Hematol Oncol Stem Cell Ther (2016) 9, 64–70

Available at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/hemonc

ORIGINAL RESEARCH REPORT

Therapeutic approaches for treating hemophilia A using embryonic stem cells

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Received 18 January 2016; accepted 9 April 2016
Available online 22 April 2016

KEYWORDS
Cell therapy;
Embryonic stem cell;
Hemophilia A;
Mouse model

Abstract
Hemophilia A is an X-linked rescessive bleeding disorder that results from *F8* gene aberrations. Previously, we established embryonic stem (ES) cells (tet-226aa/N6-Ainv18) that secrete human factor VIII (hFVIII) by introducing the human *F8* gene in mouse Ainv18 ES cells. Here, we explored the potential of cell transplantation therapy for hemophilia A using the ES cells. Transplant tet-226aa/N6-Ainv18 ES cells were injected into the spleens of severe combined immunodeficiency (SCID) mice, carbon tetrachloride (CCl_4)-pretreated wild-type mice, and CCl_4-pretreated hemophilia A mice. *F8* expression was induced by doxycycline in drinking water, and hFVIII-antigen production was assessed in all cell transplantation experiments. Injecting the ES cells into SCID mice resulted in an enhanced expression of the hFVIII antigen; however, teratoma generation was confirmed in the spleen. Transplantation of ES cells into wild-type mice after CCl_4-induced liver injury facilitated survival and engraftment of transplanted cells without teratoma formation, resulting in hFVIII production in the plasma. Although CCl_4 was lethal to most hemophilia A mice, therapeutic levels of FVIII activity, as well as the hFVIII antigen, were detected in surviving hemophilia A mice after cell transplantation. Immunolocalization results for hFVIII suggested that transplanted ES cells might be engrafted at

Abbreviations: Bry, brachyury; CCl_4, carbon tetrachloride; DAPI, 4′,6-diamidino-2-phenylindole; Dox, doxycycline; EB, embryoid body; ES, embryonic stem; FVIII, factor VIII; FVIII:Ag, factor VIII antigen; FVIII:C, factor VIII activity; FIX, factor IX; GFP, green fluorescent protein; H&E, hematoxylin and eosin; hFVIII, human factor VIII; iPS, induced pluripotent stem; LSEC, liver sinusoidal endothelial cell; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; tet, tetracycline; VWF, von Willebrand factor; WT, wild type

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http://dx.doi.org/10.1016/j.hemonc.2016.04.002

the periportal area in the liver. Although the development of a safer induction method for liver regeneration is required, our results suggested the potential for developing an effective ES-cell transplantation therapeutic model for treating hemophilia A in the future.

Introduction

Hemophilia A is an inherited X-linked, recessive hemorrhagic disorder caused by a deficiency of clotting factor VIII (FVIII) and is commonly treated by FVIII replacement. Concentrated FVIII products are derived from human plasma or produced recombinantly. Advances in viral-screening and -inactivation methods have improved the safety of plasma-derived products. Furthermore, the infectious risk of recombinant FVIII products is lowered by excluding human- or animal-derived proteins from cell culture solutions [1]. However, FVIII concentrates remain extraordinarily expensive, and frequent injections are required for FVIII replenishment, especially with severe hemophilia A patients.

Since hemophilia A is a single-gene disorder and small increases in FVIII levels exert a curative influence, the disease is a good candidate for gene therapy. However, problems are associated with gene therapy, such as the development of leukemia and decreased target-protein expression following cytotoxic CD8 T-cell induction against viral vector-derived capsid epitopes [2]. Nevertheless, a single injection of an adeno-associated virus serotype 8 vector in patients with severe hemophilia B caused prolonged factor IX (FIX) overexpression and clinical improvement [3]. However, viral vectors employed in gene therapy cannot deliver the *F8* gene due to its large size. Therefore, truncated *F8* gene variants, such as a B-domain-deleted variant, have been used, but successful hemophilia A gene therapy using viral vectors has not been achieved clinically. A recent study using a hemophilia A mouse model demonstrated that transducing the entire *F8* gene via the piggyBac vector improved clotting activity [4]. However, as the vector preferentially integrates near transcriptional start sites [5], insertional mutagenesis and genotoxicity remain significant concerns.

As the liver is the major site of FVIII synthesis, liver transplantation is effective in hemophilia A patients [6]; however, the lack of donor organs hinders clinical applications. Recent studies revealed that the main source of FVIII is liver sinusoidal endothelial cells (LSECs), and transplantation experiments using murine or human LSECs in hemophilia A mice demonstrated symptomatic improvement [7]. However, such transplantation requires a donor liver from which LSECs can be isolated, and contamination with different donor cells remains a potential problem. Several therapies have been investigated using cells capable of differentiating into LSECs [8—11].

Considering their potential for multilineage development and illimitable proliferation potential, embryonic stem (ES) cells may enable cell transplantation therapy. Fair et al. [12] reported that injecting mouse ES cells into the livers of hemophilia B mice corrected FIX deficiency. However, there have been few reports describing cell therapy for hemophilia A using ES cells. Previously, we established mouse Ainv18 ES cells (tet-226aa/N6-Ainv18), which secrete human FVIII (hFVIII) by introducing the human *F8* gene [13]. Here, we investigated ES-cell transplantation therapy against hemophilia A.

Materials and methods

Mice

All animal experiments were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and national regulations after approval from the institutional animal-care committee. Severe combined immunodeficiency (SCID) mice and wild-type (WT) C57/BL6 mice were purchased from CLEA Japan (Tokyo, Japan). *F8*-deficient (hemophilia A) mice were provided by Professor Yoichi Sakata (Jichi Medical University, Shimotsuke, Japan) and back-crossed for over eight generations with the C57/BL6 background as previously described [14]. Mouse blood was collected by retro-orbital bleeding at the indicated times and placed into 3.8% sodium citrate buffer. Immediately after blood sampling, plasma was isolated by centrifugation to evaluate FVIII activity (FVIII:C) and human FVIII-antigen (hFVIII:Ag).

FVIII assay

Mouse plasma samples were diluted with Owren's Veronal Buffer (Sysmex, Kobe, Japan). FVIII:C was measured in a one-stage aPTT clotting assay in a KC10A coagulometer (Amelung, Lemgo, Germany) using human FVIII-deficient plasma (George King Biomedical, Overland Park, KS, USA). Activated partial thromboplastin and $CaCl_2$ were purchased from bioMerieux (Durham, NC, USA). hFVIII:Ag was quantified using the VisuLize FVIII Antigen ELISA Kit (Affinity Biologicals, Ancaster, ON, Canada). For measurements of both FVIII:C and hFVIII:Ag, a standard curve was generated using normal human plasma (Sysmex). The detection limits of the FVIII:C and FVIII:Ag assays were 0.1% and 2 ng/mL, respectively.

ES cells

Production of tet-226aa/N6 ES cells was described previously [13]. Briefly, for efficient production of the active FVIII protein, we employed 226aa/N6-*F8* cDNA [15]. The 226aa/N6-*F8* construct encodes a shortened B-domain of 226 amino acids with six N-linked oligosaccharides. Green fluorescent protein (GFP)—brachyury (Bry) Ainv18 ES cells, established by targeting the GFP cDNA to the Bry locus in Ainv18 ES cells [16], were transfected with the 226aa/N6-plox targeting plasmids, yielding tet-226aa/N6 ES cells.