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Genetic modification of bone-marrow mesenchymal stem cells and hematopoietic cells with human coagulation factor IX-expressing plasmids

Mohammad Reza Sam [a, b, *], Azadeh Sadat Azadbakhsh [a, b], Farrah Farokhi [b], Kobra Rezazadeh [a], Sohrab Sam [a], Alireza Zomorodipour [c], Aliakbar Haddad-Mashadrizeh [d], Nowruz Delirezh [e], Aram Mokarizadeh [f]

[a] Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Urmia, Iran
[b] Department of Histology and Embryology, Faculty of Science, Urmia University, Urmia, Iran
[c] Department of Molecular Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
[d] Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
[e] Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
[f] Department of Immunology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran

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ABSTRACT

Ex-vivo gene therapy of hemophilias requires suitable bioreactors for secretion of hFIX into the circulation and stem cells hold great potentials in this regard. Viral vectors are widely manipulated and used to transfer hFIX gene into stem cells. However, little attention has been paid to the manipulation of hFIX transgene itself. Concurrently, the efficacy of such a therapeutic approach depends on determination of which vectors give maximal transgene expression. With this in mind, TF-1 (primary hematopoietic lineage) and rat-bone marrow mesenchymal stem cells (BMSCs) were transfected with five *hFIX*-expressing plasmids containing different combinations of two human β-globin (*hBG*) introns inside the *hFIX*-cDNA and Kozak element and hFIX expression was evaluated by different methods.

In BMSCs and TF-1 cells, the highest hFIX level was obtained from the intron-less and *hBG* intron-I,II containing plasmids respectively. The highest hFIX activity was obtained from the cells that carrying the *hBG* intron-I,II containing plasmids. BMSCs were able to produce higher hFIX by 1.4 to 4.7-fold increase with activity by 2.4 to 4.4-fold increase compared to TF-1 cells transfected with the same constructs. BMSCs and TF-1 cells could be effectively bioengineered without the use of viral vectors and *hFIX* minigene containing *hBG* introns could represent a particular interest in stem cell-based gene therapy of hemophilias.

1. Introduction

Hemophilia B is an X-linked bleeding disorder results from the absence or low level of FIX in plasma [1].This genetic disease causes high mortality in patients and its treatment requires frequent infusion of normal hFIX, produced either from human plasma or recombinant expression systems [1].

The high cost of replacement therapy, risk of blood-borne pathogens transmission, formation of inhibitors, allergic and thrombosis reactions, caused attention to other treatments, including gene and cell-based therapy of hemophilias [2–4].

Two types of strategies have been considered so far for treatment of hemophilias. A first strategy, consist of removing cells from the patients, modifying the cells *ex vivo* and injection of genetically modified cells expressing the therapeutic protein to the patients

* Corresponding author. Department of Cellular and Molecular Biotechnology, Institute of Biotechnology, Urmia University, Urmia, P. O. Box 165, Iran. Tel./fax: +98 4433440199.
E-mail addresses: m.sam@urmia.ac.ir, s_mohammadreza@yahoo.com (M.R. Sam).

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[4].The second strategy, involved a direct injection of a vector containing the transgene in target tissues or in the circulation [5].

Several potential target cells were shown to be able to produce an active hFIX such as hepatocytes [6], myoblasts [7], keratinocytes [8,9], endothelial cells [10], bone-marrow stromal cells [11] and hematopoietic stem cells (HSCs) [12].

Among examined cells, hepatocytes are the main cellular host for expression of functional hFIX. These cells induce antigen-specific tolerance with successful delivery of synthesized FIX protein into the circulation [13]. Thus, they are attractive candidate for cell-based gene therapy approaches. Unfortunately, the isolation of sufficient transplantable hepatocytes depends on and restricted by different conditions and viral liver infections in hemophilias. Therefore, attention to other suitable bioreactors for production and delivery of hFIX into the circulation is essential. HSCs and BMSCs are attractive target cells in this regard. HSCs have capacity for self-renewal with immediate access to the blood circulation by virtue of their progeny representing all blood cell types, induction of antigen-specific tolerance, ability to trans-differentiate into non-hematopoietic cells such as hepatocytes and relative ease of transfusing *ex vivo*-generated stem cell-derived cells [12,14,15]. BMSCs have several unique properties which make them ideally suited for cell-based therapy of hemophilias. These include; relative ease of isolation from various tissues, the ability to self-renew and differentiate into multiple lineages, the ability to be extensively expanded in culture without loss of differentiation capacity and lower immunogenicity in transplant procedure with anti-inflammatory properties [16,17].

Despite the improvement of recombinant viral vectors, little attention has been paid to the bioengineering of transgene itself and generation of genetically modified cells using plasmid vectors. In this respect, introduction of first intron of hFIX into *hFIX*-cDNA increased hFIX production compared to the intron-less cDNA [18]. In our previous study, *hBG* intron-II and *hBG* intron-I,II were introduced at their corresponding positions inside the *hFIX*-cDNA and increased hFIX production by 5 to 25-fold increase in comparison with the intron-less cDNA in Chinese hamster ovary (CHO) cells [19].

Human β-globin gene expressed at high level in hematopoietic cells and experimental studies showed that the *hBG* introns play critical roles in the expression of the corresponding gene [20]. The enhancer-like activities of the *hBG* introns on the expressions of different transgenes have also been shown [21–23]. With this in mind, we assumed that *hBG* introns would improve the hFIX expression in hematopoietic and mesenchymal expression systems.

To examine this assumption, in our bioengineering strategy, five *hFIX*-expressing plasmids with different combinations of two *hBG* introns inside *hFIX*-cDNA and Kozak element were constructed and introduced into the TF-1 cells as a model for primary hematopoietic cells and BMSCs.

Our main goals in the present study were to evaluate the potential of nonviral FIX gene transfer into hematopoietic and mesenchymal stem cells for expression, storage and secretion of biologically active hFIX from the *hBG* introns containing constructs *in vitro*.

2. Materials and methods

2.1. Bacterial strain, cells, plasmids and primers

DH5α strain of *Escherichia coli* (Strategene, La Jolla, CA, USA) was used as the bacterial host for various cloning and sub-cloning steps. TF-1 cell-line as a model for primary hematopoietic cells was obtained from the National Cell Bank of Iran (NCBI; Pasture Institute of Iran). BMSCs were isolated from the tibias and the femora of rats and used as expression host. Plasmid pET26-hFIX was used for cloning and sub-cloning steps as well as source of the *hFIX*-cDNA. Plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was used for construction of the *hFIX*-expressing plasmids. The primers used for polymerase chain reactions (PCRs) were synthesized by MWG Biotech (Ebersberg, Germany).

2.2. Media, enzymes, chemicals, antibodies and kits

Luria-Bertani medium was used for bacterial growth in which either ampicillin (100 μg/ml) or Kanamaycin (30 μg/ml) was added when required, to maintain selection pressure. RPMI-1640, fetal bovine serum (FBS), bovine serum albumin (BSA) and L-glutamine were obtained from Gibco-BRL Life Technology (Karlsrahe, Germany). Penicillin G and streptomycin were purchased from Sigma-Aldrich (Munich, Germany). All the enzymes used for molecular techniques in addition to kits for PCR product purification, plasmid isolation, RNA preparation, X-tremeGENE HP transfection reagent and protease inhibitors were purchased from Roche (Mannheim, Germany). Interleukin-3 (IL-3) was purchased from R & D systems (Minneapolis, USA). The kit for the cloning of PCR products (InsT/Aclone) was obtained from Fermentas (Burlington, Ontario, Canada). The enzyme-linked immunosorbent assay (ELISA) kit for the measurement of the hFIX antigen (Asserachrom, hFIX:Ag) and FIX-deficient plasma were purchased from Diagnostica Stago (Asnieres sur Seine, France). One-step reverse transcription-PCR (RT-PCR) kit (Accupower RT/PCR PreMix) was purchased from Bioneer (Alameda, USA). Monoclonal antibodies against antigens CD90, CD44 and CD45 were purchased from eBioscience (San Diego, USA). Monoclonal antibody against CD29 was purchased from Abcam, USA.

2.3. DNA manipulation techniques

DNA manipulation techniques were carried out based on standard protocols. *Cis*-regulatory elements used to construction of *hFIX* expression cassettes are shown in Table 1.

2.4. Construction of hFIX expressing plasmids

The first plasmid, p.KhFIX carrying an intron-less *hFIX*-cDNA was used both as the parental *hFIX*-expressing plasmid for the construction of *hBG* intron-containing plasmids and as a control *hFIX*-expressing plasmid. The four other constructed plasmids, namely p.KhFIX-I (carry *hBG* intron-I), p.hFIX-II (carry *hBG* intron-II), p.KhFIX-I, II and p.hFIX-I, II (carry *hBG* intron-I, II) represent the second generations of the p.KhFIX plasmid (Table 1).

Detailed construction of the intron-less and *hBG* intron-containing plasmids were described previously [19].

2.5. Verification of the recombinant plasmids

Verification of various plasmids constructed during cloning and sub-cloning steps was done by restriction digestion followed by nucleotide sequence analysis of both strands of the inserted fragments in the *hFIX*-expressing plasmids; using ABI 373A automated sequencer (MWG-Germany). All expression cassettes preserved the consensus splice donor, acceptor and lariat branch point elements at the 5′ and 3′ end of introns.

2.6. Ethics statements

Prior to study, all procedures were approved by our institution's animal welfare regulatory committee.