

Repression of factor VIII inhibitor development with apoptotic factor VIII-expressing embryonic stem cells

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Abstract

Development of factor VIII (fVIII)-neutralizing antibodies, called inhibitors, is a challenging problem in the management of hemophilia A patients. We explored the possibility of pretreatment with apoptotic fVIII-expressing embryonic stem (ES) cells to prevent the development of fVIII inhibitors. Murine ES cells integrated with the human *F8* gene were differentiated into embryoid bodies, dissociated to a single cell suspension, subjected to hypo-osmotic shock to induce apoptosis, and intraperitoneally injected into hemophilia A mice. Inhibitors were induced by periodic intraperitoneal injections of recombinant human fVIII (rhfVIII). In the groups in which intraperitoneal injections of rhfVIII began at 1-3 weeks after pretreatment, the titers of inhibitors were significantly lower after the third administration of rhfVIII compared with that in the control group in which apoptotic Ainv18 ES cells (without the human *F8* gene) were used for pretreatment, and continued to show lower levels until the sixth administration of rhfVIII. These results suggest that pretreatment with apoptotic hfVIII-expressing ES cells might be promising for the prevention of fVIII inhibitor development in hemophilia A patients.

Introduction

Hemophilia A is an X-linked bleeding disorder resulting from an abnormality in the coagulation factor VIII (fVIII) gene. Hemophilia A patients require life-long supplementation with intravenous fVIII. The risk of infectious diseases has dramatically decreased as a result of the development of highly pure plasma-derived and

recombinant fVIII. Nonetheless, development of fVIII-neutralizing antibodies, called fVIII inhibitors, remains a challenging problem for the treatment of hemophilia A. Approximately 25-35% of severe type hemophilia A patients develop inhibitors that reduce or completely negate the benefits of replacement therapy.^{1,2} Furthermore, a major issue is that all gene therapy trials for hemophilia are subject to the risk of developing inhibitory antibodies.³ Although immune tolerance induction therapy (ITI) using high amounts of fVIII is effective for eradicating inhibitors, the extremely high cost required for this type of therapy hampers its worldwide application. Moreover, ITI is ineffective in about 30% of hemophilia A patients with inhibitors.⁴ Thus, it is desirable to develop a novel method to prevent the development of fVIII inhibitors. To this end, several attempts have been made as follows. Madoiwa *et al.* successfully induced immune tolerance for human fVIII (hfVIII) by intrathymic injection of hfVIII into neonatal hemophilia A mice.⁵ Furthermore, induction of long-term fVIII tolerance can be achieved by administration of lentiviral vectors carrying a canine fVIII transgene to neonatal hemophilia A mice.⁶ Although these approaches were effective, they have several disadvantages such as difficulties in the procedures or the risk of viral vectors causing adverse genetic modification of patients.

The use of apoptotic cells for immune tolerance induction is in line with the current perception that dendritic cells induce peripheral tolerance by capturing cells that normally die during cell turnover. Because apoptotic cells are immunologically *silent*,⁷ they exert a tolerogenic influence in adaptive immune responses.^{4,8-10} Therefore, a method to prevent antibody production with apoptotic cells has an immunological rationale and is sufficiently promising based on recent studies.^{11,12} However, such an approach has several disadvantages that should be overcome for practical use in the clinical setting of hemophilia A treatment. One of the most concerning issues is the source of fVIII-expressing cells. While the most appropriate cells are autologous cells from an immunological viewpoint, patient-derived cells have a genetically abnormal *F8* gene. Thus, *ex vivo* transfection of the *F8* gene into patient-derived fibroblasts has provided a breakthrough for this approach. Su *et al.* demonstrated that injection of fVIII expression vector-modified apoptotic syngeneic fibroblasts achieves suppression of fVIII inhibitor development in hemophilia A mice.¹² However, the significant issues in this approach are the complex procedures such as collection of fibroblasts from each patient and transfection of the *F8* gene into these cells. Therefore, fVIII-expressing cells derived from histocompatible stem cells [such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells] will be the most suitable.

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Although we have observed that iPS cells can be differentiated into liver-like populations that show a strong expression level of fVIII mRNA,¹³ fVIII protein expression levels have not yet been examined. Therefore, we attempted to use genetically engineered (hfVIII-transgenic) ES cells as fVIII-expressing stem cells. We explored the possibility of fVIII inhibitor prevention by pretreatment with apoptotic fVIII-secreting ES cells in anticipation of the common and broad usage of iPS cells.

Materials and Methods

Mice

fVIII-deficient mice carrying a stop mutation in exon 16 of the *F8* gene were generously given to us by Prof. Yoichi Sakata (Research Division of Cell and Molecular Medicine, Jichi Medical University, Shimotsuke, Japan) and used as a model of hemophilia A.¹⁴ These hemophilic mice were mated with C57BL/6 mice for at least eight generations to create C57BL/6 fVIII knockout mice. The genotypes of hemophilic mice were

confirmed by polymerase chain reaction analysis of genomic DNA extracted from peripheral leukocytes.¹⁴ The mice were maintained as an inbred colony and were between 8 and 12 weeks of age at the beginning of the study. All mice were housed under specific pathogen-free conditions in the animal facility of Nara Medical University Research Center. Blood samples were obtained by orbital plexus bleeding and anticoagulated (9:1) with 0.105 M sodium citrate. Plasma samples were obtained by centrifugation of the blood at 3600 g for 10 min at room temperature, divided into aliquots, and frozen at -80°C until analysis. All experiments using mice were approved by the Nara Medical University Animal Use Committee and performed in accordance with the applicable guidelines and regulations.

Induction of factor VIII inhibitors

Development of fVIII inhibitors in C57BL/6 fVIII knockout mice was induced by weekly intraperitoneal administrations of 4 IU recombinant hfVIII (rhfVIII; Kogenate FS, Bayer Yakuhin, Osaka, Japan) (approximately 100 IU/kg body weight).

Assay for factor VIII inhibitors

The titers of fVIII inhibitors were assessed by the Bethesda assay using the obtained plasma samples according to a previous report with minor modification.^{15,16} In brief, 50 μL of doubling diluted mouse plasma with Owren's Veronal Buffer (more diluted if needed) was incubated with 50 μL of normal pooled human plasma at 37°C for 2 h. Residual human fVIII activity was measured in a one-stage assay using 50 μL of FVIII-deficient human plasma (Sysmex, Kobe, Japan) and a 50 μL sample from the previous incubation. Samples were mixed with 100 μL of phospholipid activator (APTT-

SLA, Sysmex), incubated at 37°C for 3 min, and then mixed with 100 μL of 20 mM CaCl_2 . Clotting times were measured with a coagulometer (KC 10, Amelung, Lemgo, Germany). Coagrol N (Sysmex) was diluted with Owren's Veronal Buffer to produce a standard curve of fVIII activity. The measurements were made in the linear portion of the response range.

Cell preparations

Murine Tet-WT-F8 ES cells, in which human F8 gene expression was induced by the tetracycline analog doxycycline, were prepared as described previously.¹⁷ Briefly, Ainv18 ES cells were transfected with the WT-F8-plox targeting plasmid (wild-type human fVIII gene-loading, lox-targeting plasmid) by electroporation to yield tet-WT-F8,¹⁸ after which the transfectants were selected with G418 as described previously.¹⁹ Tet-WT-F8 ES cells were maintained on mouse embryonic fibroblasts as a feeder layer, passaged twice on gelatin-coated dishes, and then induced to form embryoid bodies (EBs) for 6 days. The EBs were stimulated with doxycycline for the last 2 days. Then, EBs were dissociated to a cell suspension by digestion with 0.25% trypsin/EDTA. As a control, Ainv18 ES cells without the human F8 gene were treated in the same manner.

Apoptosis of the dissociated ES cells was induced according to the osmotic shock method by Liu *et al.*²⁰ Briefly, 1×10^6 cells were washed in RPMI 1640 twice, and resuspended in 1 mL hypertonic medium (0.5 M sucrose, 10% wt/vol polyethylene glycol 1000, and 10 mM HEPES in RPMI 1640, pH 7.2) for 10 min at 37°C . Then, 10 mL prewarmed hypotonic medium (40% H_2O , and 60% RPMI 1640) was added, and the cells were incubated for an additional 2 min at 37°C . Immediately after the incubation, the cells were

centrifuged, washed twice with ice-cold phosphate-buffered saline (PBS), and used as apoptotic cells. Induction of apoptosis of the dissociated ES cells was confirmed by flow cytometry analysis with annexin V/7-amino-actinomycin D staining (*data not shown*).

Administration of apoptotic embryonic stem cells

Dissociated and apoptosis-induced Tet-WT-F8 ES cells or control Ainv18 ES cells in 200 μL PBS (2×10^5 cells) were intraperitoneally injected into hemophilia A mice. Intraperitoneal injections of rhfVIII for the induction of fVIII inhibitor development were started at 3 days, 1, 2, and 3 weeks after administration of the apoptotic Tet-WT-F8 ES cells (group D3, W1, W2, and W3, respectively) and after administration of Ainv18 ES cells (control). After the first intraperitoneal injection of rhfVIII, blood sampling was performed just prior to the next intraperitoneal injection of rhfVIII (Figure 1).

Statistical analysis

Data were presented as the mean \pm standard error of the mean (SEM). Differences between groups were assessed by the Kruskal-Wallis test, followed by Dunn's multiple-comparison post hoc analysis. A P value of less than 0.05 was defined as statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Development of factor VIII inhibitors in hemophilia A mice

Figure 2 shows the progressive increase of the fVIII inhibitor titer with weekly intraperi-

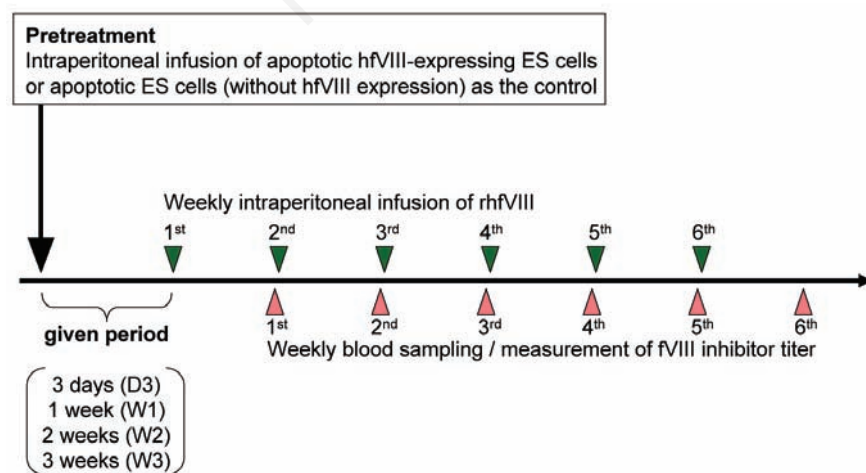


Figure 1. Timeline of the experimental procedure.

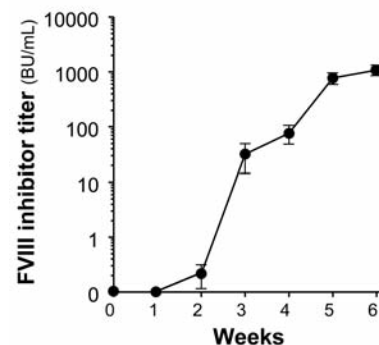


Figure 2. Progressive increase of factor VIII (fVIII) inhibitor titers in hemophilia A mice by weekly intraperitoneal administrations of recombinant human fVIII (rhfVIII) ($n=6-8$). Inhibitor titers reached a plateau after the fifth administration of rhfVIII.

toneal administration of rhfVIII to non-pretreated hemophilia A mice (n=8). The fVIII inhibitor titers appeared after the second administration of rhfVIII (0.22±0.38 BU/mL). Then, inhibitor titers increased with every following rhfVIII administration and reached 1687±309 BU/mL after the six administrations.

Intraperitoneal injection of apoptotic factor VIII-expressing cells attenuates the development of factor VIII inhibitors

The fVIII inhibitor titers were undetected in both treated and untreated groups before intravenous rhfVIII administration. Those of pretreatment groups at each blood collection point are shown in Figure 3. Pretreatment with apoptotic Ainv18 ES cells (without the human *F8* gene), regardless of the period between pretreatment and the first administration of rhfVIII, resulted in no difference in fVIII inhibitor development compared with that in non-pretreatment groups (data not shown). The fVIII inhibitor titers of a group in which intraperitoneal injections of rhfVIII were started at 1 week after administration of Ainv18 ES cells were shown in Figure 3 as a representative control group (Cont).

The fVIII inhibitor titers observed in pretreatment group D3 appeared higher than that in the control group after the second blood sampling but without significance. However, in W1, W2, and W3 groups, the fVIII inhibitor titers were significantly lower than that in the control group at the third blood sampling ($P<0.05$, $P<0.01$, and $P<0.05$, respectively). The results obtained during the rest of the observation period also showed a similar tendency of lower fVIII inhibitor titers in W1, W2, and W3 groups than those in the control group.

Discussion

In the current study, we examined the possibility of pretreatment with hfVIII-expressing ES cells for the prevention of fVIII inhibitor development. The efficacy for the reduction of fVIII inhibitor titers depended on the period between pretreatment with apoptotic hfVIII-expressing ES cells and the first administration of rhfVIII. In the D3 group, pretreatment with apoptotic hfVIII-expressing ES cells resulted in rising inhibitor titers rather than a reduction. However, in groups with a greater than 1 week interval, significant suppressive effects on fVIII inhibitor development were achieved by the pretreatment. These observations suggest that a specified period is required to suppress immune response to fVIII by prior injection of apoptotic hfVIII-expressing ES cells.

The molecular weight of the hfVIII protein is

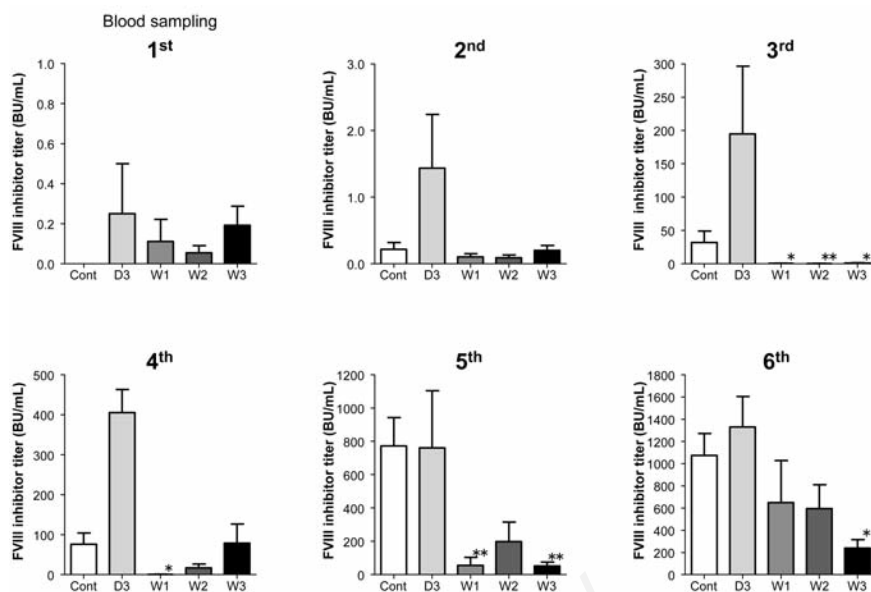


Figure 3. Effects of intraperitoneal injection of apoptotic factor VIII (fVIII)-expressing embryonic stem cells on fVIII inhibitor development. Intraperitoneal administrations of recombinant human fVIII (rhfVIII) for the induction of fVIII inhibitor development were started at 3 days, 1, 2, and 3 weeks after administration of the apoptotic cells (D3, W1, W2, and W3 groups, respectively) (n=4-8) and 1 week after administration of Ainv18 cells (Cont) (n=6). Data are presented as the mean ± standard error of the mean. * $P<0.05$, ** $P<0.01$, compared with the control group.

very large, as much as 280 kDa, and multiple epitopes in this protein are recognized by fVIII inhibitors.²¹ Thus, it is a critical issue to clarify whether the fVIII inhibitors that bind multiple epitopes of the fVIII protein can be suppressed by apoptotic hfVIII-expressing cells. Lei *et al.* found that full-length antigen expressed by B cells can induce immune tolerance for multiple epitopes in a target protein,²² because proteolytic cleavage of the protein, selection of the resulting peptides, and peptide presentation are executed by the host's own APCs. Our successful results in the present study support this previous notion. We have no need for identification of the precise peptide epitopes of the fVIII inhibitors or the establishment of ES cells that express each epitope corresponding to a fVIII inhibitor.

The mechanism by which pretreatment with apoptotic hfVIII-expressing ES cells suppresses fVIII inhibitor development remains to be elucidated. Although further investigations are required to address this issue, it might be attributed to the inhibitory effects of the apoptotic ES cells on the proliferation of fVIII-responsive effector T cells by antigen-specific CD4⁺CD25⁺ regulatory T cells as described by Su *et al.*¹²

In this study, rhfVIII was injected not intravenously but intraperitoneally. A previous report describes that inhibitor titer elevated to 122.5 BU/mL after five biweekly intravenous injections of 50 U/kg rhfVIII (total amount of 250 U/kg) in hemophilia mouse.⁵ In our study,

inhibitor titer did not exceed 122.5 BU/mL until total amount of administered rhfVIII reached 500 U/kg, suggesting that our protocol may be inefficient compared to that of the previous report. The difference of administration route as well as applied dose and administration interval may exert influence. Large size of molecules are absorbed slowly through the peritoneal lymphoid system through the stomata of the milky spot.²³ As intraperitoneally injected fVIII with a molecular size of more than 280 kDa would be therefore absorbed more slowly than intravenously injected fVIII, immune response against rhfVIII might wane. Furthermore, the peritoneal immune system, which contains distinct immune cells such as B1 cells,²⁴ may respond to foreign rhfVIII differently from the circulating immune system. Apoptotic ES cells were also administered intraperitoneally. Intraperitoneal administration of apoptotic cells could be applied as intraperitoneal chemotherapy. A comparative controlled study of administration routes, intravenous and intraperitoneal should be required to clarify which route is more effective. Furthermore, modification of the protocol for administration of apoptotic fVIII-expressing ES cells, including changes of the administering cell dose or administration frequency, would be required for complete suppression of fVIII inhibitor development.

Thus, our methodology is not necessarily directly applicable to hemophilia patients with

inhibitors. Nevertheless, our study opens up a whole new avenue for the prevention of fVIII inhibitor development. Although the ES cells used in this study were genetically engineered (hfVIII transgenic), iPS cell-derived fVIII-secreting cells will be established in the near future.²⁵ In addition, establishment of an iPS cell bank may allow the prevalence of this approach for fVIII inhibitor prevention.²⁶

Conclusions

Our results in this study suggest the effectiveness of pretreatment of apoptotic fVIII-expressing ES cells on repression of factor VIII inhibitor development. In addition, a specific period might be required to induce immune tolerance by pretreatment with such apoptotic cells. We believe that this approach has potential as a future preventive therapy against fVIII inhibitor development in hemophilia A patients.

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