Adoptive transfer of CD4$^+$CD25$^+$ regulatory T cells into syngeneic hemophilia A mice

Single-cell suspensions were prepared and pooled from the spleens of hemophilia A mice without intrathymic administration of FVIII (non-IT mice) or those with intrathymic administration of FVIII (FVIII-IT mice). The pooled cells were purified to obtain CD4$^+$CD25$^+$ T-cell populations as described above. A total of 0.5 × 10^6 CD4$^+$CD25$^+$ T cells per body in 100 μL of PBS was injected into syngeneic naïve hemophilia A mice via the jugular vein. Mice were challenged with repeated intravenous stimulation by 0.05 U g^{-1} BW FVIII every 2 weeks, and inhibitory antibody titers were followed over time.

Statistical analysis

Two-tailed unpaired *t*-tests with 95% confidence intervals were performed using SPSS software (SPSS, Chicago, IL, USA). Mean values were considered to be statistically significant if the *P*-values were below 0.05.

Results

Effect of intrathymic administration of FVIII on anti-FVIII inhibitory antibody formation in hemophilia A mice

We performed histologic analysis of hemophilia A mice 1 day after direct thymic injection of human recombinant FVIII. We analyzed thymus sections 5 days after thymic injection with hematoxylin and eosin staining, and confirmed that there was no bleeding or histologic change in thymic structure, similar to what was seen with untreated mice (Fig. 1C). Then, we studied the impact of intrathymic injection of FVIII on the immune response in hemophilia A mice. For this, we administrated FVIII or albumin as control antigen into the thymus of naïve hemophilia A mice, and analyzed anti-FVIII inhibitory antibody formation after repeated intravenous stimulation with FVIII (0.05 U g^{-1} body weight). All non-IT mice developed high titers of anti-FVIII antibodies (n = 22, 122.5 ± 27.6 BU mL^{-1}; Fig. 2B), confirming that human FVIII is highly immunogenic in hemophilic mice [3,19]. In addition, mice that had been given intrathymic injections of human albumin (Alb-IT mice) showed high titers of antibody against FVIII (n = 10, 129.0 ± 40.6 BU mL^{-1}, Fig. 2C). By contrast, FVIII-IT mice had undetectable or low titers of anti-FVIII antibodies (n = 18, 9.4 ± 2.3 BU mL^{-1}; Fig. 2D). Moreover, FVIII-IT mice did not develop of high titers of anti-FVIII antibodies even after the boosted immune challenges with a combination of FVIII and Freund's adjuvant (20.1 ± 8.1 BU mL^{-1}, n = 5; Fig. 2E). As shown in Fig. 2F, mice treated on day 7, day 14 and day 70 developed high titers of anti-FVIII antibodies after the fifth intravenous stimulation with FVIII: 118.3 ± 34.9 BU mL^{-1} (n = 15), 113.9 ± 31.5 BU mL^{-1} (n = 10), and 120.5 ± 37.6 BU mL^{-1} (n = 16), respectively. Several researchers have demonstrated that induction of antigen-specific tolerance by intrathymic inoculation of soluble antigens is dose-dependent, and that an optimal dose of soluble antigen is required to induce antigen-specific unresponsiveness [20,21]. We injected FVIII into the thymus of neonatal hemophilia A mice at variable doses (0.005–0.5 U g^{-1} BW), and followed this with repeated intravenous stimulation with FVIII at 10, 12, 14, 16 and 18 weeks. As shown in Fig. 2G, intrathymic administration of 0.005, 0.05 and 0.5 U g^{-1} BW resulted in lower titers of anti-FVIII inhibitory antibodies (24.5 ± 4.4, 9.4 ± 2.3, and 18.9 ± 4.8 BU mL^{-1}, respectively) than those seen without thymic treatment. Interestingly, mice injected intrathymically with 0.005 U g^{-1} BW of FVIII developed significantly higher titers of anti-FVIII antibodies than those injected with 0.05 U g^{-1} BW of FVIII (P = 0.036), suggesting that there is some dose-dependency in the ability to induce immune tolerance. Taken together, these findings show that the intrathymic injection of FVIII antigen within 3 days after birth minimizes neutralizing antibody formation in FVIII-deficient mice. To determine whether the suppression of antibody against FVIII was specific, FVIII-IT mice and non-IT mice were immunized intraperitoneally with TT vaccine 2 weeks after the final challenge with FVIII. As shown in Fig. 2H, FVIII-IT mice were able to mount a T-cell-dependent immune response to a different antigen, and the antibody response was similar to that in non-IT mice. These results indicated that the immune suppression observed in our mouse model was FVIII-specific.

Fig. 2. Effect of intrathymic administration of FVIII on anti-FVIII inhibitory antibody formation in hemophilia A mice. Normal B6 control mice [(A), n = 5], and hemophilia A mice without thymic treatment [(B), n = 22], or with thymic injection of human albumin [(C), n = 10] or human FVIII [(D), n = 18] within 3 days after birth, were injected intravenously with human FVIII (0.05 U g^{-1} body weight) at 10, 12, 14, 16 and 18 weeks. The anti-FVIII inhibitor titer was determined by Bethesda assay. (E) FVIII-deficient mice with prior intrathymic injection of FVIII were repeatedly stimulated with FVIII and Freund's adjuvant every 2 weeks, and anti-FVIII inhibitor titers were measured. (F) The initial intrathymic injection of FVIII was given within 3 days (n = 22), 7 days (n = 15), 14 days (n = 10) or 70 days (n = 18) after birth. Each mouse was then given repeated intravenous injections at 10, 12, 14, 16 and 18 weeks. The mice were bled on day 4 after the fifth treatment, and anti-FVIII inhibitor titers were measured. Bars show means. $^{**}P$ < 0.03 (G) Zero unit per gram body weight (saline control, n = 18), 0.005 U g^{-1} body weight (saline control, n = 10), 0.05 U g^{-1} body weight (saline control, n = 22) or 0.5 U g^{-1} body weight (saline control, n = 11) of FVIII were injected into the thymus within 3 days after birth, and the mice were then treated with 0.05 U g^{-1} body weight of FVIII at 10, 12, 14, 16 and 18 weeks. The mice were bled on day 4 after the fifth treatment, and anti-FVIII inhibitor titers were measured by Bethesda assay. Bars show means. $^*P$ < 0.05, $^{**}P$ < 0.03 (H) Hemophilia A mice without (n = 14, open bar) or with (n = 15, closed bar) thymic administration of FVIII were given five doses of intravenous FVIII (0.05 U g^{-1} body weight) every 2 weeks. Each mouse was injected intraperitoneally with one Limit of flocculation (Lf) unit per body of tetanus toxoid vaccine 2 weeks after the last challenge with FVIII. Plasma samples were obtained 3 weeks after the tetanus toxoid injection. Anti-tetanus toxoid antibody titers were measured by enzyme-linked immunosorbent assay as described in Materials and methods. Data are shown as the means ± standard deviations.

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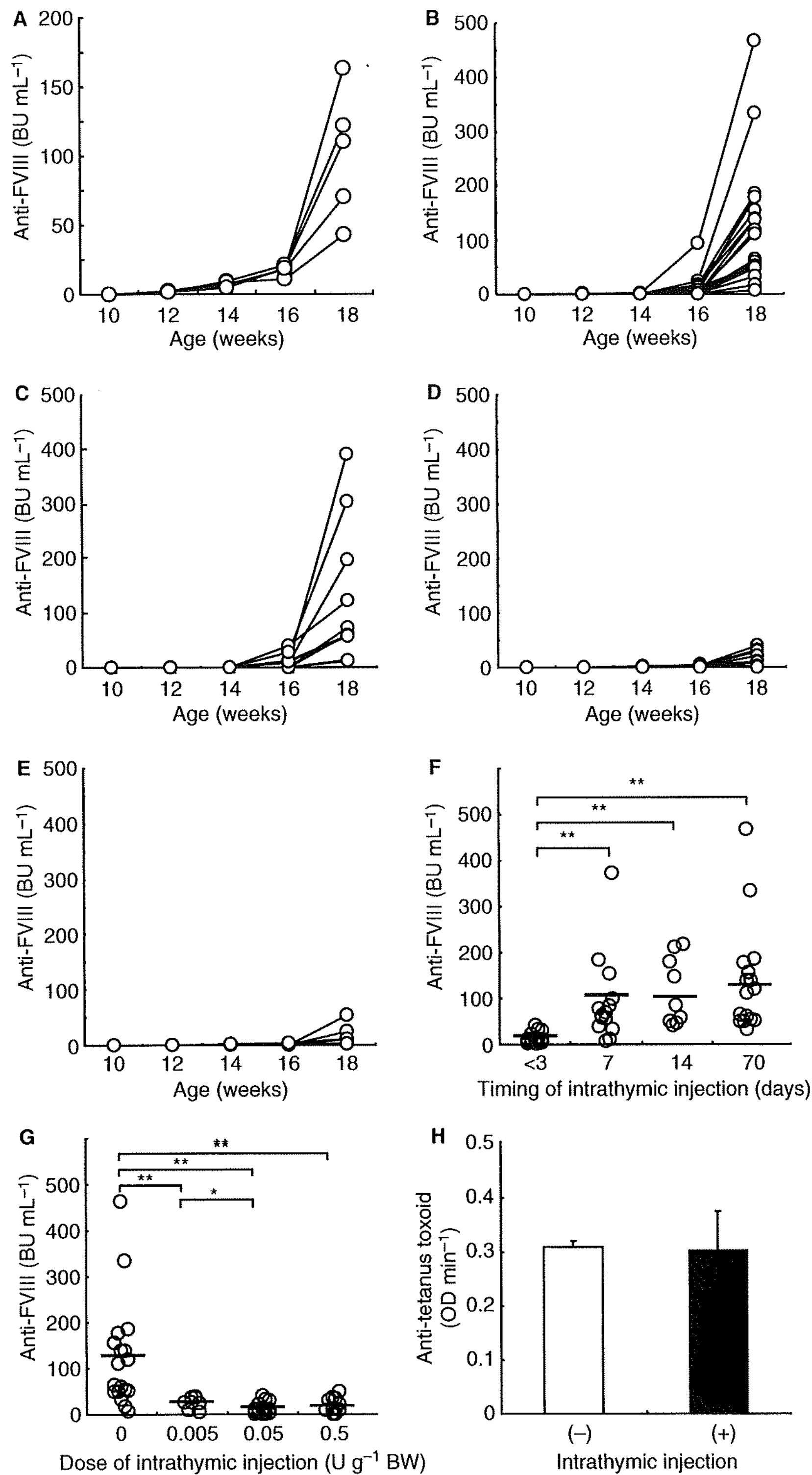

Effect of intrathymic administration of FVIII on anti-FVIII IgG formation in hemophilia A mice

We treated hemophilia A mice with repeated injections of human FVIII, and measured anti-FVIII IgG titers after the fifth injection at 16 weeks. IgG antibodies against FVIII were significantly inhibited in FVIII-IT mice as compared with non-IT mice (14.9 ± 11.2 μg mL^{-1} vs. 665.16 ± 225.4 μg mL^{-1}, $P = 0.0038$) (Fig. 3A). As these mice are on a B6 background, we used C57BL/6J mice as normal controls for experiments on the development of anti-FVIII IgG. The B6 control mice developed high titers of anti-FVIII inhibitory antibodies (101.6 ± 46.4 BU mL^{-1}; Fig. 2A). Levels of anti-FVIII IgG of FVIII-stimulated B6 control mice (409.9 ± 84.8 μg mL^{-1})

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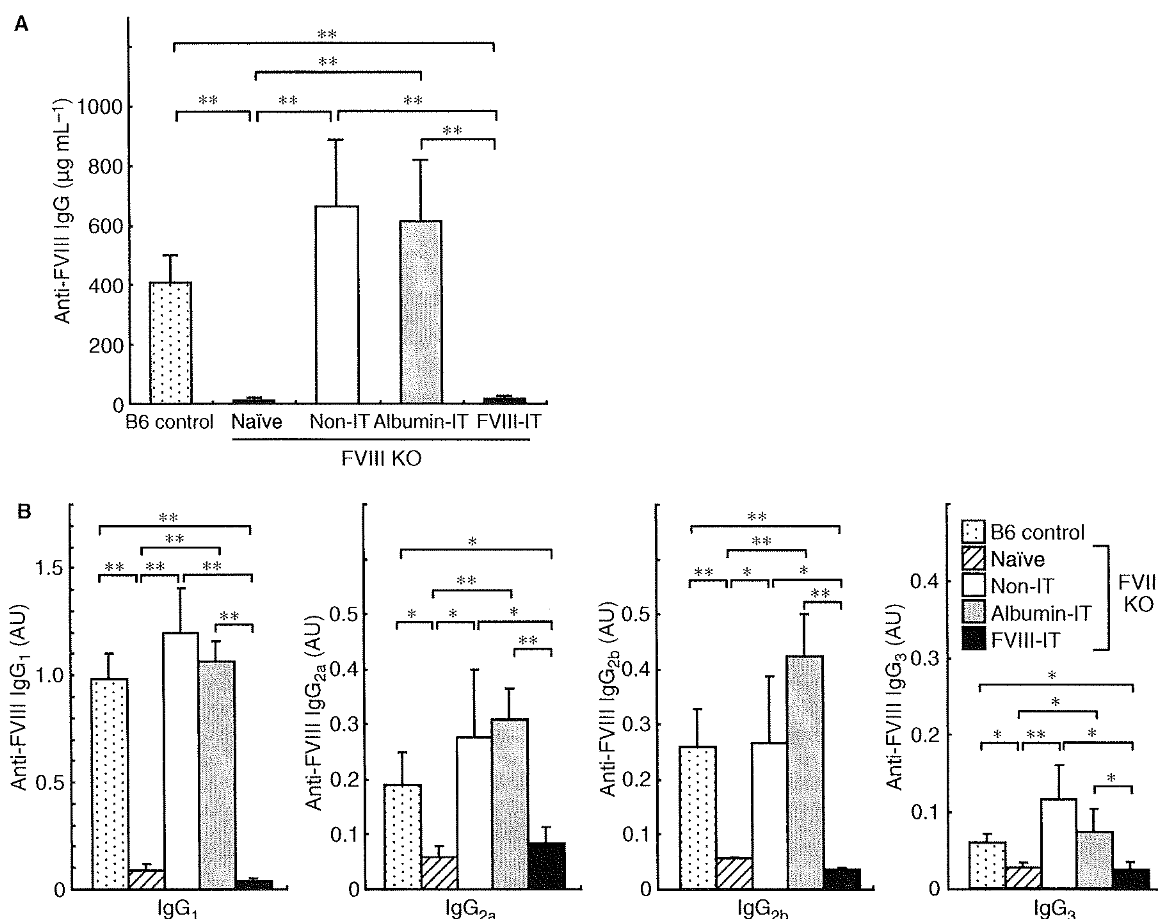

Fig. 3. Effect of intrathymic administration of FVIII on anti-FVIII IgG formation in hemophilia A mice. Normal B6 control mice (B6 control mice, $n = 5$), naïve FVIII-deficient mice (naïve mice, $n = 5$), FVIII-deficient mice without thymic treatment (non-IT mice, $n = 5$) and mice with prior thymic injection of human albumin (Albumin-IT mice, $n = 5$) or human FVIII (FVIII-IT mice, $n = 5$) were injected intravenously with human FVIII every 2 weeks. Each of the mice was bled on day 4 after the fifth stimulation, and total anti-human FVIII IgGs (A) and their titers of IgG subclasses (B) were measured by enzyme-linked immunosorbent assay as described in Materials and methods. The values [(A), μg mL^{-1}; (B), AU] represent the means ± standard deviations. *$P < 0.05$; **$P < 0.03$.

were significantly higher than those of FVIII-deficient naïve (5.0 ± 5.6 μg mL^{-1}, background values) or FVIII-IT mice. In addition, Alb-IT mice also produced significant amounts of anti-FVIII IgG antibodies (616.4 ± 207.9 μg mL^{-1}). All IgG isotypes of anti-FVIII antibodies in B6 control, Alb-IT and non-IT mice significantly increased as compared with those in FVIII-IT mice (Fig. 3B). These results suggest that intrathymic injection of FVIII efficiently suppressed the formation of antibodies against FVIII in hemophilia A mice.

The anti-FVIII specific CD4$^+$ T-cell proliferative response is blocked by intrathymic administration of FVIII

To evaluate whether intrathymic administration of FVIII had direct suppressive effect on FVIII-specific CD4$^+$ T cells, we analyzed the CD4$^+$ T-cell proliferative response to *in vitro* FVIII stimulation in the presence or absence of non-IT mouse-derived antigen-presenting cells (Fig. 4A). As shown in Fig. 4B, CD4$^+$ T cells isolated from B6 control, naïve, non-IT, Alb-IT or FVIII-IT mice did not proliferate at any concentration of FVIII when they were cultured without antigen-presenting cells. Moreover, the antigen-presenting cells alone did not respond to the stimulation with FVIII. The CD4$^+$ T cells of B6 control, non-IT and Alb-IT mice showed dose-dependent proliferation in response to FVIII when they were cocultured with the antigen-presenting cells (Fig. 4C). By contrast, the CD4$^+$ T cells isolated from FVIII-IT mice did not show any proliferative response to FVIII, even if they were cocultured with antigen-presenting cells, indicating that the intrathymic administration of FVIII could be important for the prevention of an immune response to FVIII.

Cytokine responses are suppressed by intrathymic administration of FVIII

The CD4$^+$ T cells from B6 control (Fig. 5A), non-IT (Fig. 5C) and Alb-IT (Fig. 5D) mice produced significant amounts of IL-2, IL-12 and IFN-γ in response to FVIII stimulation. In contrast, the levels of IL-4 and IL-10 in

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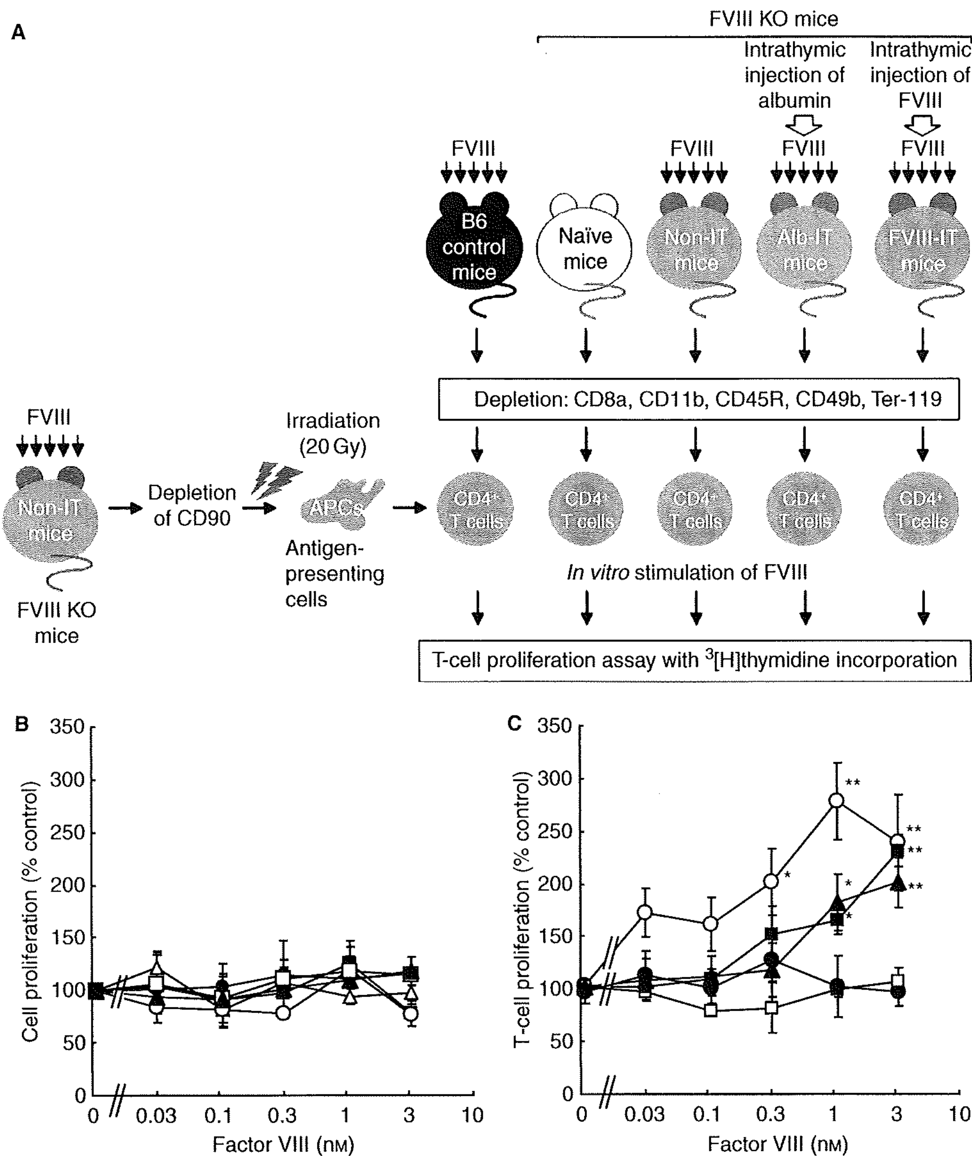

Fig. 4. The anti-FVIII specific T-cell proliferative response is blocked by intrathymic administration of FVIII. (A) B6 control mice, mice without intrathymic administration of FVIII (non-IT mice), mice that had been given intrathymic injections of human albumin (Alb-IT mice) and mice that had been given intrathymic injections of FVIII (FVIII-IT) mice were intravenously injected with human FVIII every 2 weeks. The $CD4^+$ T cells were isolated from each of the mice, as well as from naïve FVIII-deficient mice, by depletion of CD8a, CD11b, CD45R, CD49b and Ter119 cells with a cell sorting system. The antigen-presenting cells were isolated from non-IT mice after repeated intravenous stimulation with FVIII with depletion of CD90 cells, followed by 20 Gy of irradiation. Each group of $CD4^+$ T cells was stimulated with FVIII in the absence or presence of the antigen-presenting cells. (B) The proliferation of $CD4^+$ T cells from B6 control mice (closed triangles, n = 5), naïve mice (open squares, n = 5), non-IT mice (open circles, n = 7), Alb-IT mice (closed squares, n = 5) and FVIII-IT mice (closed circles, n = 7) was analyzed under *in vitro* stimulation with FVIII (0–3 nmol L^{-1}) in the absence of antigen-presenting cells, as described in Materials and methods. The proliferation of antigen-presenting cells alone was also analyzed (open triangles, n = 5). (C) The proliferation of $CD4^+$ T cells from B6 control mice (closed triangles, n = 5), naïve mice (open squares, n = 5), non-IT mice (open circles, n = 7), Alb-IT mice (closed squares, n = 5), and FVIII-IT mice (closed circles, n = 7) was analyzed under *in vitro* stimulation with FVIII (0–3 nmol L^{-1}) in the presence of the non-IT mouse-derived antigen-presenting cells. Data are shown as the means ± standard deviations. *$P < 0.05$, **$P < 0.03$, when compared with the proliferation in the absence of FVIII.

these mice did not change even after addition of FVIII. FVIII-IT mice spontaneously produced higher amounts of IL-4 (71.2 ± 40.5 pg mL^{-1}; Fig. 5E) than non-IT mice (14.7 ± 6.8 pg mL^{-1}, P = 0.016; Fig. 5C) or Alb-IT mice (26.6 ± 2.9 pg mL^{-1}, P = 0.035; Fig. 5D). IL-4 has been shown to be the dominant cytokine required for the development of a Th2 phenotype from naïve $CD4^+$ T cells [22]. The population of $CD4^+$ T cells or antigen-presenting cells may be heterogeneous and contain a subpopulation of cells (such as Th3 cells) capable of producing IL-4 in our system [23]. Alternatively, IL-4, as a regulatory cytokine, might tend to suppress Th1 responses and enhance

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regulatory T-cell function in FVIII-IT mice [24]. In addition, the FVIII-IT-mouse-derived $CD4^+$ T cells did not increase their production of the cytokines IL-2, IL-12, IFN-γ and IL-10 (Fig. 5E). These results suggest that the FVIII-specific Th1 cytokine response is suppressed by intrathymic administration of FVIII.

Intrathymic administration of FVIII induces antigen-specific regulatory T cells

To evaluate the role of FVIII-specific regulatory T cells in the induction of thymic tolerance, we analyzed $CD4^+CD25^+$ T cells and $FoxP3^+$ cells after *in vitro* stimulation with FVIII

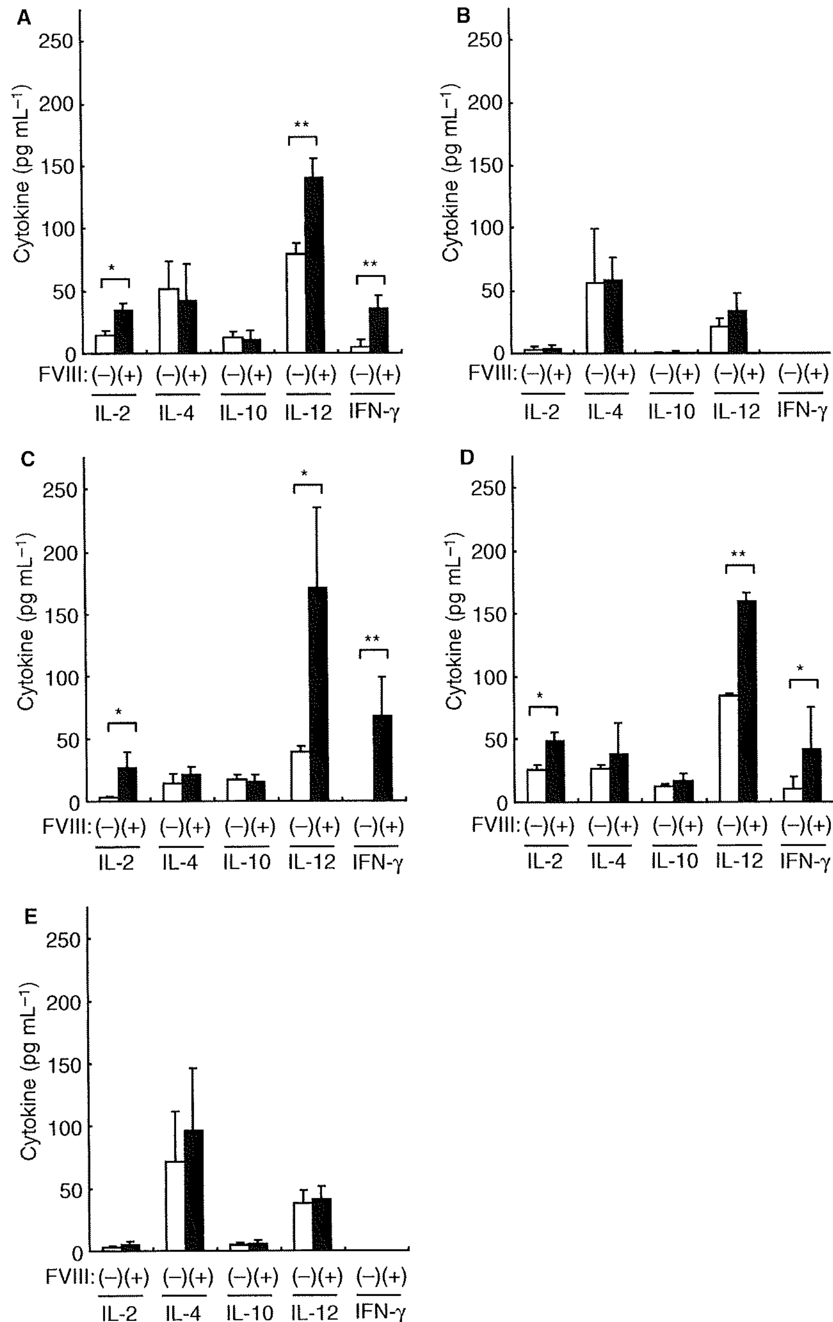

Fig. 5. The Th1 cytokine response is suppressed by intrathymic administration of FVIII. The $CD4^+$ T cells isolated from normal B6 control mice [(A), $n = 5$], FVIII-deficient mice without intrathymic administration of FVIII (non-IT mice) [(C), $n = 6$], mice that had been given intrathymic injections of human albumin [(D), $n = 5$] and mice that had been given intrathymic injections of FVIII [(E), $n = 8$] after repeated intravenous stimulation with FVIII were mixed with the antigen-presenting cells derived from non-IT mice with FVIII stimulation. The $CD4^+$ T cells from FVIII-deficient naïve mice were also examined with the antigen-presenting cells [(B), $n = 5$]. Each group of cells was cultured in the absence (open bars) or presence of 3 nmol L^{-1} FVIII (closed bars), and its cytokine production [interleukin (IL)-2, IL-4, IL-10, IL-12, and interferon-γ (IFN-γ)] were analyzed by enzyme-linked immunosorbent assay as described in Materials and methods. The values (pg mL^{-1}) represent the means ± standard deviations. $*P < 0.05$; $**P < 0.03$.

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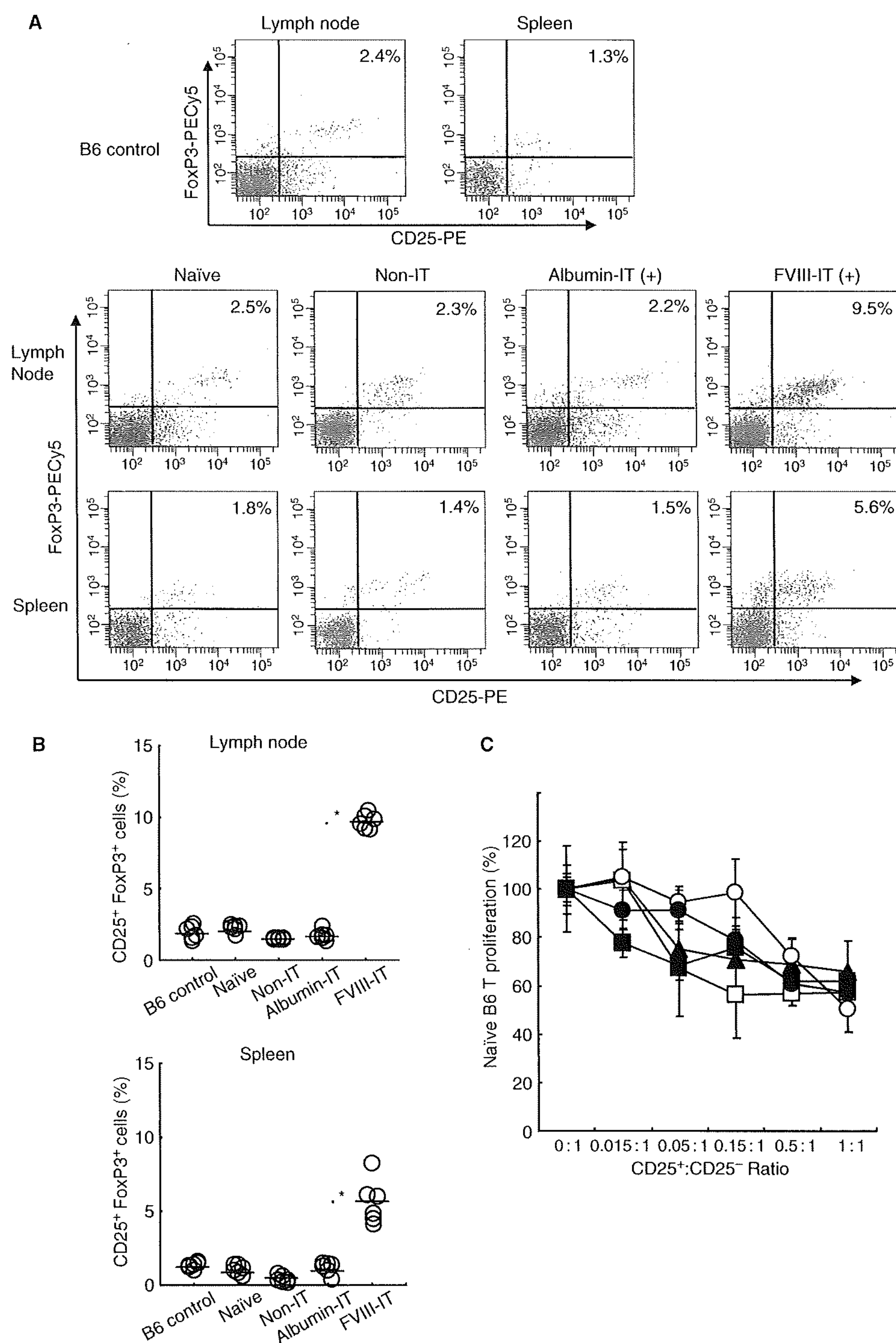

Fig. 6. Injection of FVIII into the thymus increases the numbers of FVIII-specific $CD25^+FoxP3^+$ cells. (A) The lymph node and spleen-derived $CD4^+$ T cells of normal B6 control mice, FVIII-deficient mice without intrathymic administration of FVIII (non-IT mice), mice that had been given intrathymic injections of human albumin (albumin-IT mice) or mice that had been given intrathymic injections of FVIII (FVIII-IT mice) with repeated intravenous FVIII stimulation were cultured with antigen-presenting cells from FVIII-stimulated non-IT mice in the presence of 3 nmol L^{-1} FVIII for 72 h. The $CD4^+$ T cells of FVIII-deficient naïve mice were also cultured with antigen-presenting cells and FVIII antigen. The percentage of $CD25^+FoxP3^+$ cells among $CD4^+$ T cells was analyzed by flow cytometry as described in Materials and methods. (B) The frequencies of FVIII-specific-$CD25^+FoxP3^+$ cells among $CD4^+$ T cells isolated from the lymph nodes and spleens of B6 control mice (n = 6), naïve mice (n = 6), non-IT mice (n = 6), albumin-IT mice (n = 6) or FVIII-IT mice (n = 6) were analyzed. $^{**}P$ < 0.03 as compared with those of other groups. (C) The standard regulatory T-cell inhibition assay. Under stimulation by anti-mouse CD3 antibodies (BD Biosciences), the proliferation of naïve normal B6-derived $CD4^+CD25^-$ T cells was analyzed in the presence of $CD4^+CD25^+$ T cells from B6 control mice (closed triangles, n = 5), FVIII-deficient naïve mice (open squares, n = 5), non-IT mice (open circles, n = 5), albumin-IT mice (closed squares, n = 5), or FVIII-IT mice (closed circles, n = 5), at a variety of ratios. PE, phycoerythrin.

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

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

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

Discussion

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

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

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

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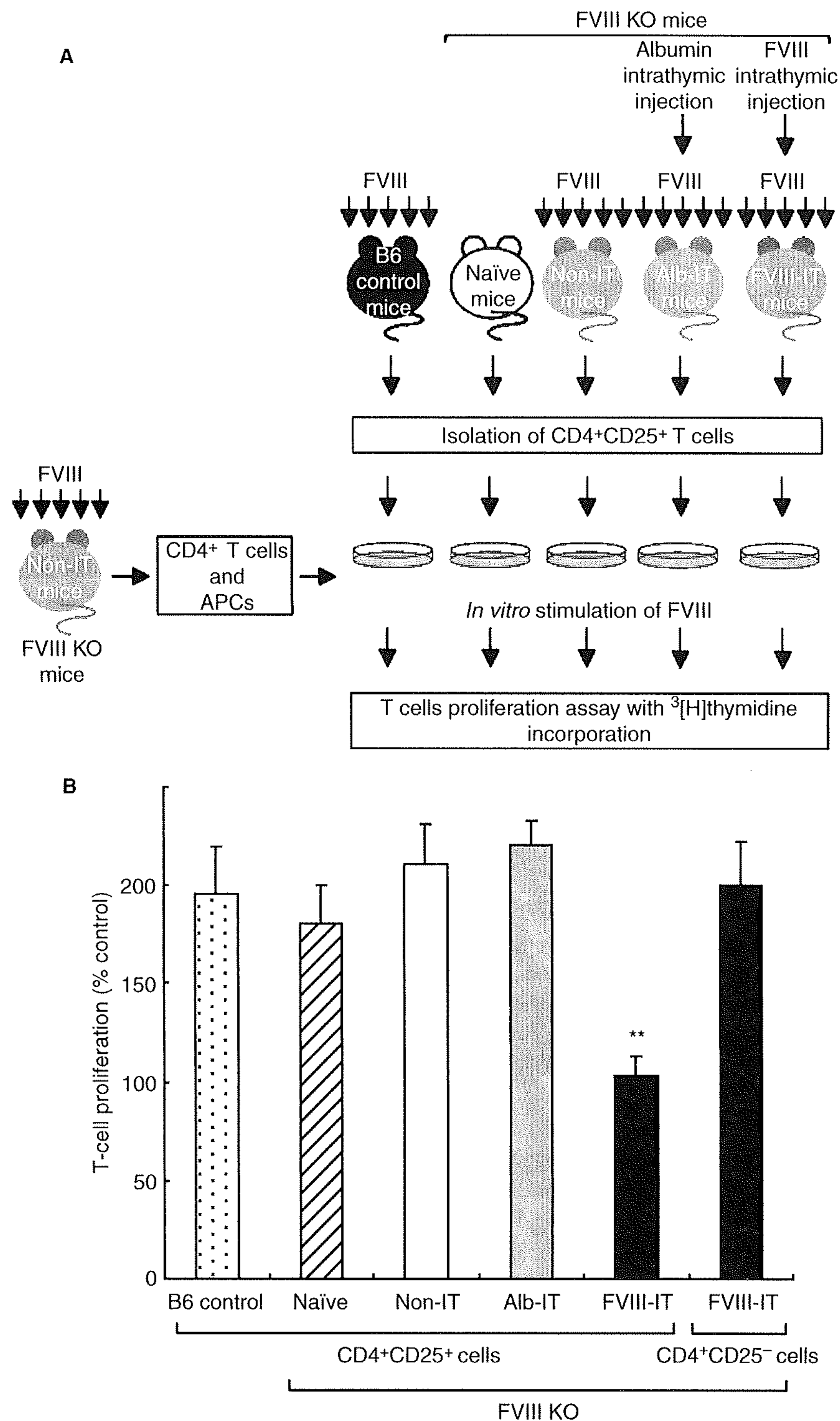

Fig. 7. $CD4^+CD25^+$ T cells isolated from thymus-injected mice inhibit the FVIII-immunized $CD4^+$ T-cell proliferative response. (A) $CD4^+CD25^+$ T cells were isolated from spleens of B6 control mice, FVIII-deficient mice without intrathymic administration of FVIII (non-IT mice), mice that had been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

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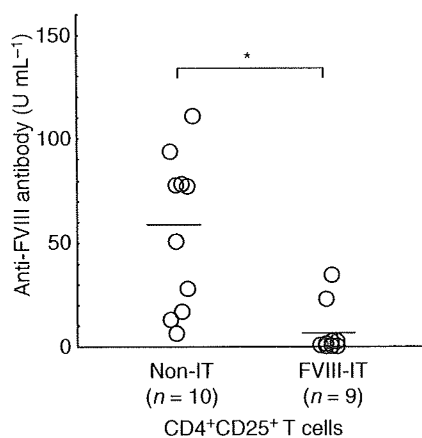

Fig. 8. Adoptive transfer of $CD4^+CD25^+$ regulatory T cells into syngeneic hemophilia A mice. The $CD4^+CD25^+$ T cells were prepared from the spleens of mice without intrathymic administration of FVIII (non-IT mice) or mice that had been given intrathymic injections of FVIII (FVIII-IT mice). A total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

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

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

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

Addendum

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

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Disclosure of Conflict of Interests

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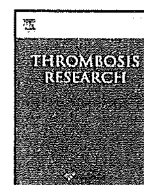

Regular Article

Prevalence of genetic mutations in protein S, protein C and antithrombin genes in Japanese patients with deep vein thrombosis☆

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ABSTRACT

Introduction: Genetic deficiencies of *PROS1*, *PROC*, and *SERPINC1* (antithrombin) are risk factors for deep vein thrombosis (DVT). Diagnosis of the inherited deficiencies of these three genes is sometimes difficult because of the phenotypic variability. This study was undertaken to reveal the frequency of nonsynonymous mutations of these three genes in Japanese DVT patients.
Patients/Methods: One hundred seventy-three DVT patients were registered by the Sub-group of Blood Coagulation Abnormality, from the Study Group of Research on Measures for Intractable Diseases. We sequenced the entire coding regions of the three genes in all DNA samples and identified the nonsynonymous mutations.
Results and Conclusions: For *PROS1* we identified 15 nonsynonymous mutations in 28 DVT patients; for *PROC*, 10 nonsynonymous mutations in 17 patients; and for *SERPINC1*, 13 nonsynonymous mutations in 14 patients. Five patients had two mutations in *PROS1* and *PROC*, and all of them had *PROS1* K196E mutation. We previously identified one patient with a large *PROS1* gene deletion. Thus, 55 out of 173 patients (32%) carried at least one genetic defect in the three genes. The *PROS1* K196E mutation found in 15 Japanese DVT patients was the most prevalent. Mutations of *PROC* K193del and V339M were the second, each found in four patients. Our data suggested that the *PROC* K193del mutation caused the loss of the anticoagulant activity but not the amidolytic activity. Our effort is the first DNA resequencing study to identify the genetic variations in DVT patients without any consideration of their plasma activities and antigens. To minimize selection bias in a future evaluation of the contribution of genetic deficiency to DVT, we must recruit patients consecutively.
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Introduction

Genetic deficiencies of protein S, protein C, and antithrombin (*PROS1*, *PROC*, *SERPINC1*) are well known risk factors for deep vein thrombosis (DVT) [1–4]. These rare deficiencies are present in less than 10% of Caucasian patients with DVT [5]. In the Caucasian population, two common genetic mutations, factor V Leiden and prothrombin G20210A, have been recognized as additional causes of DVT [6]. These two common genetic mutations have extreme difference in prevalence among various ethnic groups and are absent in the Japanese population [7]. There is growing evidence that within different ethnic groups, mutations associated with disease arise with different frequencies [8]. Recently, it was found that protein S mutation K196E is a genetic risk factor for DVT in the Japanese population [9,10]. We estimated the allele frequency to be 0.009 based on the observation that 66 out of 3,651 Japanese in the general population carried the mutant E allele [9] and that heterozygous carriers showed 16% lower plasma protein S activity than wild-type individuals [11].

Abbreviations: DVT, deep vein thrombosis; PCR, polymerase chain reaction.
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The prevalence of inherited genetic deficiencies for DVT has been intensively studied so far. In unselected Caucasian patients with DVT, rare mutations in *PROS1*, *PROC*, and *SERPINC1* were observed in about 8% of patients; in selected DVT patients, these mutations were observed in about 13% [6,12]. When two common mutations, factor V Leiden and prothrombin G20210A, were included, the prevalence was increased to more than 30% of unselected DVT patients and in 41-69% of selected DVT patients [6,12]. Most of the genetic analyses reported so far have been examined in DVT patients with low plasma activity, and no sequencing efforts for DVT patients without consideration of their activity and antigen levels have been undertaken. Thus, the prevalence of inherited mutations in *PROS1*, *PROC*, and *SERPINC1* in patients with DVT might be underestimated.

To identify genetic risk factors for DVT in Japanese, we previously enrolled 161 Japanese DVT patients. We genotyped five functional genetic variations in patients with DVT and in the general population and identified protein S mutation K196E as a genetic risk factor [9]. In the present study, we sequenced the entire coding regions of *PROS1*, *PROC*, and *SERPINC1* in DNA samples of the previously enrolled 159 Japanese DVT patients and additional 14 DVT patients without consideration of their plasma activities and antigens. As a result, we identified various nonsynonymous mutations in the three genes. We had previously identified one patient with a large *PROS1* gene deletion [13]. In total, 55 out of the 173 enrolled patients (32%) carried at least one mutation in either *PROS1*, *PROC*, or *SERPINC1*. This is the first DNA sequencing study to identify the genetic variations in DVT patients without any consideration of their plasma activities and antigens.

Materials and Methods

Patients

One hundred and seventy-three DVT patients were enrolled by the Study Group of Research on Measures for Intractable Diseases working under the auspices of the Ministry of Health, Labor and Welfare of Japan, as described previously [9]. The patients consisted of the previously enrolled 159 Japanese DVT patients and additional 14 DVT patients. Diagnosis of DVT was made by ultrasonography, radioisotope venography, and magnetic resonant imaging angiography. Patients had a mean age of 46.6 years (range 22-87). In 16% of patients, thrombosis was recurrent, and in 13%, thrombosis had occurred in family members. The protocol of this study has been approved by the Ethical Review Committee of each institute. Only those who gave written informed consent for genetic analyses were included in this study.

Direct DNA sequencing of PROS1, PROC, and SERPINC1

We sequenced all exons, flanking regions, and promoter regions of *PROS1*, *PROC*, and *SERPINC1* in 173 DVT patients [14]. The method of direct sequencing using the 96-capillary 3730xl DNA Analyzer (Applied Biosystems Japan, Tokyo, Japan) has been described previously [15,16]. Information on the primers and polymerase chain reaction (PCR) conditions is available on request. The obtained sequences were examined for the presence of mutations using NAMIHEI (version 1.0; Mitsui Knowledge Industry, Tokyo, Japan) and Sequencher software (version 4.2.2; Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [17]. We have adopted the numbering standards of the Nomenclature Working Group, wherein the A of the ATG of the initiator Met codon is denoted as nucleotide + 1, and the initial Met residue is denoted amino acid + 1 [18].

Activity measurements in patients with nonsynonymous mutations

Protein S anticoagulant activity was measured as cofactor activity for activated protein C on the basis of the activated partial thromboplastin time assay. Protein C amidolytic activity was measured using the chromogenic substrate and Protac derived from *Agkistrodon contortrix* as the activator. Antithrombin activity was measured as a heparin cofactor activity using the chromogenic substrate. The assay has been performed at each institution.

Statistical analysis

Comparisons between mutation carriers and non-carriers were analyzed by t-test. Differences with P values less than 0.05 were considered statistically significant.

Results

Nonsynonymous mutations in PROS1

We sequenced all exons, flanking regions, and approximately 200 bp of the promoter region in *PROS1*, and identified 15 nonsynonymous mutations in 173 Japanese patients with DVT (Table 1). These mutations included 10 missense mutations, 3 splice-site mutations, and 2 frameshift mutations. The K196E mutation was prevalent with

Table 1
List of nonsynonymous mutations identified in the *PROS1* gene in 173 Japanese DVT patients.

Relative position from ATG		Nuc.	Region/ Domain	Amino acid change	Major homo	Hetero	Minor homo	Total	Minor allele freq.	5' Near Seq. 20 bp	3' Near Seq. 20 bp
Genomic DNA	cDNA										
46342	77-1	G/C	Int1	Splicing	172	1	0	173	0.003	taaactgattgtttccttca	ttttgtcaaagcaacaggct
49510	259	G/C	Ex3/TSR	Val87Leu	171	2	0	173	0.006	atttttatccaaaatactta	gtaagttcaaaacatctcaa
63044	260-1	G/A	Int3	Splicing	172	1	0	173	0.003	tatgtttgtttttattttca	tttgtcttcgctcttttcaa
63086	301	C/T	Ex4/TSR	Arg101Cys	172	1	0	173	0.003	ctgggttattcactgctgca	gtcagtcaactaatgcttat
67676	416	C/T	Ex5/EGF1	Ala139Val	172	1	0	173	0.003	gagctgcaaagatggaaaag	ttcttttacttgcacttgta
67951	586	A/G	Ex6/EGF2	Lys196Glu	158	13	2	173	0.049	gttttgttatgctttcaaat	agaaagattgtaaaggtaag
75209	756	T/G	Ex8/EGF4	Cys252Trp	172	1	0	173	0.003	gaatgctctgagaacatgtg	gctcagctttgtgtcaatta
80726	1064	G/A	Ex10/SHBG	Arg355His	172	1	0	173	0.003	gtggctcctgattgcacttc	tggtggaaagattgaagttc
80732	1070	G/T	Ex10/SHBG	Gly357Val	172	1	0	173	0.003	cctgattgcacttcgtggtg	aaagattgaagttcagctta
80763	1101	1/2*	Ex10/SHBG	Thr368TyrfsX9	172	1	0	173	0.003	gttcagcttaagaatgaaca	acatccaaaatcacaactgg
80810_80811	1148_1149	1/2**	Ex10/SHBG	Asn384GlyfsX9	172	1	0	173	0.003	tgttattaataatggtctat	aatatggtacgtttgcagat
87338	1247	C/A	Ex11/SHBG	Pro416Gln	172	1	0	173	0.003	acctggacccctttttaagc	ggaaaatggattgctggaaa
89016	1486	G/T	Ex12/SHBG	Asp496Tyr	172	1	0	173	0.003	gaattgctcaatttcacata	attatagtaagtgattttcc
96772	1858	G/A	Ex14/SHBG	Gly620Ser	172	1	0	173	0.003	caaaagtggccacatacctg	gtggccttccaggtatctgc
96785	1870+1	G/A	Int14	Splicing	172	1	0	173	0.003	atacctgggtggccttccag	tatctgcttactttttcttc

*1: T, 2: TT, T insertion, **1: (GG)1, 2: (GG)2, GG insertion.
The A of the ATG of the initiator Met codon is denoted nucleotide + 1, and the initial Met residue is denoted amino acid + 1.
Int: intron, Ex: exon, TSR: thrombin sensitive region, EGF: epidermal growth factor like domain, SHBG: sex hormone binding globulin region.
Thr368TyrfsX9: Frameshift mutation at Thr368 to Tyr resulting in the stop codon after 9 amino acids. Asn384GlyfsX9: Frameshift mutation at Asn384 to Gly resulting in the stop codon after 9 amino acids.
The nucleotide sequence (GenBank Accession ID: NC_000003.9) was used as a reference sequence.

Table 2
List of nonsynonymous mutations identified in the *PROC* gene in 173 Japanese DVT patients.

Relative position from ATG		Nuc.	Region/ Domain	Amino acid change	Major homo	Hetero	Minor homo	Total	Minor allele freq.	5' Near Seq. 20 bp	3' Near Seq. 20 bp
Genomic DNA	cDNA										
1469	199	G/A	Ex3/Gla	Glu67Lys	172	1	0	173	0.003	tagaggagatctgtgacttc	aggaggccaaggaaattttc
3229	400	G/T	Ex5/EGF1	Glu134stop	172	1	0	173	0.003	agggccgcttctgccagcgc	gtgagggggagaggtggatg
3377	446	A/C	Ex6/EGF2	His149Pro	172	1	0	173	0.003	ggacaacggcggctgcacgc	ttactgcctagaggaggtgg
6184_6186	577_579	AAG/–	Ex7/LC tail	Lys193del	169	4	0	173	0.012	cctggaagcggatggagaag	cgcagtcacctgaaacgaga
6188	581	G/A	Ex7/LC tail	Arg194His	172	1	0	173	0.003	gaagcggatggagaagaagc	cagtcacctgaaacgagaca
6238	631	C/T	Ex7/AP	Arg211Trp	171	2	0	173	0.006	aagaagaccaagtagatccg	ggctcattgatgggaagatg
6266	659	G/A	Ex7/Cat	Arg220Gln	172	1	0	173	0.003	tgatgggaagatgaccaggc	gggagacagcccctggcagg
8429	811	C/T	Ex9/Cat	Arg271Trp	172	1	0	173	0.003	ctgcaggagagtatgacctg	ggcgctgggagaagtgggag
8633	1015	G/A	Ex9/Cat	Val339Met	169	4	0	173	0.012	aggccggccaggagaccctc	tgacgggctggggctaccac
8661	1043	G/A	Ex9/Cat	Arg348Gln	172	1	0	173	0.003	ctggggctaccacagcagcc	agagaaggaggccaagagaa

The A of the ATG of the initiator Met codon is denoted nucleotide + 1, and the initial Met residue is denoted amino acid + 1.
Ex: exon, Gla: Gla domain, EGF: epidermal growth factor like domain, LC tail: C-terminal portion in the light chain, AP: activation peptide, Cat: catalytic domain.

an allele frequency of 0.049 including 2 homozygotes and 13 heterozygotes, as has already been reported [9]. Two patients had the V87L mutation. Thirteen other nonsynonymous mutations were found in each of 13 patients. One patient had two mutations, R101C and K196E. To determine whether the two mutations were located on the same allele, PCR products including the two mutations were subcloned into a plasmid and 9 independent clones were sequenced. Each clone contained one mutation, indicating that the patient was a compound heterozygote with R101C and K196E mutations. Another patient had P416Q and D496Y mutations. We sequenced subcloned PCR products and found that the two mutations were on the same allele. We had previously examined whether the large *PROS1* gene deletion was present in the DVT patients and found one patient with the large *PROS1* gene deletion [13]. In all, 29 patients accounting for 17% of the 173 patients carried one or two nonsynonymous mutations or the large gene deletion in the *PROS1* gene and 15 patients accounting for 9% had the K196E mutation.

Nonsynonymous mutations in PROC

We sequenced all exons and flanking regions in *PROC*. The initial ATG codon of *PROC* is located in exon 2. We sequenced approximately 330 bp of the upstream of exon 1 as the putative promoter regions. We identified 10 nonsynonymous mutations, including 8 missense mutations, 1 nonsense mutation, and 1 in-frame deletion (Table 2). Three nonsynonymous mutations were found in more than one patient: K193del in 4 patients, R211W in 2 patients, and V339M in 4 patients. In all, 17 patients accounting for 10% of our 173 patients carried nonsynonymous mutations of the *PROC* gene.

Nonsynonymous mutations in SERPINC1

We sequenced all exons, flanking regions, and approximately 1.0 kb of the promoter region in *SERPINC1*. We identified 13 nonsynonymous mutations, including 8 missense mutations, 2 frameshift mutations, 2 nonsense mutations, and 1 splice-site mutation (Table 3). One frameshift mutation, L256fsX9, was found in 2 patients. In all, 14 patients accounting for 8% of our 173 patients carried nonsynonymous mutations of the *SERPINC1* gene.

Activity of protein S, protein C, and antithrombin in patients with nonsynonymous mutations

We have measured plasma activities of protein S, protein C, and antithrombin in patients with nonsynonymous mutations (Table 4). Heterozygous patients with the *PROS1* K196E mutation showed low protein S anticoagulant activity and homozygote of this mutation showed even lower activity. We measured the protein C amidolytic activity in three patients with the *PROC* K193del mutation and found that all showed more than 90% activity. Previous studies reported that the protein C amidolytic activity in patients with this mutation was normal but the anticoagulant activity was low [10,19]. Three patients with the *PROC* V339M mutation showed low protein C amidolytic activity but one patient showed the normal level (Table 4).

Table 3
List of nonsynonymous mutations identified in the *SERPINC1* gene in 173 Japanese DVT patients.

Relative position from ATG		Nuc.	Region	Amino acid change	Major homo	Hetero	Minor homo	Total	Minor allele freq.	5' Near Seq. 20 bp	3' Near Seq. 20 bp
Genomic DNA	cDNA										
2346_2348	47_49	3/2*	Ex2	Tyr17IlefsX14	172	1	0	173	0.003	tttgtctccttgcaggaagg	atcttttgtccttgctgctc
5317	480	C/G	Ex3	Cys160Trp	172	1	0	173	0.003	ttctttgccaaactgaactg	cgactctatcgaaaagccaa
6429	685	C/T	Ex4	Arg229stop	172	1	0	173	0.003	tgtccaataagaccgaaggc	gaatcaccgatgtcattccc
6481	737	T/A	Ex4	Val246Glu	172	1	0	173	0.003	caatgagctcactgttctgg	gctggttaacaccatttact
6506	762	G/C	Ex4	Lys254Asn	172	1	0	173	0.003	gttaacaccatttacttcaa	gtactcagaatggccctgga
7322	767	1/2*	Ex5	Leu256fsX9	171	2	0	173	0.006	tgtgattctcttccagggcc	gtggaagtcaaagttcagcc
7368	813	C/G	Ex5	Phe271Leu	172	1	0	173	0.003	aacacaaggaaggaactgtt	tacaaggctgatggagagtc
7431	876	T/A	Ex5	Tyr292stop	172	1	0	173	0.003	caggaaggcaagttccgtta	cggcgcgtggctgaaggcac
7435	880	C/T	Ex5	Arg294Cys	172	1	0	173	0.003	aaggcaagttccgttatcgg	gcgtggctgaaggcacccag
9745	1154-1	G/A	Int5	Splicing	172	1	0	173	0.003	cttctccggtcttccttcca	gtattgttgcagaaggccga
9791	1199	T/C	Ex6	Phe400Ser	172	1	0	173	0.003	cctctatgtctcagatgcat	ccataaggcatttcttgagg
13253	1277	C/T	Ex7	Ser426Leu	172	1	0	173	0.003	tgttgtgattgctggccgtt	gctaaaccccaacagggtga
13291	1315	C/A	Ex7	Pro439Thr	172	1	0	173	0.003	tgactttcaaggccaacagg	ctttcctggttttataaga

*3: TTT, 2: TT, T deletion, Int: intron, Ex: exon.
The A of the ATG of the initiator Met codon is denoted nucleotide + 1, and the initial Met residue is denoted amino acid + 1.
Thr17IlefsX14: Frameshift mutation at Thr17 to Ile resulting in the stop codon after 14 amino acids. Leu256fsX9: Frameshift mutation at Leu256 resulting in the stop codon after 9 amino acids.
The nucleotide sequence (GenBank Accession ID: NC_000001.9) was used as a reference sequence.

Table 4
Activity levels in patients with nonsynonymous mutations *PROS1* mutation.

PROS1 mutation	anticoagulant activity, %
Splicing, intron 3	<10
Ala139Val	28
Lys196Glu, hetero*	60, 49
Lys196Glu, homo	39
Gly357Val	31
Pro416Gln/Asp496Tyr**	26
Gly620Ser	<10
Splicing, intron 14	18
PROC mutation	**amidolytic activity, %**
Glu67Lys	70
Glu134stop	54
His149Pro	44
Lys193del***	90, 112, 130
Arg194His	77
Arg211Trp	35
Arg220Gln	64
Arg271Trp	77
Val339Met****	28, 40, 38, 77
Arg348Gln	127
SERPINC1 mutation	**activity, %**
Tyr17IlefsX14	44
Cys160Trp	51
Arg229stop	52
Val246Glu	53
Lys254Asn	50
Leu256fsX9*	52, 52
Phe271Leu	56
Tyr292stop	48
Arg294Cys	96
Splicing, intron 5	50
Phe400Ser	53
Ser426Leu	113
Pro439Thr	58

*Two patients, **Pro416Gln and Asp496Tyr were on the different alleles, ***Three patients, ****Four patients.

Patients with mutations in two genes

Five patients had nonsynonymous mutations in both *PROS1* and *PROC* (Table 5). All had the *PROS1* K196E mutation. As described, two patients had two different mutations in the *PROS1* gene and one patient had the large *PROS1* gene deletion [13]. Thus, as the results of extensive sequencing of the three genes in 173 DVT patients, 24 patients carried nonsynonymous mutations only in *PROS1*, 12 carried nonsynonymous mutations only in *PROC*, and 14 carried nonsynonymous mutations only in *SERPINC1* (Table 6). Thus, in total, 55 patients accounting for 32% of the 173 patients carried one or two nonsynonymous mutations in three genes (Table 6).

Comparison of the first onset age of DVT between mutation carriers and non-carriers

We compared the first onset age of DVT between mutation carriers and non-carriers. The onset age of mutation carriers (n = 55: 44.7 +/- 16.5 years old, mean +/- SD) was significantly earlier than that of non-carriers (n = 118, 52.6 +/- 16.1 years old, p = 0.0031) (Table 7). Family history of DVT in mutation carriers (12 out of 41, percent of family history: 29%) was significantly higher than that in non-carriers (8 out of 93, percent of family history: 9%, p = 0.0034).

Table 5
Five patients with nonsynonymous mutations in two genes.

PROS1	PROC	DVT onset age, year	Family history
Lys196Glu	Lys193del	57	unavailable
Lys196Glu	Arg221Trp	40	no
Lys196Glu	Arg271Trp	39	yes
Lys196Glu	Val339Met	25	yes
Lys196Glu	Val339Met	55	no

Table 6
Number of patients with nonsynonymous mutations.

mutated gene	number of patients
PROS1	24*
PROC	12
SERPINC1	14
PROS1+PROC	5**
Total	55

*Ten patients had Lys196Glu mutation and one patient had a large gene deletion. ** Five patients had *PROS1* Lys196Glu mutation.

Discussion

We sequenced *PROS1*, *PROC*, and *SERPINC1* genes in DNA samples from 173 Japanese DVT patients and found that 54 patients carried 38 nonsynonymous mutations such as missense, frameshift, splice-site, in-frame, and nonsense mutations. We had already identified one patient with the large *PROS1* gene deletion among our DVT patients [13]. Thus, in our DVT population, about 32% patients (55 out of 173) carried nonsynonymous mutations or the large gene deletion in at least one of the above three genes. This is the first DNA sequencing study to identify the genetic mutations in DVT patients without any consideration of their plasma activities and antigens.

We have previously measured plasma activities of protein S, protein C, antithrombin, and plasminogen in a general population consisting of about 4,500 individuals [20–22] and found that the standard deviations of antithrombin and plasminogen were relatively small (12.3% and 15.9%, respectively) whereas those of protein S and protein C were large (20.8% and 21.1%, respectively) [7]. Kurtosis for these activities in the general population occurred in the descending order of plasminogen, antithrombin, protein C, and protein S; thus, the distribution of protein S activity was the broadest. We also found age and gender differences for these activities. The activities of all proteins were significantly reduced in men older than 50 years. Decreases in protein C and protein S activity were particularly noticeable. Females in their 30's and 40's showed about 20% lower protein S activity than males. In particular, the plasma protein S activity can be influenced by many factors including age, gender, hormonal status, and disease [1,5,7,23]. Thus, the range of normal values for determination of risk can not be defined clearly, indicating the limitation of an activity assay for anticoagulant deficiency. DNA sequencing is an alternative approach to defining risk for DVT.

The sequencing of three genes revealed that the *PROS1* K196E mutation was the most prevalent (allele frequency of 0.049) in Japanese DVT patients. Mutations of *PROC* K193del and V339M were the second most prevalent in Japanese DVT patients, each found in four patients. The K193del and V339M mutations have been referred to as K150del or K151del and V297M, respectively, when the amino terminal Ala of the protein C light chain was designated as +1 [10,19,24]. These three deleterious mutations are concentrated in the Japanese population and contribute to the development of DVT. So far, the *PROS1* K196E and *PROC* K193del mutations have been found only in the Japanese population.

Table 7
Comparison of the first onset age of DVT between mutation carriers and non-carriers.

	mutation carriers	non-carriers
number	55*	118
Onset age, year, mean±SD	44.7 ± 16.5	52.6 ± 16.1

*Five had mutations in *PROS1* and *PROC*. Two were homozygotes for *PROS1* Lys196Glu. One was a compound heterozygote for *PROS1* Lys196Glu and Arg101Cys.
p = 0.0031.

In the present study, we found that the protein C amidolytic activities in patients with the V339M mutation were generally lower than the normal level but those in patients with the K193del mutation were within the normal range (Table 4). It has been reported that the protein C anticoagulant activity in patients with the K193del mutation was actually low [10]. Therefore, we considered that the K193del mutation in the protein C molecule causes the loss of anticoagulant activity but not the amidolytic activity. We would suggest that the protein C amidolytic activity measurement might miss the identification of patients with K193del. The K193 residue is located at the sixth position from the C-terminus in the light chain of protein C and we cannot estimate how this residue functions in the protein C molecule.

We identified 38 nonsynonymous mutations in this study, 27 of which were missense mutations. In contrast to frameshift, splice-site, and nonsense mutations, the functional effects of missense mutations are not predictable [25]. Therefore, the effects of the genetic mutations identified in the present study might be overestimated. As shown in Table 4, some of the plasma samples of patients with the nonsynonymous mutations showed their activities within the normal range. A functional analysis of the mutants with missense mutations is needed for the definite elucidation of the deficiency. In particular, biochemical characterization of the protein C mutant with the second most prevalent mutation, K193del, is important.

Limitation of the study: There might have been a selection bias for recruitment of DVT patients. The patients in this study were not recruited consecutively. Patients with overt genetic deficiency of one of these three genes might be concentrated in our study population. Therefore, in a future study, we will need to recruit patients consecutively and classify DVT patients into unselected and selected groups to evaluate the contribution of genetic deficiency to DVT.

Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

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State-of-the-Art Article

Venous thromboembolic risk and protein S deficiency: ethnic difference and remaining issues

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Abstract Protein S deficiency is an autosomal dominant disorder that results from mutations in the protein S gene (PROS1). Inherited deficiency of protein S constitutes a risk factor for venous thromboembolism. Protein S functions as a nonenzymatic cofactor for activated protein C in the proteolytic degradation of coagulation factors Ⅴa and Ⅷa. The frequency of protein S deficiency seems to differ between populations. More than 200 rare mutations in PROS1 have been identified in patients with protein S deficiency. Among the prevalent mutations within PROS1, the S460P substitution (known as Heerlen polymorphism) detected in Caucasians and the K196E substitution (known as protein S Tokushima) found in Japanese have been intensively studied for their structures and potential functions in the disorder of protein S deficiency. Until now, causative mutations in PROS1 have been found in only approximately 50% of cases with protein S deficiency. Co-segregation analysis of microsatellite haplotypes with protein S deficiency in families with protein S deficiency suggests that the causative defects in the PROS1 mutation-negative patients are located in or close to the PROS1 gene. Large PROS1 gene deletions have been identified in 3 out of 9 PROS1 mutation-negative Swedish VTE families with protein S deficiency and 1 out of 6 PROS1 mutation-negative Japanese patients with protein S deficiency. Intensive sequencing of the entire PROS1 gene, including introns, may be needed to identify the cryptic mutations in those patients, and these efforts might uncover the pathogenesis of protein S deficiency. (***J Geriatr Cardiol*** **2009; 6:12-20)**

Key words protein S deficiency; PROS1; thrombophilia; mutation; genetic defects

Introduction

Thrombin generation is critical to hemostasis and thrombosis; it is therefore tightly and synergistically regulated by two independent anticoagulant systems. One system is known as the protein C anticoagulant pathway, and the other as the heparan sulfate-dependent protease inhibitor system.[1,2] In the protein C anticoagulant system, activation of both factor Ⅹ and prothrombin is down-regulated by activated protein C (APC) through proteolytic inactivation of activated factors Ⅴ(FⅤa) and Ⅷ (FⅧa). Protein S is a vitamin K-dependent plasma protein that suppresses blood coagulation by serving as a cofactor for APC and promoting the factor Ⅹa inhibition by tissue factor pathway inhibitor.[3]

Genetic or acquired deficiency of protein S is one of the major risk factors for venous thromboembolism (VTE). Individuals with hereditary protein S deficiency have a predisposition to recurrent VTE.[4] The activity assay for plasma protein S in the general population and patients with VTE showed that the prevalence of protein S deficiency is much higher in Japanese than in Caucasian,[5-9] although we must take into consideration the different assay methods and different criteria for the deficiency. In addition, the K196E mutation in the protein S molecule with decreased activity shows a relatively high prevalence in the Japanese population.[10-13]

Screening for mutations in individuals with protein S deficiency has been performed in a number of studies, and more than 200 rare mutations have been reported thus far.[14,15] Although many laboratories have intensively analyzed the PROS1 gene to identify the causative mutation for protein S deficiency, mutations were only found in approximately 50% of the patients and the rest remained to be determined. Most of the genetic defects identified thus far are missense, nonsense, and frameshift mutations, and these are scattered throughout the coding region of the gene. Several large gene deletions have also been described.

In this review, characteristics of protein S, thrombotic diseases caused by protein S deficiency, and the genomic changes underlying protein S deficiency are focused on. We have adopted the recommendations of the Human Genome Organization and the Nomenclature Working Group throughout this article, in which A of the initiation codon ATG is denoted as nucleotide +1 and the first translated Met is amino acid +1.

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Physiological roles of protein S in anticoagulant pathway

Genomic structure of human PROS1 gene

The human PROS1 gene is located near the centromere

on chromosome 3q11.2. It spans approximately 101 kb of genomic DNA and comprises 15 exons and 14 introns. The human genome contains two protein S genes on chromosome 3: the active PROS1 gene and a closely related pseudo gene (PROSP). PROSP is located at 3p11-1 and shows 96.5% homology to exons 2 to 15 of the PROS1 gene. PROSP does not encode protein S.

Characteristics of protein S

Protein S is a 69-kD vitamin K-dependent glycoprotein that in its N-terminal domain has 11 γ-carboxyglutamic acids, abbreviated as Gla. Mature protein S has a discrete domain structure: a Gla domain (residues 42-87), a region sensitive to cleavage by thrombin (residues 88-283), four domains homologous to epidermal growth factor (EGF-like domains, residues 76-242), and a region homologous to sex hormone binding globulin (residues 284-676). Protein S is mainly produced in the liver and is also synthesized in endothelial cells, Leydig cells of the testis, megakaryocytes, osteoblasts, and the central nervous system.[16-18]

Physiological role of protein S

The blood coagulation cascade is regulated by two independent pathways, the protein C anticoagulant pathway and the heparan sulfate-dependent protease inhibitor system (Fig.1).[1,2] Protein C is proteolytically activated by thrombin bound to thrombomodulin on the endothelial cell surface, and the resulting APC proteolytically degrades procoagulant FVa and FVIIIa in the presence of protein S, thereby suppressing the coagulation. Thus, both protein C and protein S are anticoagulant proteins (Fig. 1).[19,20] In addition to the APC-dependent anticoagulant activity, protein S has been recently reported to inhibit the tissue factor pathway by stimulating factor Xa inhibition by tissue factor pathway inhibitor.[21] Protein S may also be involved in the regulation of cell growth and in the prevention of inflammation. [22, 23]

Protein S exists in two forms in plasma: the free form and the bound form with C4b binding protein (C4BP) (Fig. 2). The free form has anticoagulant activity and the C4BP bound form does not. Quite recently, a kinetic study has revealed that the protein S-C4BP complex expresses low but significant APC-catalyzed proteolysis and to some extent stimulates APC-catalyzed proteolysis of FVa.[3] Approximately 40% of plasma protein S circulates as the free form, while the remaining 60% is the bound form. Human C4BP exists in several forms in plasma. C4BP contains either 6 or 7 α-chains and either one or no β-chain held together by disulfide bonds.[24] The β-chain contains the protein S binding site. Thus, protein S binds to β-chain containing C4BP, known as C4BPβ$^+$, and does not bind to C4BP with no β-chain. Protein S and C4BPβ$^+$ form a 1:1 stoichiometric complex. C4BP is an acute-phase protein. The concentrations of C4BP dramatically increase up to 400% during inflammatory disorders. This rise is largely due to an increase in the C4BPa form, and the concentrations of the protein S-C4BPβ+ complex are altered mildly.[25]

Classification of protein S deficiency

Protein S deficiency is classified into three types. Type I is characterized by low levels of both total and free protein S (quantitative deficiency).[14] Type II entails reduced activity of protein S but normal levels of free and total protein S

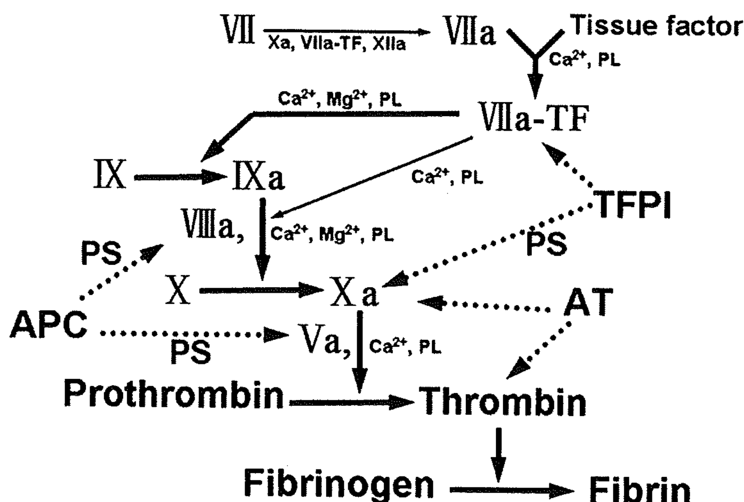

Figure 1 Blood coagulation and regulation

An extrinsic coagulation pathway is initiated by the binding of activated factor VII (VIIa) with cell-surface procoagulant protein, tissue factor (TF). The coagulation reaction is amplified on the negative phospholipid (PL) surface, such as with activated platelets in the presence of cofactor proteins. In this amplification reaction, activated factors VIII (VIIIa) and V (Va) facilitate the reaction, leading to the massive production of multi-functional protease, thrombin. Activated protein C (APC), generated by the proteolytic activation of protein C (PC) by thrombin-thrombomodulin complex, proteolytically cleaves VIIIa and Va and regulates their activities with the help of protein S (PS). Thus, protein C and protein S serve as anticoagulant proteins. Protease inhibitors, tissue factor pathway inhibitor (TFPI) and antithrombin (AT), can bind to the heparan sulfate glycosaminoglycan on the endothelium and regulate the coagulation reaction. Protein S inhibits TF activity by promoting the inhibition of Xa by TFPI.

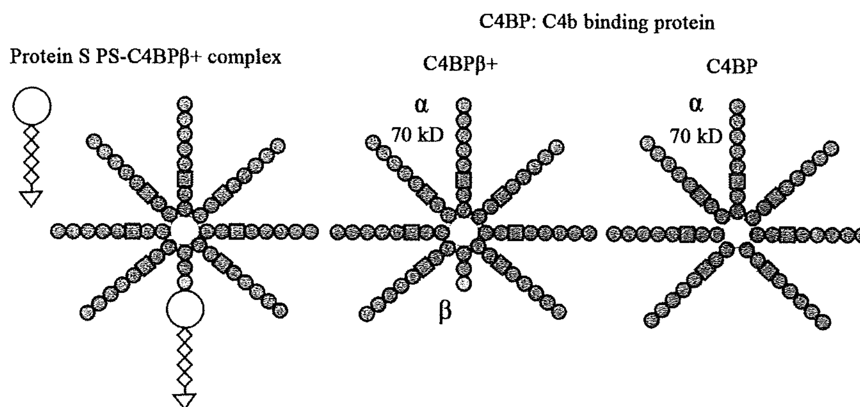

Figure 2 Protein S-C4BPβ+ complex

Protein S exists in two forms in plasma: the free form and the bound form with C4BP. C4BP contains either 6 or 7 α-chains and either one or no β-chain held together by disulfide bonds. The β-chain contains the protein S binding site. Thus, protein S binds to β-chain containing C4BP, known as C4BPβ+

(qualitative deficiency). Type III deficiency is characterized by low levels of free protein S and normal levels of total protein S. Type I and type III deficiencies have been shown to coexist in many families[26,27] indicating that these two types are phenotypic variants of the same genetic disease.

Diagnosis of protein S deficiency is complicated by the inter- and intra-individual variation in plasma protein S levels and by the overlap in levels between unaffected individuals and those having heterozygous deficiencies. To obtain the actual protein S activity in the plasma samples, the temperature of the assay is very important. Activity assay at the room temperature gave consistent protein S activity, but increased temperatures such as 37℃ gave a false high activity.[28] Several factors, including age, sex, hormonal state, pregnancy, liver disease, and inflammation, can influence plasma protein S levels.[25,29-31] In particular, the protein S activity of pregnant women is consistently low,[32] which hampers the definite identification of protein S deficiency in pregnant women.

Protein S deficiency and thrombotic diseases

With venous thrombosis diseases

Inherited protein S deficiency is an autosomal dominant disorder. Many studies have indicated that hereditary protein S deficiency is a confirmed risk factor of VTE, as described below. Individuals with hereditary protein S deficiency have a predisposition to recurrent VTE. Protein S deficiency was found in approximately 5% of VTE patients and 1-2% of consecutive patients with the first episode of VTE.[19] A population-based, patient-control study showed that protein C and antithrombin deficiency are clearly associated with an increase in thrombosis risk, but that total protein S or free protein S levels are not associated with VTE risk.[33] In a case-control study, individuals with low free protein S levels had a relative risk of 2.4 for VTE.[34] This paradox suggests that the screening methods for determining protein S deficiency do not effectively identify most patients.[35]

Thrombosis risk for individuals with hereditary protein S deficiency (risk ratio, 8.5) was significantly higher than that for normal individuals and was much higher than that for individuals with factor V Leiden mutation.[36] A retrospective study in a single extended family consisting of 122 members, including 44 mutation carriers of the PROS1 G336V mutation, has been reported.[37] The G336V mutation in this family was a strong independent risk factor for VTE with a hazard ratio of 11.5. A study using a cohort of patients from a single center where the diagnosis was confirmed at the genetic level showed that, in relatives, a low free protein S level is the most reliable predictor of a PROS1 gene defect. First-degree relatives with a PROS1 gene defect were found to have a 5-fold higher risk of thrombosis than those with a normal PROS1 gene and no other recognized thrombophilic defect.[38]

A large multicenter prospective follow-up study showed that relatives with hereditary protein S deficiency had experienced a VTE with an incidence of 7.1 per 1,000 person years and that the risk of VTE associated with hereditary protein S deficiency was 26.1.[39] The highest incidence per 1,000 person years was found in relatives with combined defects, and the lowest incidence was found in those with the FV Leiden mutation, indicating considerable differences in the lifetime risk of VTE observed among individuals with different thrombophilic defects.

With arterial thrombosis diseases

The relationship between protein S deficiency and arterial thrombosis is less obvious. A few patients with arterial occlusions showed protein S deficiency. Therefore, hereditary protein S deficiency may be a risk factor for the development of arterial thrombosis, especially cerebrovascular occlusion.[40-42] More extensive studies are needed to

demonstrate whether or not protein S deficiency is a risk factor for the development of arterial thrombosis.

Frequency of hereditary protein S deficiency in different ethnicities

The frequency of protein S deficiency seems to be quite different between Caucasians and Japanese. Heterozygous protein S deficiency is present in approximately 2-5% in Caucasian thrombosis patients,[19,43] whereas the prevalence in the general Caucasian population has been estimated to be between 0.16% and 0.21%.[5,44] Protein S deficiency in Japanese both in the general population and in patients with VTE is much higher. The prevalence of protein S deficiency, defined as low protein S activity, in the Japanese general population has been estimated to be 1.12% in men and 1.60% in women.[9] The reasons for the frequency difference of protein S deficiency between Caucasians and Japanese were not clear. The use of different assay methods and a different cut-off value for protein S deficiency may explain the differences. There are no available data regarding the frequency of protein S deficiency in other east-Asian countries; it is therefore not clear whether the difference is intrinsic to the relation between Japanese and Caucasian populations.

Genetic changes underlying protein S deficiency

The large size of the gene and the presence of a pseudogene (PROSP) complicate the genetic analysis of the PROS1 gene. Nevertheless, screening for PROS1 gene mutations has been performed in a number of studies. To date, more than 200 rare mutations in PROS1 have been identified as causative PROS1 gene defects.[14] Most of the genetic defects are missense, nonsense, or frameshift mutations scattered throughout the coding region of the gene.

Two missense mutations, the S460P mutation (known as Heerlen polymorphism) found in Caucasians and the K196E mutation (known as protein S Tokushima) found in Japanese, have been intensively studied.

Protein S S460P polymorphism (Heerlen polymorphism)

A S460P missense mutation known as the Heerlen polymorphism is present in the sex hormone-binding globulin domain of protein S molecule. The protein S mutant molecule carrying Pro at position 460 is characterized by a lower molecular weight than normal protein S molecule due to the loss of the N-glycosylation consensus sequence at N458. Many studies have indicated that the Heerlen polymorphism is not a risk factor for thrombosis.[45-47]

Protein S K196E as a risk factor for VTE in Japanese

Two independent case-control studies carried out in Japan have indicated that PROS1 K196E is a genetic risk factor for VTE.[12,48] We have summarized the characteristics of PROS1 K196E in Table 1. One of the studies was large scale, including 161 VTE patients and 3,655 population-based controls.[12] In this study, five functional genetic variants, including PROS1 K196E, plasminogen A610T, ADAMTS13 P475S, factor XII -4C>T, and plasminogen activator inhibitor-1 4G/5G, were evaluated by comparing their frequencies among VTE patients and controls. It was found that only PROS1 K196E was a genetic risk factor for VTE, with an odds ratio of 5.58.[12] Another study reached the same conclusion, showing PROS1 K196E to be a risk factor for VTE with an odds ratio of 3.74.[48]

The protein S K196E mutation is a missense mutation causing Lys196 to be replaced by Glu, formerly known as protein S Tokushima, and referred to as the K155E mutation by the nomenclature system of mature protein numbering. It is present in the second EGF-like domain of the protein S molecule. This mutation was originally identified independently in two different Japanese patients with VTE.[10,11] The allele frequency is approximately 0.9% in the Japanese population, which means that 1 out of 55 Japanese carries the mutation as a heterozygote.[10,12,48] Thus, a substantial number of Japanese carry the mutant E allele for protein S and are at risk of developing VTE. The protein S K196E mutation has thus far been identified only in Japanese, but may be present in eastern Asian populations as well. A genetic study of this mutation should be carried out in other eastern Asian populations.

Functional analysis of the protein S K196E mutant

In vitro studies using the recombinant proteins have shown that mutant protein S with Glu196 had impaired APC cofactor function.[49,50] Plasma protein S activities in carriers of the K196E mutation show reduced activity, but antigen levels are within normal limits.[10,51,52] In the Japanese general population, 34 out of 1,862 individuals were heterozygous for the K196E mutation. They showed a 16% mean reduction in plasma protein S activity, indicating that it is not as dramatic as conventional protein S deficiency.[52] Actually, the measurable protein S activities of the carriers were not always reduced but were intensively overlapped with those of normal individuals. This result indicated that protein S activity itself is not a proper tool for detecting this type of protein S deficiency. Therefore, genetic analysis or other direct discrimination methods must be used for identification of protein S K196E carriers.[13] Homozygote carriers with this mutation have not been identified in the general population thus far, but they have in the VTE group.

Failure of detection of causative mutations in protein S-deficient families

It is widely recognized that causative mutations in PROS1 in patients with protein S deficiency are only found in approximately 50% of cases. The proportion of cases where no mutations are detected varies widely between studies.[35] When all cases are pooled together, PROS1 gene