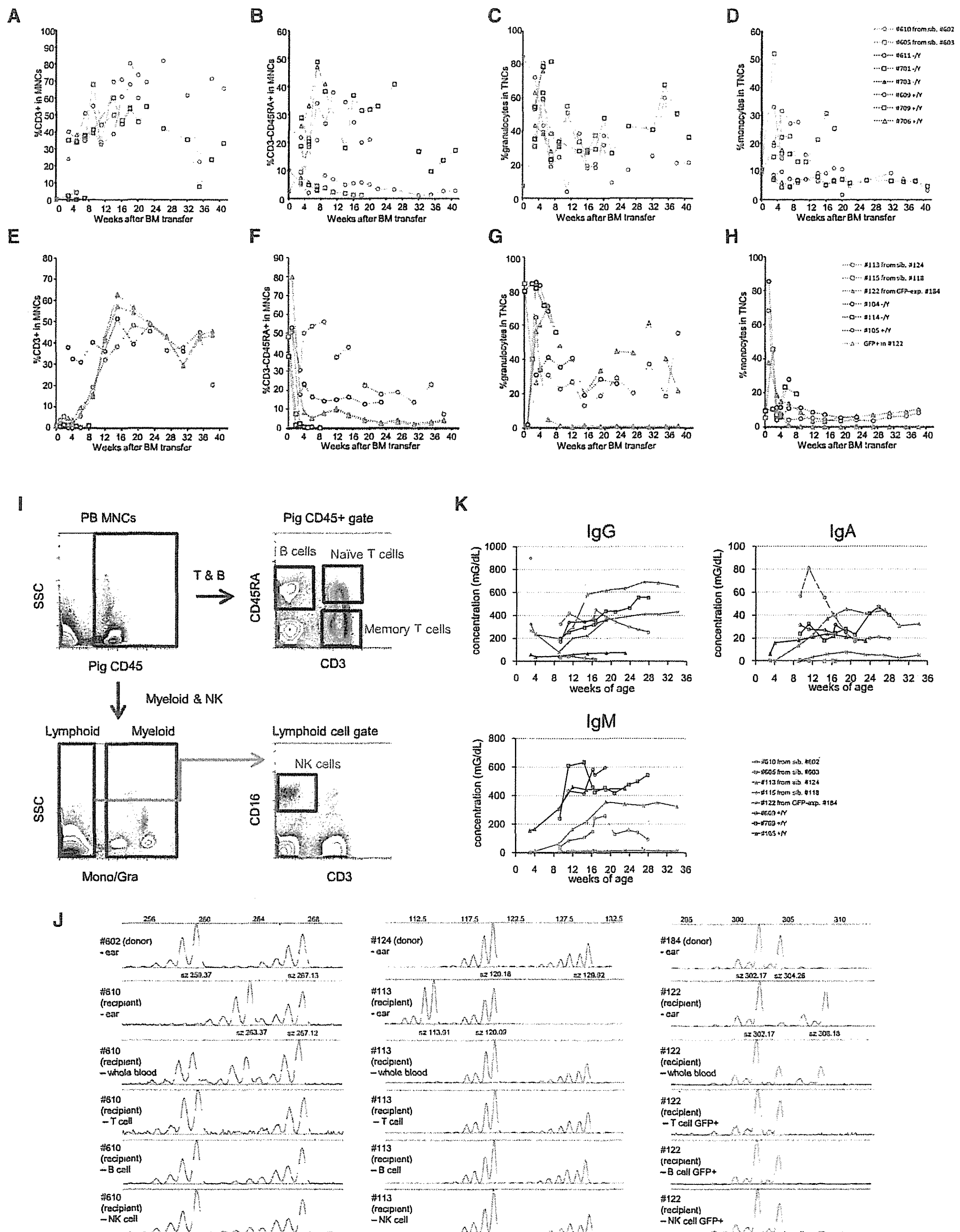


Figure 2. Allogeneic Bone Marrow Transfer into *Il2rg*$^{-/Y}$ Males

(A–H) Flow cytometric quantification of each subtype of leukocytes in *Il2rg*$^{-/Y}$ males (red lines) at different times after BM transfer. Controls are WT littermates (blue lines) and *Il2rg*$^{-/Y}$ males (black lines) without BM transfer. (A)–(D) correspond to BMT without conditioning, the case for #605 and #610. (E)–(H) correspond to BMT with conditioning, the case for #105, #113, and #122. (A) and (E) show the proportion of CD3^{+} T cells in PB at different times after BM transfer. (B) and (F) show proportion of CD45RA^{+}CD3^{-} B cells in PB at different times after BM transfer. (C) and (G) show proportion of granulocytes in PB at different times after BM transfer. (D) and (H) show proportion of monocytes in PB at different times after BM transfer.

were transferred to a *Il2rg*$^{-/Y}$ male (#122) with conditioning. Recipient #115 died 15 days after BM transfer (BMT), possibly due to conditioning toxicity, and recipient #605 died of presumptive pneumonia (not shown) 140 days posttransplantation; nevertheless, it survived longer than *Il2rg*$^{-/Y}$ controls without BMT, which died within 54 days. The remaining three recipients have survived >516 days (#610) and >321 days (#113 and #122) posttransplantation, with PB T cell populations exhibiting similar dynamics (Figures 2A and 2E); T cell counts increased ~6 weeks posttransplantation and remained high. Following early variability, PB B cell counts equilibrated at detectably low levels. The exception was #113, in which B cell counts were clearly higher than those of WT controls until 16 weeks posttransplantation, gradually decreasing thereafter to a level comparable with other recipients (Figures 2B and 2F). Compared to WT controls, recipient #610 PB contained similar T cell counts and a diminished but substantial number of B and NK cells 42 weeks posttransplantation (Figures 2A, 2B, and 2I). Similar profiles were observed in #113 and 122 (Figures 2E and 2F; not shown for NK). Surviving recipients and WT littermates had comparable granulocyte and monocyte numbers (Figures 2C, 2D, 2G, and 2H).

The provenance of immune cells in surviving recipients was examined by microsatellite marker analysis of genomic DNA from the ear, whole blood, and sorted T, B, and NK cells. GFP fluorescence of immune cells was also detected in the case of #122 (Figures 2E–2H and 2J). Lymphoid lineages were totally of donor origin in all recipients. Myeloid lineages were mainly of donor origin in #113 and of mixed donor/host origin in #122 and #610. Thus, our conditioning regimen enabled donor-derived myeloid lineage reconstitution in #113, but not #122. All surviving recipient PB contained IgG, IgA, and IgM (Figure 2K), albeit at varying levels, strongly suggesting that humoral immunity had been reconstituted and that donor-derived recipient B cells produced antibodies. Thus, allogeneic BM transplantation to *Il2rg*$^{-/Y}$ SCID pigs reproducibly resulted in enduring functional donor cell engraftment and reconstituted acquired immunity.

Assuming that the allogeneic model reported here reflects the behavior of cells transplanted from different species, SCID pigs promise to become a valuable tool in xenogeneic transplantation studies of human stem cells, such as hematopoietic, embryonic, and induced pluripotent stem cells (Takahashi et al., 2007). In particular, they promise to serve as platforms for the evaluation of therapeutic outcomes over several years, possibly after further genetic manipulation such as disruption of recombination activating genes 1 or 2 (*Rag1*, *Rag2*), which play critical roles in both cellular and humoral immunity (Shinkai et al., 1992) and may facilitate efficient human stem cell engraftment. Preliminary xenotransplantation of human BM cells to porcine *Il2rg*$^{-/Y}$ recipients in the absence of preconditioning permitted limited engraftment, underscoring the importance of further genetic manipulation, and optimized preconditioning (not shown). The porcine SCID model described here therefore represents an essential step toward the translational evaluation of human stem cells for long-term clinical applications.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2012.04.021.

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(I) Identification of immune subsets in recipient *Il2rg*$^{-/Y}$ male, #610, 12 weeks posttransfer as CD45RA$^+$CD3$^-$ B cells, CD3$^+$CD45RA$^+$ naive T cells, CD3$^+$CD45RA$^-$ memory T cells, and CD3$^-$CD16$^+$ NK cells among nonmyeloid and myeloid cells.

(J) Representative microsatellite PCR analyses to distinguish between donor and recipient DNA illustrated by the discriminatory marker (SW1263 for #610, SWR1367 for # 113, and SW24 for #122).

(K) Changes of IgG (left), IgA (middle), and IgM levels in serum from recipients (red lines) and wild-type controls (blue lines).

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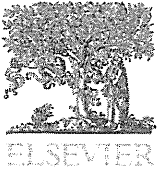

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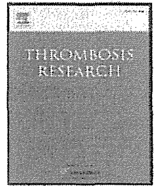

Regular Article

Lack of association between serum paraoxonase-1 activity and residual platelet aggregation during dual anti-platelet therapy

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High residual platelet aggregability during thienopyridine treatment occurs because of low levels of the active drug metabolite, and is associated with an increased rate of major adverse cardiovascular events. Recent findings suggest that paraoxonase-1 (PON1) is a major determinant for clopidogrel efficacy. The aim of this study was to assess the impact of serum PON1 activity on platelet aggregability in thienopyridine-treated patients. In 72 patients receiving treatment with aspirin and ticlopidine after acute coronary syndrome, various laboratory data including the formation of platelet aggregations induced by agonists were compared with serum PON1 activities, measured as paraoxonase and homocysteine thiolactone hydrolase (HTLase). Serum paraoxonase activity was significantly associated with HTLase activity ($R=0.4487$, $P<0.0001$). These PON1 activities were not correlated with any parameters for platelet aggregation, hypertension, sleep apnea, and diabetes mellitus. In contrast, serum PON1 activities seemed to be involved in cardiac function, with brain natriuretic peptide and ejection fraction being significantly correlated with serum HTLase activity ($R=-0.2767$, $P=0.0214$) and paraoxonase activity ($R=0.2558$, $P=0.0339$), respectively. Paraoxonase activity also demonstrated a significant association with increased levels of ankle-brachial index ($R=0.267$, $P=0.0255$). Serum PON1 activities did not influence platelet aggregability during treatment with thienopyridine. However, they might modulate cardiac function after acute coronary syndrome and progression of atherosclerosis.

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Introduction

The concept of antiplatelet resistance, particularly poor responsiveness to thienopyridine, has received increasing attention in recent years because of its reported involvement in cardiovascular events after percutaneous coronary artery intervention (PCI) [1-3]. Thienopyridines such as clopidogrel and ticlopidine are rapidly absorbed prodrugs, and must therefore be converted to an active metabolite to exert their inhibitory actions at the target $P2Y_{12}$ ADP nucleotide receptor on platelets. This conversion is *via* a two-step process involving the hepatic cytochrome P450 (CYP) enzyme pathway [4]. Resistance to clopidogrel was thought to result mainly from decreased CYP function leading to reduced active metabolite production [4]. Indeed, individuals carrying the loss-of-function polymorphism of the *CYP2C19* allele had significantly lower levels of the active metabolite of clopidogrel, and a higher rate of major adverse cardiovascular events [5,6]. Drug interaction with the CYP2C19 inhibitor, omeprazole, might also reduce the production of active metabolites [7,8].

Very recently, it was reported that paraoxonase-1 (PON1) is a major and essential factor in the production of active metabolites from clopidogrel [9]. PON1 hydrolyses 2-oxoclopidogrel (an oxidative metabolite of clopidogrel) to form the final active metabolite, a thiol derivative of clopidogrel (Supplemental Fig. 1) [9]. PON1 is a high-density lipoprotein-associated enzyme that prevents oxidative modification of low-density lipoprotein [10]. The *PON1* genotype (Q192 allele) has significant dose-dependent associations with decreased levels of serum PON1 activity and with increased levels of oxidative stress [11]. PON1 has multiple enzyme activities including paraoxonase, arylesterase, and thiolactonase (Supplemental Fig. 1). Although the full range of endogenous substrates hydrolysed by PON1 remains to be elucidated, PON1 has been shown to produce homocysteine from homocysteine thiolactone *via* its homocysteine thiolactone hydrolase (HTLase) activity [12].

Abbreviations: PON1, paraoxonase-1; HTLase, homocysteine thiolactone hydrolase; PCI, percutaneous coronary artery intervention; CYP, cytochrome P450; BNP, brain natriuretic peptide.

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We have previously investigated the mechanisms and clinical backgrounds that determine residual platelet aggregability, and attempted to ascertain whether platelet aggregability is involved in systemic thrombogenicity during dual antiplatelet therapy [13]. Using this previous population, we have retrospectively measured actual serum PON1 activities, measured as paraoxonase and HTLase, in 72 patients treated with ticlopidine and aspirin, and assessed the correlation between PON1 and platelet aggregability.

Methods

Patients

The institutional review board approved all study protocols, and informed consent was obtained from all participants. The design and protocol of this study has been described previously [13]. Briefly, we enrolled consecutive hospitalized patients from July 2006 to April 2007 who were treated by PCI because of symptomatic coronary artery disease. After normalization of cardiac enzymes, patients underwent blood sampling, ankle-brachial index monitoring and cardiorespiratory monitoring.

Blood collection and platelet aggregation

Platelet aggregation was assessed as described previously [14]. A fasting venous sample was carefully collected, and platelet-rich plasma was obtained by centrifugation. The aggregation response was measured based on the light scattering intensities obtained with a PA-200 platelet aggregation analyzer (Kowa Co. Ltd., Tokyo, Japan). This device is particularly sensitive for detecting and classifying the size of platelet aggregates (small, medium, and large) [14]. Platelet aggregation was stimulated with collagen (Hormon-Chemie, Munich, Germany), ADP (MC Medical Co., Tokyo, Japan) and thrombin receptor-activating peptide (TRAP; Invitrogen Co., Carlsbad, CA), a specific agonist for protease activating receptor-1. Blood samples (serum and plasma) were stored at −80 °C until analysis.

Laboratory testing

Plasma levels of plasminogen activator inhibitor-1 antigen, D-dimer, E-selectin and soluble fibrin were assayed using an automated latex agglutination assay (LPIA-S500; Mitsubishi Chemical Medience Co., Tokyo, Japan) based on conjugated monoclonal antibodies. The concentrations of brain natriuretic peptide (BNP) (ShionoRIA BNP kit; Shionogi USA, Inc. Florham Park, NJ) were measured by SRL Inc. (Tokyo, Japan).

Measurement of serum PON1 activities

We quantified paraoxonase and HTLase activities as a measure of serum PON1 activity (Supplemental Fig. 1). Serum paraoxonase activity was measured by using paraoxon as a substrate (Fully Automated Paraoxonase Activity Measurement Kit, Rel Assay Diagnostics, Gaziantep, Turkey). HTLase activity was measured by a hydrolysis of γ-thiobutyrolactone (Alfresa Auto HTLase, Alfresa Pharma Corp., Osaka, Japan). HTLase hydrolyzes the lactone ring of the substrate γ-thiobutyrolactone, producing free thiols that are detected using Ellman's reagent (DTNB; 5,5'-dithiobis (2-nitrobenzoic acid)). Assay reproducibility was high (coefficient of variation was less than 6%).

Statistical analysis

Statistical analyses were performed using Prism v5 (GraphPad software, Inc, La Jolla, CA). The associations between the individual parameters were calculated using Spearman's correlation method. All reported P values are two-sided; a P value of less than 0.05 was considered to indicate statistical significance.

Results

Patients

Of the 85 patients from our previous study, we selected 72 patients taking 100 mg / day of aspirin and 200 mg / day of ticlopidine after acute coronary syndrome. Base line characteristics of the study population are summarized in Table 1.

Lack of correlation of serum PON1 activities with platelet aggregation

We initially examined serum PON1 activities (measured by paraoxonase and HTLase activity). As show in Fig. 1, serum HTLase activity, but not paraoxonase activity, appeared to be normally distributed across the study population (HTLase: 130.3 ± 36.7 U/L; paraoxonase: 62.65 ± 25.27 U/L). These PON1 activities were significantly correlated ($R = 0.4487$, $P < 0.0001$). To examine whether serum PON1 activities determine platelet aggregability during dual antiplatelet therapy, serum PON1 activities were compared with several parameters of platelet aggregation. However, none of these parameters was significantly associated with PON1 activities (Fig. 2 and Table 2).

Correlation between serum PON-1 activities and cardiac function

We next compared serum PON1 activities with parameters for hypertension, sleep apnea, diabetes mellitus, hyperlipidemia, blood coagulation, arteriosclerosis, and cardiac dysfunction. Using linear regression analysis, we determined that only HDL cholesterol and BNP were correlated with HTLase activity (Table 3). Paraoxonase activity was associated with triglyceride, D-dimer, ankle-brachial index, and ejection fraction (Table 3). The medication including use of diuretics, angiotensin II receptor blocker, angiotensin converting enzyme inhibitor, beta blocker, calcium channel blocker, or statin did not demonstrate a significant association with serum PON1 activities (Supplemental Table 1). These data suggest that decreased levels of PON1 activity might lead to the acceleration of atherosclerosis and cardiac dysfunction after acute coronary syndrome.

Table 1
Characteristics of the study population.

Variables	Total subjects (n = 72)
Age, years	62.15 ± 11.62
Men, n (%)	57 (80)
BMI, kg/m^2	25.11 ± 3.514
Systolic blood pressure (mmHg)	125.3 ± 21.08
Diastolic blood pressure (mmHg)	76.6 ± 11.28
Pulse rate (/min)	72.39 ± 14.59
Blood sugar (mg/dl)	118.3 ± 50.43
HbA1c (%)	6.76 ± 1.891
Triglyceride (mg/dl)	130.2 ± 53.01
Total cholesterol (mg/dl)	167.7 ± 36.77
LDL cholesterol (mg/dl)	100.4 ± 30.35
HDL cholesterol (mg/dl)	41.3 ± 12.72
CPK max (U/L)	2,194 ± 2,211
BNP (pg/ml)	151.4 ± 183.6
Concomitant medications	
Antiplatelet agents, n (%)	
Aspirin + Ticlopidine	72 (100)
Antihypertensive medication, n (%)	66 (91.7)
Statin, n (%)	55 (76.4)
NSAIDs, n (%)	0 (0)

Data for continuous variables are expressed as the mean ± SD. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BNP, brain natriuretic peptide; NSAID, non-steroidal anti-inflammatory drug.

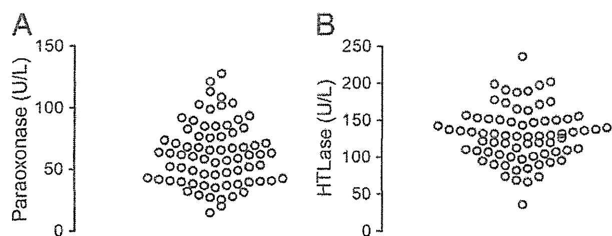

Fig. 1. Serum paraoxonase and HTLase activities in the study population.

Discussion

Inhibition of the $P2Y_{12}$ nucleotide receptor, an ADP receptor on platelets, is currently the gold-standard therapy for the prevention of ischemic events in patients undergoing PCI [15,16]. Although the second-generation thienopyridine, clopidogrel, is recommended by a number of current clinical guidelines, the inter-individual variability of its efficacy is a major drawback in its clinical use [17]. Better understanding of this variability in the efficacy of clopidogrel and other thienopyridines is vital at a time when the number of PCIs is increasingly rapidly. The loss-of-function polymorphism of the CYP2C19 allele has attracted attention as a potential factor in clopidogrel efficacy [4-6], while an elegant recent study suggested that PON1 is a major determinant in the production of the final active metabolite of clopidogrel [9]. In this study, we measured serum two PON1 activities in acute coronary syndrome and compared them with platelet aggregability in patients receiving dual antiplatelet therapy. We could identify no correlation between PON1 activities and any parameter for platelet aggregation in our population.

Several explanations may exist for the discrepancy between our result and the previous report. First, genetic divergence between Caucasian and Japanese patients might affect the result. The Japanese population is reported to express predominantly the 192R allele of *PON1* (192QQ: 18.2%; 192QR: 40.9%; 192RR: 40.9%) [18], whereas the Caucasian population in a large cohort study tended to express the 192Q variant (192QQ: 46.3%; 192QR: 43.9%; 192RR: 9.8%) [11]. The Q allele of *PON1* genotype was significantly and dose-dependently associated with decreased serum PON1 activity, whereby 192QQ, 192QR and 192RR had comparatively low, intermediate and high PON1 activity, respectively [11]. In contrast, the frequency of polymorphism for *CYP2C19*, a key enzyme in clopidogrel oxidation, varies among races, with loss-of-function polymorphisms reportedly being more common in Asian patients [19,20]. However, even in a genetically homogenous population, the *CYP2C19* allele was reported to account for only 12% of the variability in clopidogrel efficacy, whereas the *PON1*

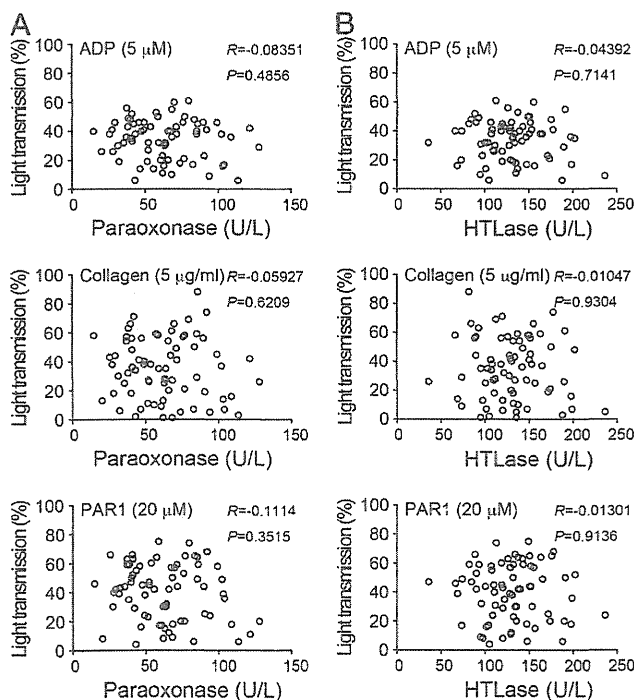

Fig. 2. Association between PON1 activities and platelet aggregation. Platelet aggregation induced by 5 μM ADP, or 5 μg / ml of collagen, or 20 μM TRAP was assessed by aggregometry, and was expressed as light transmission (%). Serum paraoxonase activities (U / L) (A) and HTLase activities (U / L) (B) were compared with platelet aggregation using Spearman's rank correlation coefficient.

Table 2
Correlation between serum paraoxonase activities and platelet aggregation.

	Paraoxonase		HTLase	
	R	*P* value	*R*	*P* value
ADP 2 μM-LT	−0.1252	0.2945	−0.03844	0.7486
ADP 2 μM-Small	−0.2083	0.0791	−0.07811	0.5143
ADP 2 μM- Med	−0.1335	0.2636	−0.01776	0.8823
ADP 2 μM-Large	−0.03798	0.7514	0.085	0.4777
ADP 5 μM-LT	−0.08351	0.4856	−0.04392	0.7141
ADP 5 μM-Small	−0.2212	0.0619	−0.09755	0.415
ADP 5 μM- Med	−0.2317	0.0501	−0.1055	0.3776
ADP 5 μM-Large	−0.1406	0.2389	−0.06589	0.5824
Coll 1 μg/ml-LT	−0.1072	0.37	0.02695	0.8222
Coll 1 μg/ml-Small	−0.1524	0.2012	0.06594	0.5821
Coll 1 μg/ml- Med	−0.1174	0.3262	0.04772	0.6906
Coll 1 μg/ml-Large	−0.00214	0.9857	0.04327	0.7182
Coll 5 μg/ml-LT	−0.05927	0.6209	−0.01047	0.9304
Coll 5 μg/ml-Small	−0.1489	0.212	−0.1001	0.4029
Coll 5 μg/ml- Med	−0.1269	0.2881	−0.03555	0.7669
Coll 5 μg/ml-Large	−0.1113	0.352	0.007927	0.9473
TRAP 20 μM-LT	−0.1114	0.3515	0.01301	0.9136
TRAP 20 μM -Small	−0.1585	0.1835	−0.1742	0.1434
TRAP 20 μM - Med	−0.09187	0.4427	−0.04854	0.6855
TRAP 20 μM -Large	−0.05235	0.6623	0.03127	0.7943

LT, light transmission; Small, small aggregates; Med, medium aggregates; Large, Large aggregates; Coll, collagen; TRAP, thrombin receptor-activating peptide (SFLLRN). *$P<0.05$.

Table 3
Correlation between paraoxonase activities and other laboratory data.

	Paraoxonase		HTLase	
	R	*P* value	*R*	*P* value
SBP	−0.0953	0.443	−0.118	0.3417
DBP	−0.121	0.3294	−0.00797	0.949
HR	−0.1096	0.3774	0.02895	0.8161
AHI	0.06843	0.5735	0.05563	0.6474
Blood suger	−0.06266	0.609	0.06714	0.5836
HbAlc	−0.08087	0.5121	−0.09806	0.4263
Triglyceride	0.2958	0.0129*	0.00273	0.9821
Total cholesterol	0.01199	0.9215	0.1166	0.3365
LDL cholesterol	−0.0158	0.8975	0.05602	0.6475
HDL cholesterol	−0.01299	0.915	0.2646	0.0269*
PAI-1	−0.08214	0.4928	0.01523	0.899
E-selectin	−0.0007075	0.9953	−0.1618	0.1746
Soluble Fibrin	−0.03224	0.788	−0.08483	0.4787
D-dimer	−0.2348	0.0471*	−0.2229	0.0598
max CPK	−0.1691	0.1616	−0.02521	0.8359
BNP	−0.1306	0.2849	−0.2767	0.0214*
Pulse wave velocity	−0.1665	0.1682	−0.04456	0.7141
ABI	0.267	0.0255*	−0.01957	0.8722
Ejection fraction	0.2558	0.0339*	0.1632	0.1803

SBP, systolic blood pressure; DBP, diastolic blood pressure; AHI, apnea-hypopnea index; LDL, low-density lipoprotein; HDL, high-density lipopurotein; PAI-1, plasminogen activator inhibitor-1; BNP, brain natriuretic peptide; ABI, ankle-brachial index. *$P<0.05$.

Q192R polymorphism was estimated to be responsible for 72.5% of the variability in ADP-stimulated platelet aggregation after clopidogrel administration [9]. It is therefore important that we clarify which polymorphism combinations (of *PON1* and *CYP2C19*) are the most relevant in the metabolism of thienopyridines in our population.

The first-generation thienopyridine, ticlopidine, was used instead of clopidogrel in our study because ticlopidine was the only approved drug for acute coronary syndrome in Japan during our study period. We acknowledge the possibility that the rate-limiting enzyme for ticlopidine metabolism to its active metabolite may differ from that of clopidogrel. All thienopyridines including ticlopidine, clopidogrel, and prasugrel are prodrugs that need to be converted into active metabolite through the formation of thiolactone metabolites (2-oxo-ticlopidine, 2-oxo-cloipdogrel, and prasugrel thiolactone, respectively (see Supplemental Fig. 1)) [4]. The free active thiol of these active metabolites forms disulfide bonds with, and therefore binds irreversibly to, cysteine residues Cys17 and Cys270 of $P2Y_{12}$ [21]. It is of great importance, therefore, to understand whether thiolactone metabolites of all thienopyridines are hydrolyzed mainly by PON1, or are instead oxidized by CYP.

We found correlations between PON1 activities and cardiac function in our study population. PON1 has a protective effect against oxidation of lipoproteins, and a *PON1* polymorphism (the 192Q allele) that produces decreased levels of PON1 activity was associated with systemic oxidative stress and higher rates of major cardiovascular events [11]. It is possible that decreased levels of PON1 activities enhance the progression of atherosclerosis in the coronary artery, resulting in decreased cardiac function after acute coronary syndromes. Indeed, reduced paraoxonase activity was significantly associated with a decreased ankle-brachial index in our study. Further studies are needed to assess the possible mechanisms and biological effect of PON1, particularly the severity of its effects on cardiac function after coronary artery disease.

Some limitations in this study merit discussion. First, we could assess platelet function testing in the patients treated with ticlopidine, but not clopidogrel. We cannot exclude the possibility that results may differ with other thienopyridines, as described above. In addition, we assessed only the correlation between serum PON1 activities and platelet response to ticlopidine, and we did not assess gene polymorphisms. Although it is accepted that serum PON1 activities are determined by *PON1* polymorphism, more data regarding genetic variation in *CYP* and *PON1* may have extended our findings relating to the mechanism(s) of the platelet response during dual antiplatelet therapy. Finally, the analysis reported here is *post hoc* analysis of a previously reported population and the number of participants is limited. We previously estimated that at least 62–85 participants would be required for the study ($\alpha = 0.05$, $\beta = 0.20$, and expected correlation coefficient, $R = 0.30$–0.35) [13]. Weak association due to β-error may affect the strength of any conclusions based on these data.

Conclusions

The current study has demonstrated that serum PON1 activities did not influence platelet aggregation in patients receiving thienopyridine treatment, but was involved in cardiac function. Our data suggest the need for a re-evaluation of the importance of PON1 (and/or CYP) in the production of active metabolites from thienopyridines. We may also need to consider how expression of the rate-limiting enzymes for thienopyridine metabolism differs between individual drugs and racial populations. During the preparation of this article, it was reported that no association exists between *PON1* genotype and platelet response to clopidogrel and stent thrombosis in a *post hoc* analysis of prospective studies [22]. Further large-scale prospective studies are required to determine which enzyme (PON1 or CYP) is critical for the production of active metabolites from thienopyridines, and therefore for cardiovascular events during thienopyridine administration.

Supplementary materials related to this article can be found online at doi:10.1016/j.thromres.2011.10.033.

Conflict of interests statement

T.O. has received financial support from Daiichi Sankyo. The other authors declare that they have no competing interest.

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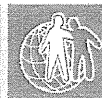
Haemophilia



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ORIGINAL ARTICLE *Laboratory science*

Immune response against serial infusion of factor VIII antigen through an implantable venous-access device system in haemophilia A mice

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Summary. Haemophilia A is a life long bleeding disorder caused by an inherited deficiency of factor VIII (FVIII). About 30% of haemophilia A patients develop neutralizing antibodies as a consequence of treatment with FVIII concentrates. Immune tolerance protocols for the eradication of inhibitors require daily delivery of intravenous FVIII. We evaluated the immune responses to serial intravenous administration of FVIII in preimmunized haemophilia A mice. We introduced an implantable venous-access device (iVAD) system into haemophilia A mice to facilitate sequential infusion of FVIII. After preimmunization with FVIII, the haemophilia A mice were subjected to serial intravenous administration of FVIII through the iVAD system. In all mice with serial infusion of FVIII, high titers of anti-FVIII inhibitory antibodies developed at 10 exposure days (EDs). However, the anti-FVIII IgG titers were decreased after 150 EDs of sequential low-dose infusion of FVIII [0.05 U g^{-1} body weight (BW) five times per week]. Proliferative response to *ex vivo* FVIII stimulation was significantly suppressed in splenic $CD4^+$ T cells from mice with serial low-dose FVIII infusion compared with those from mice with high-dose FVIII infusion (0.5 U g^{-1} BW five times per week) or preimmunized mice. Moreover, splenic $CD4^+$ T cells from mice with serial low-dose infusion of FVIII failed to produce interleukin-2 and interferon-γ. These data suggest that serial infusion of FVIII could induce T-cell anergy in haemophilia A mice with inhibitor antibodies.

Keywords: anergy, factor VIII, haemophilia A mice, inhibitor, venous-access device

Introduction

Haemophilia A is a life-long bleeding disorder caused by an inherited deficiency of factor VIII (FVIII) because of mutations in the FVIII gene [1]. About 30% of severe haemophilia A patients who received replacement therapy with intravenous FVIII products develop neutralizing antibodies that inhibit the function of substituted FVIII [2,3]. Once an inhibitor develops, treatment of bleeding episodes is quite difficult due to partial or complete lack of efficacy of replacement therapy. Immune tolerance induction (ITI) therapy using regular applications of FVIII is the only strategy that has been proven successfully to combine eradication of FVIII inhibitors and induction of FVIII-specific immune tolerance [2,4].

Central venous-access devices (VADs) are often used in haemophiliacs undergoing ITI to overcome difficulties of regular venous puncture [5,6]. The fully implantable devices offer many advantages compared with external catheters, because they generally have longer useful duration with lower rate of infectious complication and cannot be accidentally displaced [7]. Although ITI approach was introduced several decades ago, little is known about the immunological mechanisms that cause down-modulation of FVIII-specific immune responses and the induction of long-lasting immune tolerance against FVIII.

In this study we introduced an implantable VAD (iVAD) system into haemophilia A mice to facilitate serial intravenous infusion of FVIII and evaluated immune responses against FVIII in preimmunized haemophilia A mice. We demonstrated that sequential administration of FVIII through the iVAD system could induce T-cell anergy in adult haemophilia A mice with inhibitors.

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Methods

Animal models

Haemophilia A mice (B6; $_{129}S_4$-$F8^{tmlKaz}$/J) with targeted destruction of exon 16 of the FVIII gene were kindly provided by Dr H.H. Kazazian Jr (University of Pennsylvania, Philadelphia, PA, USA) [8]. All mice were housed and used in a pathogen-free facility at Jichi Medical University, Shimotsuke, Tochigi, Japan. All animal experiments were performed in accordance with regulations of the Japanese Council for Animal Care; Jichi Medical University Animal Care Committee approval all animal protocols.

iVAD system and intravenous injection of FVIII

Haemophilia A mice aged 12 weeks were anaesthetized by inhalation with 2.5% isoflurane in the anaesthesia unit (Univentor, ZTN 08, Malta). An iVAD system (SoloPort; Instech Laboratories, Plymouth Meeting, PA, USA) was placed into a pocket of back skin in the chest wall of each animal (Fig. 1). The catheter was then tunneled under the skin and introduced into the superior vena cava through a cut-down site of jugular vein under a zoom stereomicroscope (Nikon, Tokyo, Japan). The entire system was flushed with saline solution after insertion. Mice were infused with intravenous recombinant human FVIII formulated with sucrose (Kogenate FS; Bayer Healthcare, Leverkusen, Germany) through the iVAD system.

Assay for FVIII inhibitors

Inhibitory FVIII antibodies were measured according to the Bethesda assay [9]. In brief, mouse plasma was serially diluted in Owren's Veronal Buffer (Dade Behring, Deerfield, IL, USA), such that the remaining FVIII activity for each sample was between 25% and 75%, and mixed 1:1 with normal pooled human plasma at 37°C for 2 h. Residual human FVIII activity was measured by one-stage assay using 50 μL of FVIII-deficient human plasma (Kokusai-Shiyaku, Kobe, Japan) and a 50-μL sample from the previous incubation on a automated coagulometer (CA-500; Sysmex, Kobe, Japan). One BU mL^{-1} was defined as the dilution of plasma containing FVIII inhibitory activity that results in 50% inhibition of FVIII activity.

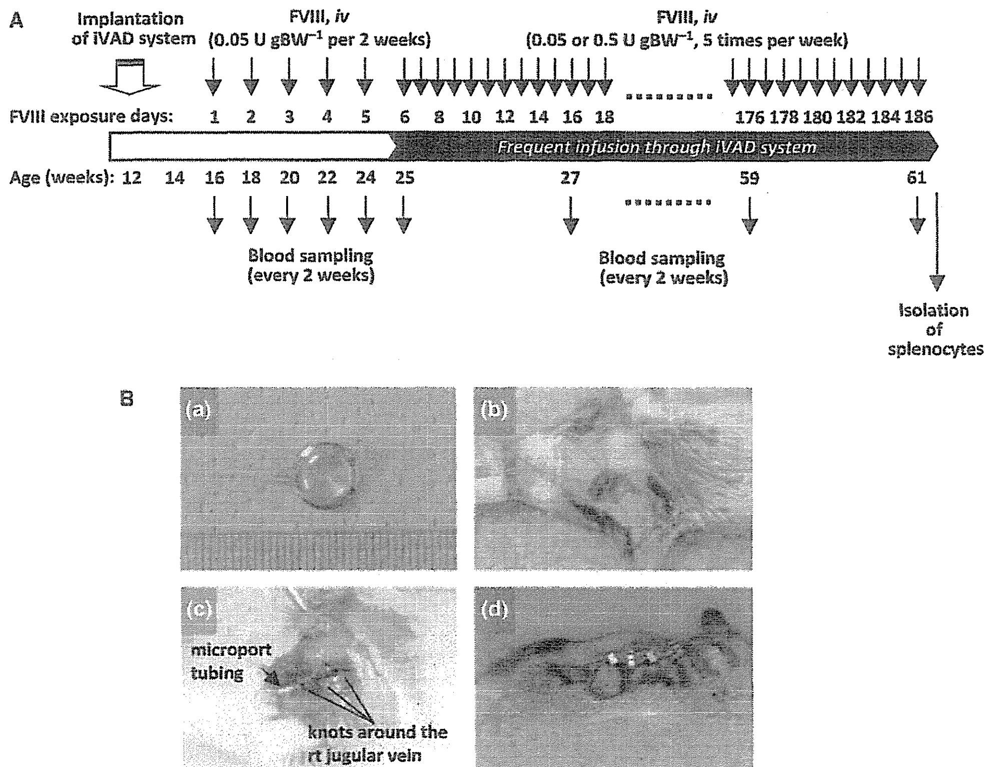

Fig. 1. Protocol for serial intravenous administration of FVIII through an implantable venous access device (iVAD) system in haemophilia A mice. A. Haemophilia A mice were implanted an iVAD system at age 12 weeks, then they were preimmunized with intravenous injection of 0.05 U g^{-1} BW recombinant human FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently administered with FVIII (0.05 or 0.5 U g^{-1} BW, five times per week) through the iVAD system. B. (a) The iVAD system consists of a stainless steel port with a molded silicon rubber and an 1.2 Fr catheter. After incision of the cervical skin in a haemophilia A mouse, the jugular vein was exposed (b), then the catheter was inserted into superior vena cava by cut-down procedure (c). The other side of the catheter was tunneled to the port that was set beneath the pocket of back skin (d).

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Anti-FVIII measurement

Anti-FVIII IgG concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in microtiter wells (Nunc, Roskilde, Denmark) coated with 1 μg mL^{-1} recombinant human FVIII. After blocking with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), serial dilutions of murine plasma were added at 4°C for 16 h. Each well was washed with 0.5% BSA in PBS containing 0.05% Tween-20. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA) was added at 37°C for 1 h. ABTS Microwell substrate (KPL, Gaithersburg, MD, USA) was added, and the absorbance at 405 nm was read. Anti-FVIII antibody concentrations were calculated from the linear portion of a standard curve obtained using antihuman FVIII monoclonal antibodies (kindly provided by Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan).

Determination of anti-FVIII IgG subclasses

Microtiter wells were coated with 1 μg mL^{-1} recombinant human FVIII in PBS for 16 h at 4°C. After blocking with 5% BSA in PBS, serial dilutions of murine plasma were added for 2 h at 37°C. The wells were washed with 0.5% BSA in PBS containing 0.05% Tween-20. The IgG1, IgG2a, IgG2b and IgG3 subtypes of anti-FVIII antibodies bound to immobilized human FVIII were determined by incubation with isotype-specific rabbit anti-mouse IgGs (Mouse Typer; BioRad, Hercules, CA, USA) for 1 h at 37°C. After washing with 0.5% BSA in PBS containing 0.05% Tween-20, the wells were incubated with goat anti-rabbit HRP conjugate for 1 h at 37°C. Substrate development was performed for 15 min at 25°C, using ABTS Microwell substrate as described above.

Proliferation assay with [^{3}H]-thymidine incorporation

Mice splenic $CD4^+$ T cells were prepared by depletion of non-$CD4^+$ T cells with the autoMACS cell sorting system (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Antigen-presenting cells were prepared from mice splenocytes by depletion of T cells using the magnetic sorting system with anti-CD90 (Thy1.2)-conjugated microbeads (Miltenyi Biotech) followed by irradiation with a single dose of 20 Gy (Gamma Cell; Norton International, ON, Canada), to prevent non-specific proliferative responses during the *in vitro* FVIII stimulation assay. To measure T-cell proliferation, 3×10^5 $CD4^+$ T cells per well were cultured with 0–3 nM human recombinant FVIII in the presence of antigen-presenting cells derived from FVIII-immunized mice (five times injection of 0.05 U g^{-1} BW FVIII, every 2 weeks) at 37°C for 72 h in complete RPMI-1640 (Gibco BRL, Rockville, MD, USA). [^{3}H]-Thymidine (Amersham Bioscience, Uppsala, Sweden) was added (0.037 MBq per well) at 37°C for 18 h. Then, cells were harvested, and [^{3}H]-thymidine incorporation was determined by scintillation counting (Top count; Packard, Meriden, CT, USA).

Cytokine assays

Splenocytes were incubated in 24-well plates at 1.0×10^6 cells per well in the absence or presence of 3 nM human recombinant FVIII at 37°C in 5% CO_2. Production of interleukin (IL)-2, IL-4, IL-12 and interferon (IFN)-γ by $CD4^+$ T cells derived from each mouse was analyzed at 72 h by ELISA kits (Biotrak ELISA System; Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. In addition, levels of IL-10 were measured at 96 h by ELISA system (Biotrak ELISA System).

Statistical analysis

Data are expressed as mean ± SE. Normally distributed variables were compared by Student's *t*-test. Variables not normally distributed were analyzed by two-sided Mann–Whitney U test. The data were considered statistically significant at P values < 0.05.

Results

Serial intravenous administration of FVIII through an iVAD system in preimmunized haemophilia A mice

We securely implanted venous access devices into haemophilia A mice at 12 weeks using a zoom microscopy, therefore, we could avoid using FVIII concentrates for haemostatic control during the procedure (Fig. 1B). After the operation-related wounds had healed, we developed immunized mice against FVIII by intravenous injection of FVIII (0.05 U g^{-1} BW) at 2-week intervals. Titers of anti-FVIII inhibitory antibodies of the mice were elevated to 100–400 BU mL^{-1} after the fifth exposure of FVIII. Thereafter, we performed serial infusion of FVIII into the preimmunized haemophilia A mice through the venous access device system. High titers (>2000 BU mL^{-1}) were developed after 10 exposure days (EDs) in mice with administration of FVIII (0.05 U g^{-1} BW five times per week) and were continued over 100–120 EDs. However, after 130–150 EDs their titers were gradually decreased despite continuing sequential stimulation of FVIII (Fig. 2a). One of the five mice was discontinued at 140 EDs because of bleeding from the site of catheter insertion (Fig. 2a; LD#3). In contrast, > 2000 BU mL^{-1} of anti-FVIII inhibitory antibodies were sustained over

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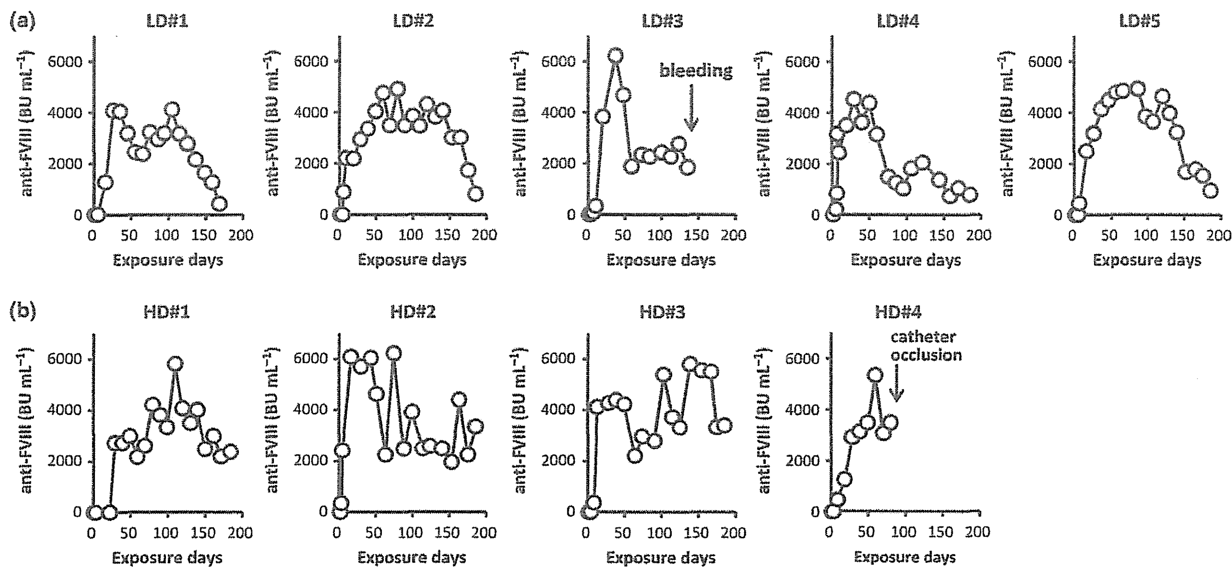

Fig. 2. Effect of serial intravenous administration of FVIII on anti-FVIII inhibitory antibody formation in preimmunized haemophilia A mice. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently administered with FVIII [(a) 0.05 U g^{-1} BW five times per week; (b) 0.5 U g^{-1} BW, five times per week] through the iVAD system. The mice were bled at every 2 weeks, and their anti-FVIII inhibitor titers were determined by Bethesda assay.

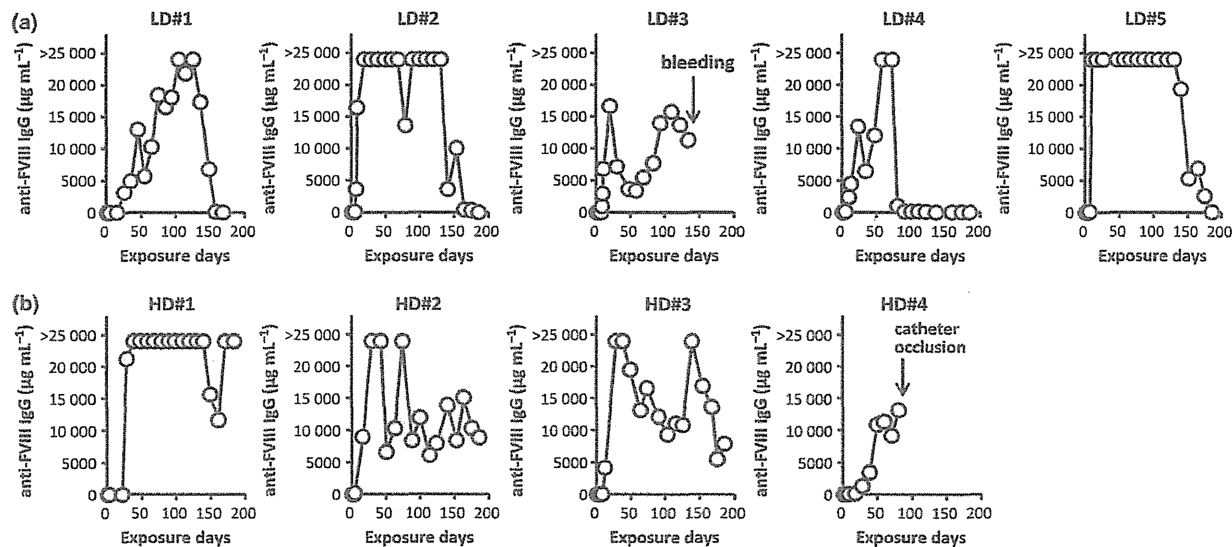

Fig. 3. Effect of repeated intravenous infusion of FVIII on FVIII-specific IgG formation in haemophilia A mice with inhibitors. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were repeatedly infused with FVIII [(a), 0.05 U g^{-1} BW, five times per week; (b) 0.5 U g^{-1} BW, 5 times per week. The mice were bled at every two weeks just before each infusion. Plasma levels of FVIII-specific IgG were measured by ELISA as described in Methods.

180 EDs in mice with serial infusion of high-dose FVIII (0.5 U g^{-1} BW five times per week) (Fig. 2b).

Effect of serial intravenous infusion of FVIII on FVIII-specific IgG and subclasses formation

Anti-FVIII IgG was detectable immediately after serial infusion of FVIII (0.05 U g^{-1} BW five times per week) in preimmunized haemophilia A mice, and were persisted for more than 80–100 EDs (Fig. 3a). Interestingly, titers against FVIII were markedly decreased after 80–150 EDs. By contrast, preimmunized mice followed by serial intravenous infusion of high-dose FVIII (0.5 U g^{-1} BW five times per week) showed high titer of anti-FVIII IgG over 150–180 EDs (Fig. 3b). One of four mice receiving sequential high-dose FVIII infusion was discontinued due to occlusion of central vein catheter at 80 EDs (Fig. 3b; HD#4). All IgG isotypes of anti-FVIII IgG

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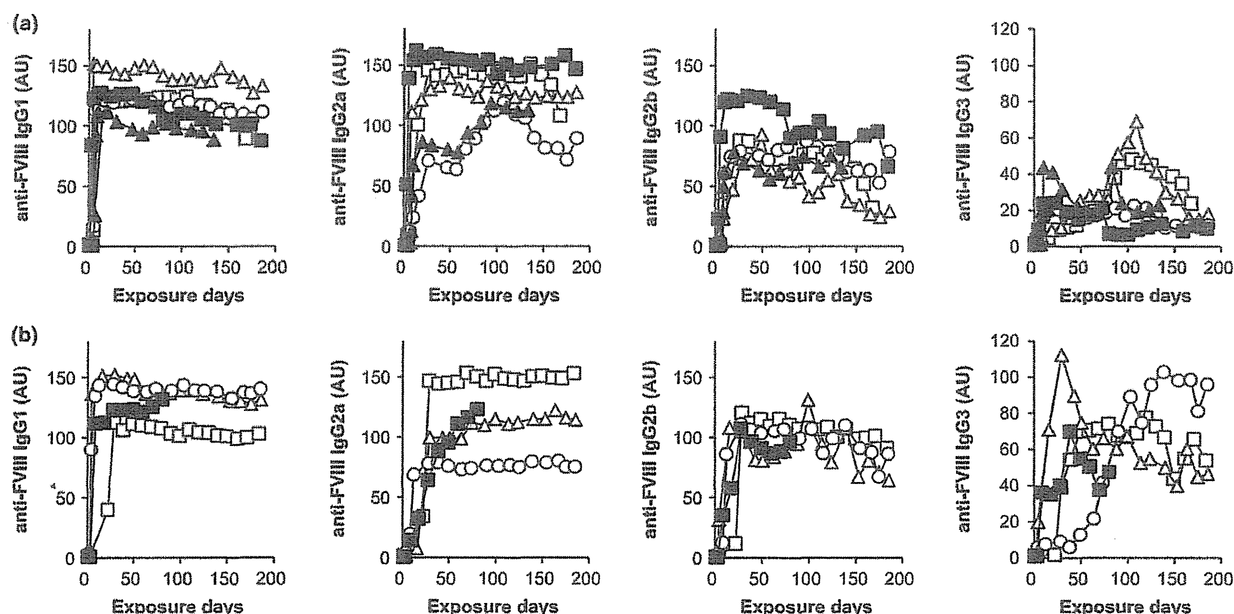

Fig. 4. Effect of serial intravenous injection on anti-FVIII IgG subclasses formation in preimmunized haemophilia A mice. (a) Haemophilia A mice (LD1, open squares; LD#2, open triangles; LD#3, closed triangles; LD#4, closed squares; LD#5, open circles) were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were repeatedly infused with FVIII (0.05 U g^{-1} BW five times per week). (b) Haemophilia A mice (HD#1, open squares; HD#2, open triangles; HD#3, open circles; HLD#4, closed squares) were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were repeatedly infused with FVIII (0.5 U g^{-1} BW five times per week). Each of the mice was bled at every two weeks just before FVIII infusion. Titers of IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) were determined by ELISA as described in Methods.

antibodies were rapidly increased after serial infusion of 0.05 U g^{-1} and 0.5 U g^{-1} BW of FVIII (Fig. 4). However, in mice with repeated administration of 0.05 U g^{-1} BW FVIII titers of IgG3 subclass antibodies were decreased after 80–100 EDs (Fig. 4b).

Effect of serial administration of FVIII on anti-factor VIII CD4⁺ T cells proliferation

Next, we evaluated whether serial infusion of FVIII exerts a suppressive effect on FVIII-specific T cells, $CD4^+$ T cells obtained after the final injection were assayed for a T-cell proliferative response to FVIII. We observed a dose-dependent $CD4^+$ T-cell proliferative response to FVIII in preimmunized mice (five times injection of FVIII every two weeks, Fig. 5). In the group with sequential infusion of 0.5 U g^{-1} BW FVIII the T cells significantly proliferated in response to FVIII stimulation. By contrast, no response was observed at any FVIII dose in $CD4^+$ T cells from the mice after serial infusion of 0.05 U g^{-1} BW FVIII.

Effect of serial infusion of FVIII on cytokine response

Mice that were immunized with FVIII every two weeks developed splenocytes, which proliferated and produced

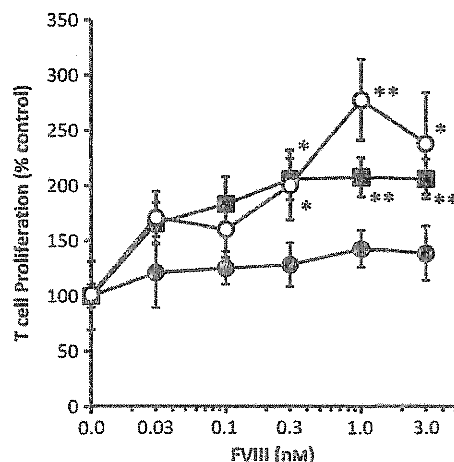

Fig. 5. Effect of repeated administration of FVIII on anti-factor VIII T-cell proliferation of haemophilia A mice. Haemophilia A mice were given intravenous injection of 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently infused with FVIII through the iVAD system. $CD4^+$ T cells of preimmunized mice (n = 5; open circles), mice with infusion of FVIII (0.05 U g^{-1} BW, five times per week; n = 4; closed circles), and mice with injection of FVIII (0.5 U g^{-1} BW, five times per week; n = 3; closed squares) were obtained three days after final immunization. The amount of ^{3}H-thymidine incorporation was measured under *in vitro* stimulation with FVIII (0–3 nM) in the presence of the FVIII-immunized mice-derived antigen-presenting cells by scintillation counting as described in the Methods. Data are means ± SD. *$P < 0.05$; **$P < 0.03$ when compared with the proliferation in the absence of FVIII.

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