HIV-mediated Expression of Btk in Hematopoietic Stem Cells is not Sufficient to Restore B Cell Function in X-linked Immunodeficient Mice

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Abstract

Mutations of Bruton’s tyrosine kinase (Btk), which is critical for B cell development and function, cause X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice. Although the severity of the clinical phenotype differs between the two species, xid mice are considered useful for evaluating treatment strategies for XLA patients. Hematopoietic stem cells (HSCs; 1~3×10^6) from xid mice were transduced with an HIV vector containing the human Btk (hBtk) gene under the control of the internal murine stem cell virus (MSCV) promoter and injected into 4-week-old xid mice. Thirty weeks later, the copy number of the integrated HIV vector was over 0.2 per cell in both bone marrow and spleen, but serum concentrations of IgM and IgG3 and the antibody response to nitrophenol (NP)-Ficoll challenge were not restored. The number of differentiated B cells (IgM^+IgD^−) was increased, while the peritoneal B1 cell count remained low. These results indicate that HIV-mediated expression of hBtk in bone marrow stem cells partially promotes B cell development, but is not sufficient for the restoration of B cell function in xid mice.


Key words: gene therapy, HIV vector, XLA, Btk

Introduction

Mutations in Bruton’s tyrosine kinase (Btk) result in X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice. Btk belongs to the src-related family of non-receptor protein tyrosine kinases that are involved in signal transduction pathways activated by growth or differentiation factors and is crucial for B cell development and function. B cell development is blocked at the pre-B stage in patients with XLA, causing a deficiency of mature B cells and all isotypes of serum immunoglobulin (Ig)^2, which results in susceptibility to recurrent and severe bacterial infections^3. The highly conserved Arg28 in Btk is mutated to Cys in the xid mouse, which is an immunodeficient CBA/N strain^4. The phenotype of xid mice is milder than that of most XLA patients. The number of peripheral B cells in xid mice is 30~50% less than that of normal CBA/J mice with the mature IgM^+IgD^− population being the most depleted^5 and IgM and IgG3 levels are severely reduced^6. The xid mouse cannot produce antibodies
to T cell independent antigens but titers of antibodies to T cell dependent antigens are nearly normal. The phenotypes of Btk<sup>−/−</sup> knockout and xid mice are identical. The milder phenotype of xid mice might be caused by an alternative signaling pathway. Nevertheless, xid mice represent an important model for studying the pathogenesis of XLA and for evaluating treatment strategies for patients with XLA.

Regular high-dose intravenous immunoglobulin (IVIg) replacement therapy can improve the clinical course of XLA patients. However, more intensive therapy might be required to fully prevent the onset of bronchiectasis, chronic sinusitis, and nonbacterial, particularly enteroviral infections. IVIg replacement and antibiotic therapy is expensive and may result in infection with unknown viruses due to contaminated IVIg. Therefore, an alternative therapeutic strategy based on genetically corrected hematopoietic stem cells (HSCs) from XLA patients would be advantageous.

Recent studies support the feasibility of gene therapy for XLA. Competitive reconstitution experiments in xid mice have shown that B cell precursors with normal Btk have a selective advantage in proliferation or survival over Btk-deficient precursors. The phenotype of Btk<sup>−/−</sup> mice has been corrected by a human Btk (hBtk) cDNA transgene driven by the murine major histocompatibility complex (MHC) class II Ea gene locus control region (LCR), which activate gene expression in B cells throughout B cell differentiation and also in various tissues. However, a preliminary attempt at retroviral mediated stem cell gene therapy was unsuccessful. In the present study, we generated a novel HIV vector containing hBtk cDNA driven by the internal murine stem cell virus (MSCV) promoter and examined the therapeutic effect of hBtk expression in the xid mouse.

Materials and Methods

(1) Plasmid Construction

Total RNA was extracted from human promyelocytic HL-60 cells using the RNeasy<sup>®</sup> Mini kit (QIAGEN, Valencia, CA). The primer set designed to amplify the full-length hBtk cDNA consisted of 5'-CTCTGAGATGAAATA-3' and 5'-ATCAAAACCCCTCCCTCCATCT-3'. The PCR conditions were 94°C for 5 minutes, and then 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 minutes followed by a final extension at 72°C for 5 minutes. The 2.15 kb hBtk cDNA fragment isolated from agarose gels was cloned into the pGEM<sup>®</sup>-T easy vector (Promega, Madison, WI) and directly sequenced using the Big-Dye Terminator (Perkin Elmer, Norwalk, CT). The Spn I/Sal I fragment containing hBtk cDNA was recloned into the pSP72 vector. Finally, the Xho I/Sal I fragment was inserted into the Xho I site of pMscv/HIV-CS-PRE (P+), which is a derivative of HIV-CS (provided by H. Miyoshi<sup>®</sup>) obtained by inserting a ccpp-cts sequence, the MSCV promoter and the woodchuck posttranscriptional regulatory element (WPRE) between Rev response element (RRE) and 3' LTR, to yield pMscv-hBtk/HIV-CS-PRE (P+).

(2) Production of HIV Vector

We transiently transfected 293T cells (3x10<sup>6</sup>) seeded in 10-cm dishes with the packaging construct pCMVΔ8.2, the envelope construct pMD. G (provided by D. Trono)<sup>®</sup> and the vector plasmid pMscv-hBtk/HIV-CS-PRE (P+) using CaPO<sub>4</sub> precipitation. The medium was replaced with DMEM containing 2% FCS 6 hours later and collected 48 hours thereafter (conditioned medium). Viral vectors were passed through a 0.45-μm filter, concentrated as described<sup>®</sup> and resuspended in PBS.

(3) Mice

CBA/N (xid) and CBA/J (wild-type) mice originally obtained from The Jackson Laboratory (Bar Harbor, ME), were purchased from Central Laboratories for Experimental Animals (CLEA) and Oriental Yeast Co. Ltd., respectively. The Animal Experiment Committee of the Nippon Medical School approved all aspects of the study protocol.

(4) Transduction of Mouse HSC Enriched Cells

We prepared an enriched fraction from mouse HSCs using StemSepTM Mouse Progenitor
Enrichment Cocktail (StemCell Technologies, Vancouver, Canada) and collected 1–2 x 10^6 HSC enriched cells per mouse. The cells were transduced in 6-well plates coated with recombinant fibronectin fragment CH-296 (Retronectin, Takara Shuzo). Fresh HSC enriched cells were exposed for 24 hours to HIV vector particles at a multiplicity of infection (MOI) of 30 in serum-free StemPro-34® SFM Complete Medium (GibcoBRL, Gaithersburg, MD) supplemented with Polybrene (8 µg/ml), mouse stem cell factor (SCF, 10 ng/ml) and human thrombopoietin (TPO, 100 ng/ml). Transduced cells (1–2 x 10^6/mouse) were injected into the lateral tail vein of lethally irradiated (9 Gy) 4-week-old male xid mice.

To determine transduction efficiency, 1 x 10^6 transduced HSC enriched cells were seeded in 2 ml of methylcellulose medium containing SCF, interleukin 3 (IL-3), IL-6, erythropoietin (EPO), and fetal bovine serum (FBS) in IMDM (MethoCult™ MF M3434, StemCell Technologies, Vancouver, Canada) in 6-well plates. Colonies were scored 14 days later. Integrated hBtk cDNA in selected individual methylcellulose colonies was detected by PCR using Btk-sense (5'-AGCTACCTGCATTAAGTCAG-3') and Btk-antisense (5'-CTTCTCGGAATCTGTCTTTC3') primers.

(5) Generation of Chimeric Mice

Lethally irradiated xid mice (4 weeks old) were transplanted with a mixture of 2 x 10^6 Btk^- HSC enriched cells from normal CBA/J mice and 1.8 x 10^6 Btk^- HSC enriched cells from mutant xid mice.

(6) Serum Ig Detection and Immunization in vivo

Mice were bled via tail veins and serum samples were stored in aliquots at –20°C. Total serum IgM and IgG3 antibody concentrations were determined using the Mouse IgM or IgG3 sandwich enzyme linked immunosorbent assay (ELISA) Quantitation Kit (Bethyl Laboratories, Montgomery, TX).

To measure T cell independent type II (TI-II) antigen responses, mice were injected intraperitoneally with 50 µg nitrophenol (NP)-Ficoll (Biosearch Technologies, Novato, CA) in PBS and then the NP-specific IgM titer was analyzed 10 days later by ELISA. Plates were coated with 10 µg/ml NP-BSA (Biosearch Technologies), and diluted serum samples were assayed as described above. The antibody titer was calculated as fold increase compared with average pre-immunization titers of normal CBA/J control mice.

(7) Semiquantitative PCR Analysis of Btk Sequences

The copy number of integrated vectors per cell was determined by semiquantitative PCR using an ATTO Densitograph Lane Analyzer (ATTO Corporation). B cells were purified from bone marrow (BM) and splenic mononuclear cells (MNCs) using autoMACS containing anti-B220 immunomagnetic beads (Miltenyi Biotec, Germany). The purity of selected B cells was about 95%.

Genomic DNA was isolated from B cells and non-B cells using QIAamp DNA purification columns (QIAGEN). The hBtk sequence was amplified using Btk-sense and Btk-antisense primers, 0.25 units of AmpliTaq and 0.4 µM of each primer in 50 µl using 28 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 minute. The copy number standard was generated by a serial dilution of DNA from a 3T3 based retroviral packaging cell line containing 5 copies of hBtk cDNA.

Ribonucleic acid was extracted from B cells and non-B cells of BM and splenic MNCs by RNeasy® Mini spin columns (QIAGEN) with the QIAGEN® RNase-Free DNase Set. The hBtk exon 2 was amplified by RT-PCR using Btk-sense and Btk-antisense primers and 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 minutes. The xid mutation is a C to T transition in exon 2 of mouse Btk that results in the loss of an Hha I restriction site. Digesting the 304-bp PCR product with Hha I allows the mouse wild-type, mutant-xid alleles, and hBtk cDNA to be distinguished by yielding 3 fragments of 95, 55, and 154-bp, 2 of 95, 209-bp, and 2 of 140, 162-bp, respectively. The fragments were separated on Spreadex EL 1200 gels (Elchrom Scientific AG, Switzerland) and stained with ethidium bromide. By comparing the intensity of the hBtk-specific 162-bp
and xid 209-bp fragments, the degree of hBtk gene expression was calculated in the reconstituted mice using an ATTO Densitograph Lane Analyzer (ATTO Corporation).

(8) Flow Cytometry
Single-cell suspensions from BM, spleen or peritoneal wash were depleted of red blood cells and stained with combinations of the following antibodies (PharMingen): anti-CD45R/B220 (RA3-6B2) fluorescein isothiocyanate (FITC), anti-IgM (R6-60.2) phycoerythrin (PE), anti-IgD (11-26c, 2a) FITC, anti-CD5 (53-7.3) FITC and anti-CD45/B220 (RA3-6B2) PE. Data were acquired on a FACScan (Becton Dickinson, San Jose, CA) and analyzed using CellQuest software (Becton Dickinson).

(9) Statistical Analysis
Results analyzed using Student’s t test were considered significant when \( P < 0.05 \).

Results

(1) HIV Mediated Gene Transfer into Mouse Hematopoietic Cells
We prepared hBtk cDNA by PCR cloning using total RNA from HL60 cells and inserted into the HIV vector plasmid containing the MSCV LTR as an internal promoter and WPRE. Expression of hBtk mediated by the HIV vector in HeLa cells and fibroblasts from XLA patients was confirmed by Northern and Western blotting (data not shown).

The HSC enriched fraction prepared from total BM cells of xid mice (5 weeks old) by magnetic beads selection was immediately incubated with the HIV vector. A preliminary experiment using the HIV vector containing the EGFP marker gene showed that almost 90% of HSC enriched cells were transduced under our conditions. We determined the efficiency of stable gene integration using methylcellulose colony assays. The hBtk sequence was detected in 178 of 215 (83%) individual colonies by PCR analysis, indicating that mouse HSC enriched cells were efficiently transduced with the HIV vectors.

(2) Bone Marrow Reconstitution of xid Mice
Transduced HSC enriched cells (1–3 × 10⁶) were injected into lethally irradiated 4-week-old xid mice through the tail vein. The hBtk sequence was detected by PCR in both BM and spleen in all reconstituted animals (n = 21) for up to 20–30 weeks after transplantation, indicating that the HIV vector stably transduced long-term repopulating cells in the HSC enriched fraction.

Chimeric mice that received a mixture of 2 × 10⁴ normal CBA/J mouse HSC enriched cells and 1.8 × 10⁵ mutant HSC enriched cells from xid mice served as controls. Such partial reconstitution is supposedly sufficient to restore B cell function in xid mice. We then compared the various immunological parameters of vector-treated mice that received xid HSC enriched cells transduced with the HIV vector and chimeric mice that received normal HSC enriched cells.

(3) B Cell Function in Treated xid Mice
We measured serum Ig concentrations at 6-week intervals after transplantation. The levels of serum IgM and IgG3 of chimeric mice were elevated to the normal range found in the CBA/J control mice (Fig. 1A–1B). In contrast, serum Ig levels did not significantly increase in vector-treated mice for 30 weeks after transplantation.

To evaluate TI-II antigen responsiveness, we measured NP-specific antibody titers by ELISA in mice immunized with NP-Ficoll (Fig. 1C). The levels of NP-specific IgM were almost normal in chimeric mice, whereas vector treated mice did not respond to NP-Ficoll. These results indicated that B cell function in chimeric mice reconstituted with as few as 10% normal HSC enriched cells becomes normalized. However, HIV vector mediated expression of hBtk in HSC enriched cells could not restore either serum Ig levels or the TI-II antibody response.

(4) B Cell Development in Treated xid Mice
To investigate the effect of HIV mediated expression of hBtk on B cell development in xid mice, we performed comparative flow cytometric analyses on splenic MNCs from HIV-treated or
chimeric mice at 30 weeks post-reconstitution. The numbers of peripheral IgM\textsuperscript{+}IgD\textsuperscript{-} B cells are very low in xid mice\textsuperscript{9}, although the numbers of mature B cells slowly increase with age\textsuperscript{9}, and CD5\textsuperscript{-} B1 cells are absent\textsuperscript{9}. Compared with age-matched CBA/J normal mice, the number of IgM\textsuperscript{+}IgD\textsuperscript{-} populations was significantly increased in both vector-treated and chimeric mice (Fig. 2 and Table 1), suggesting that B cell differentiation was at least partially corrected.

B1 cells reside mainly in the peritoneum and produce most of the natural IgM and IgG3 found in the serum of naïve animals. A subset of B1, B1a cells, can be distinguished from conventional (B2) B cells because they express CD5. The number of B1 (B220\textsuperscript{-}CD5\textsuperscript{+}) cells was not restored in vector-treated, whereas the number of B1 cells in chimeric mice was significantly increased relative to that in xid mice (Table 1).

(5) Expression of hBtk in Treated xid Mice
To understand why the gene therapy approach failed, we performed a detailed molecular characterization of vector-treated mice. Integrated hBtk cDNA was detected in BM and spleen of all treated animals (n = 21). Thereafter, the vector copy number per cell in both B cells and non-B cells of BM and spleen was determined by semiquantitative PCR (Table 2). We detected between 0.21 to 0.33 copies per cell in all cell populations, suggesting that the proportion of hBtk\textsuperscript{-} cells was maintained during splenic B lineage development after ex vivo gene therapy. The selective growth advantage of hBtk\textsuperscript{-} cells was not detected in this experimental system.

Since mutant mouse Btk from xid mice and hBtk could not be distinguished by Western blotting analysis using polyclonal anti-Btk antibody\textsuperscript{10}, the levels of hBtk expression in these cells were examined by semiquantitative RT-PCR. Spreadex EL 1200 gel electrophoresis of RT-PCR products
digested with HhaI can distinguish hBtk mRNA from cells transduced with the HIV vector and normal mouse Btk (mBtk) mRNA from transplanted donor cells from endogenous mutant Btk (mxBtk) mRNA in recipient xid mice (Fig. 3).

In chimeric mice that have a normal B cell function, the relative concentration of mBtk mRNA to mxBtk mRNA in splenic B cells (623 ± 17.1) was increased compared with that in BM B cells (24.8 ± 14.8) at 20 weeks after transplantation (Table 2). On the contrary, in the mice treated with the vector, the ratio of hBtk mRNA to endogenous mxBtk mRNA in splenic B cells (4.9 ± 2.5) was even lower than that in BM B cells (8.3 ± 2.9). The hBtk mRNA concentration in HIV treated xid mice is less than one tenth of the mBtk mRNA concentration in chimeric mice. The extremely low level of hBtk expression in splenic B cells might explain why B cell function was not restored after gene therapy.

**Discussion**

The present study examined the feasibility of HIV vector-mediated gene therapy in xid mice. A similar trial of stem cell gene therapy in xid mice using a classical oncoretroviral vector could not restore B
Table 2 Molecular characterization of vector-treated mice and chimeric mice
Between 0.21 to 0.33 copies per cell were detected in all cell populations by semiquantitative PCR at 20 weeks after transplantation. Therefore, it seems that the proportion of hBtk+ cells was maintained during splenic B lineage development. The relative concentration of normal Btk mRNA to mxBtk mRNA in splenic B cells was increased compared with that in BM B cells in chimeric mice, but in HIV-treated mice, the said concentration was lower than that in BM B cells. Data show mean values ± standard deviations (n = 5 ~ 6). ND indicates not done.

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<th>Transgene copy number/cell</th>
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<td></td>
<td>Bone marrow</td>
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<td>HIV treated</td>
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<td>B cells</td>
<td>0.23 ± 0.07</td>
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<td>Non-B cells</td>
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<td>Chimeric</td>
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<sup>a</sup> The relative concentrations of normal Btk mRNA to endogenous mxBtk mRNA.

Fig. 3 Expression of hBtk in B cells and non-B cells of BM and spleen of reconstituted xid mice.
RNA extracted from B cells and non-B cells of BM and spleen were analyzed by semiquantitative RT-PCR. (A) Hha I restriction maps of RT-PCR amplified cDNA from CBA/J wild type mice (mBtk), human Btk (hBtk) and mutant xid Btk (mxBtk). The xid mutation is a C to T transition in exon 2 of mBtk that results in the loss of a Hha I restriction site. Specific Hha I fragments for hBtk, mBtk, and mxBtk are 140 and 162-bp, 95, 55 and 154-bp, 95 and 209-bp, respectively. (B) Spreadex EL 1200 gel Electrophoresis of Hha I digested RT-PCR products. The fragment sizes of hBtk, mBtk and mxBtk are 162-bp, 154-bp and 209-bp, respectively. By comparing the ratio of the hBtk and endogenous mxBtk mRNA concentration, the intensity of the fragments was calculated with an ATTO Densitograph Lane Analyzer.

The disadvantages of retroviral vectors include the inability to transfer genes into non-dividing cells<sup>13</sup> and the silencing of gene expression<sup>14,15</sup>. Since HIV-based vectors can overcome these problems<sup>16</sup>, we surmised that HIV mediated gene transfer might correct the phenotype of xid mice. However, our data showed that HIV vector-mediated expression of hBtk promotes B cell function<sup>12</sup>.
development but cannot correct B cell function in xid mice.

One reason for the failure to correct the xid phenotype might be that the concentration of hBtk was too low to correct B cell function. Studies of a mouse Btk transgene driven by the Ig heavy chain enhancer and promoter in xid mice have demonstrated that B cell development and function require different levels of Btk activities and that the complete rescue of B cell function might require the transgenic expression of almost normal amounts of endogenous Btk. Our RNA analysis showed that the concentration of normal Btk mRNA in splenic B cells of chimeric mice reached at least up to 60%, while the concentration of hBtk mRNA was only 5% of that of endogenous mxBtk mRNA. Since the copy number of the hBtk transgene is about 0.2 per cell, the amount of functional hBtk in cells transduced with HIV vector is less than 25% of that in normal cells. This amount of hBtk might be insufficient to correct B cell function.

It has been recently reported that B cell development and function could be corrected in Btk−/−/Tec− double knockout mice but not in xid mice by retroviral mediated introduction of the normal hBtk gene into BM cells22. Tec and Btk belong to the Tec kinase family and have redundant functions in B cell development and activation7. Compared with xid mice, Btk−/−/Tec− mice exhibited a more severe block in B-lineage development at the earlier stage and its phenotype is more similar to XLA. Yu et al. claimed that successes in gene therapy of Btk−/−B cells is probably due to the strong selective advantage of Btk− pre B cells relative to Btk−/−B cells in this model system. In contrast, expansion of Btk− pre B cells may not occur in xid mice, because development of Btk−/−B cells is not severely impaired. According to this hypothesis, severe forms of XLA can be easily treated by stem cell gene therapy. However, the clinical severity of XLA is variable, from mild to severe, dependent on the specific mutations of Btk.22 It is important to develop therapeutic strategies for xid mice that exhibit milder phenotype.

Another point to consider is that regulated expression of Btk may be important for normal B cell development. It is known that Btk expression triggers apoptosis under some particular conditions22. Btk levels remain constant in the BM but significantly decrease when cells enter the periphery. Splenic B cells have 3-10 fold less Btk than BM B cells. Btk levels significantly increase when splenic B cells are stimulated with anti-IgM, which is consistent with the notion that B-cell functional responses require high levels of Btk expression relative to the survival or development of mature B cells23. Reconstitution experiments also support the importance of regulated Btk activity for normal B lymphopoiesis. B cell function in xid mice was completely restored by partial transplantation of normal HSCs in which the Btk gene is regulated by endogenous regulatory elements24. The B cell defect was completely corrected in transgenic mice harboring the 340 kb human genomic sequence including the 19 exons of the Btk gene and the upstream and downstream sequences21. In contrast, a Btk transgene driven by the Ig heavy chain enhancer could not rescue the normal trinitrotrophenol (TNP)-Fc response. Btk causes defects in the functional B cell response. In fact, 50% overexpression of Btk causes a small but measurable reduction in B cell receptor (BCR)-induced B cell proliferation and several TI-II antigens25. Present study indicated that HIV mediated constitutive expression Btk in HSCs was not sufficient to restore B cell function in xid mice. Stage-specific regulation of Btk expression may be required for normal B cell lymphopoiesis.

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