

INVITED REVIEW

Mesenchymal stem cell treatment for hemophilia: a review of current knowledge

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Summary. Hemophilia remains a non-curative disease, and patients are constrained to undergo repeated injections of clotting factors. In contrast, the sustained production of endogenous factors VIII (FVIII) or IX (FIX) by the patient's own cells could represent a curative treatment. Gene therapy has thus provided new hope for these patients. However, the issues surrounding the durability of expression and immune responses against gene transfer vectors remain. Cell therapy, involving stem cells expanded *in vitro*, can provide *de novo* protein synthesis and, if implanted successfully, could induce a steady-state production of low quantities of factors, which may keep the patient above the level required to prevent spontaneous bleeding. Liver-derived stem cells are already being assessed in clinical trials for inborn errors of metabolism and, in view of their capacity to produce FVIII and FIX in cell culture, they are now also being considered for clinical application in hemophilia patients.

Keywords: exosomes; hemophilia A; liver; metabolism, inborn errors; stem cells.

Introduction

Hemophilia A is the most common severe inherited bleeding disorder, affecting 1 in 5000 male births. This pathology exhibits different phenotypic expressions depending on its associated factor VIII (FVIII) plasma levels, resulting in severe (< 1%), moderate (1–5%), or mild (6–30%) forms of expression [1]. This disease originates from an inherited deficiency or dysfunction in the procoagulant

FVIII, a crucial element of the intrinsic pathway of blood coagulation involved in the conversion of factor X to Xa [2]. In severe cases, FVIII deficiency leads to spontaneous bleeding and internal hemorrhage that can cause disability and even death, if left untreated [3].

There is currently no cure for hemophilia A. The rationale behind replacement treatment is to sufficiently increase concentrations of the missing factor to arrest spontaneous and traumatic bleeds. Plasma-derived products became available in the 1970s, proving effective in controlling bleeding episodes through the development of home-therapy programs. However, concentrates derived from pooled plasma were contaminated with HIV and the hepatitis B or C virus, causing post-transfusion hepatitis and immunodeficiency in almost all hemophilia patients who received these concentrates. In 1984, the FVIII gene was successfully cloned, enabling the production of recombinant human FVIII (rFVIII) using mammalian cell cultures, and the first rFVIII went on the market in 1992 [4].

The introduction of rFVIII revolutionized hemophilia patient management by providing an effective and safe treatment of bleeding episodes. Nevertheless, there remain several issues concerning FVIII replacement therapy that have yet to be resolved. The primary complications are as follows: the short half-life of replacement products, necessitating frequent intravenous infusions; the immunogenicity of FVIII concentrates; and the affordability and availability of FVIII products [5].

During the last decades, several attempts have been made to develop a long-term 'cure', such as gene and cell therapy. Hemophilia A is a perfect candidate for gene therapy, given its monogenetic nature that can potentially be cured by continuous endogenous FVIII expression. For hemophilia treatment, by increasing circulating clotting factor levels to above 1% of normal, it may be possible to obtain a prophylactic therapeutic effect, thereby reducing risks of both mortality and morbidity.

If gene therapy is able to slightly increase clotting factor levels, it could significantly improve the clinical

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phenotype [6]. Recently, hemophilia B gene therapy has achieved promising outcomes in human clinical trials [7]. A key advantage of the development of gene therapy strategies for hemophilia B is the relatively small size of the cDNA of FIX, measuring approximately 1.4 kB of the coding sequence. This renders it amenable to insertion into different gene transfer vectors and enables the addition of numerous transcriptional regulatory elements to both improve and restrict transgene expression in selected cell types. The cDNA of FVIII is much larger than that of FIX (> 8 kB) and cannot be as readily accommodated in gene transfer vectors. Several strategies have previously been attempted to overcome this difficulty, by either deleting the B-domain or using two viral vectors [8].

Moreover, the main complication of viral vector delivery of clotting factor transgenes is the host immune responses [9]. A pre-existing immune response against capsid proteins is one of the criteria excluding hemophilia patients from gene therapy. Approximately 40% of the adult human population possess neutralizing antibodies against the adeno-associated virus (AAV)-2 [10], and these antibodies can cross-neutralize other AAV serotypes. In addition, patients receiving systemic viral vector administration develop a postgene therapy immune response. This response was shown to be associated with the destruction of cells expressing viral proteins after transduction, thereby decreasing the gene transfer's efficacy, along with the development of neutralizing antibodies against the viral vector employed as therapeutic agent, therefore preventing the possibility of vector re-administration.

Orthotopic liver transplantation has proven effective in correcting the hemophilic phenotype in hemophilia patients with decompensated hepatitis C (HCV)-cirrhosis [11]. This suggests that the liver plays a central role in producing blood-clotting factors like FVIII. The hepatic cellular compartments that produce FVIII are primarily composed of liver sinusoidal endothelial cells (LSEC) [12,13], although earlier evidence has suggested hepatocytes to be instrumental in FVIII expression [14]. Transplanting such cells that are capable of releasing FVIII *in situ* or into the circulation is an attractive approach for treating clotting factor disorders. Notably, in murine endothelial cell cultures, FVIII production was measured to amount to $0.07 \text{ IU million}^{-1} \text{ cells day}^{-1}$. If we extrapolate this to the 8×10^{10} liver endothelial cells contained in an adult human liver, the production would cover more than that required by a human adult [15]. Transplanting 2×10^6 mature LSECs, representing 10% of their endothelial compartment, via the portal vein in hemophilia A mice was shown to restore plasma FVIII activity levels to 14%–25% of normal, thereby correcting spontaneous bleeding [16].

In addition to endothelial cells, mesenchymal stem cells (MSCs) are also able to secrete FVIII in the cell culture supernatant, which makes them attractive candidates for treating hemophilia. MSCs are increasingly used in sev-

eral medical areas for repairing organs, such as the heart, bone, cartilage, and liver. These are tissue-resident cells that can be obtained from different tissues and organs, exhibiting the propensity to attach onto surfaces and proliferate *in vitro*. In certain conditions, they differentiate into mature cells, such as bone, cartilage, and adipocyte- or hepatocyte-like cells. These cells express specific surface markers, such as CD90 and CD105, and are negative for hematopoietic markers like CD45 [17]. However, MSC from different tissues may differ by other characteristics. For example, unmodified MSCs from human liver and lungs produce FVIII in the culture supernatant in much higher quantities compared to human bone marrow-derived MSCs (1.2 and 1.7% vs. 0.12%, respectively, per 10^5 cells at the 48-h time point) [18].

We similarly tested by chrometric assay factor VIII production in the supernatant of human-derived mesenchymal cells [19], cultured in large-scale in accordance with good manufacturing practices (GMP), produced FVIII measured by chrometric assay in the culture supernatant. (G. Rommelaere, S. Eeckhoudt, C. Lombard, E. Sokal, unpublished data). FVIII levels, accumulated over time, suggesting that the production exceeded degradation, despite the absence of stabilizing von Willebrand factor. This accumulation has been attributed to the fact that these cells do not store FVIII in granules while secreting most of the produced protein [18].

In animal experiments, sheep bone marrow MSCs transduced with porcine FVIII vector were shown to be effective in mitigating joint bleeding following peritoneal transplantation in sheep affected by hemophilia A. The transplanted cells were observed homing to the joints, locally expressing FVIII [20]. Similar results were found in mice, where intra-articular injections of autologous MSCs overexpressing FVIII had a positive impact on hemarthrosis and arthropathy associated with bleeding challenges [21]. In another experiment, injecting bone marrow (BM)-macrophages or BM-mesenchymal stromal cells protected hemophilia A mice from bleeding challenges after 3 days, with FVIII manifesting in the blood [16]. The effect was attributed to bone marrow mononuclear cells and MSCs, given that no donor bone marrow-derived endothelial cells or parenchymal cells were found in these animals.

Extracellular vesicles produced by stem cells

When stem cells are transplanted, a complex interrelationship very likely exists between the contribution of the direct engraftment of the cell at the site of the expected regeneration and the role of the 'material' released by the cells required for mediating engraftment, differentiation, phenotype changes, immunological response, apoptosis, and so on. This type of released material includes not only soluble molecules, such as growth factors, but also extracellular vesicles naturally secreted by the cells. Extra-

cellular vesicles are produced by most eukaryotic cells, including stem cells [22–26]. Although both terms are at times used interchangeably, microparticles (MPs) differ from exosomes on account of their size, content, and the way they are formed. MPs range from 100 to 1000 nm in size and are shed from the cell's plasma membrane as a result of cellular stress. They are enriched in molecules, such as cholesterol, integrins, and flotillins. Exosomes are membranous vesicles ranging from 30 to 100 nm in size and released following the fusion of multivesicular bodies with the plasma membrane. They are enriched in heat-shock proteins, tetraspanins, and endosome-specific proteins, such as Alix and TSG101. Both types of extracellular vesicles also contain deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and micro-RNA (miRNA) [22–26]. This cargo of proteins, lipids, and genetic material is readily transferrable from one cell to another, even between very remote cells, and can influence the phenotype and fate of target cells in transplantation [27]. The genetic material released by the donor cells enters the host cells and either modulates the expression of endogenous genes, as is the case with miRNAs, or is expressed and can contribute to the restoration of an impaired cell function, as with mRNAs [22–26]. For example, exosomes released from MSCs have been found to mediate, at least in part, the observed regenerative effects of applied MSCs in the context of cardiac tissue repair [28]. Similarly, the experimental and clinical use of MSCs and liver stem cells in the setting of alcoholic liver disorders has highlighted the role of microparticles and exosomes in regenerative processes [29]. In addition, microparticles from murine embryonic stem cells have been shown capable of reprogramming hematopoietic progenitors through RNA transfer [30]. Previous studies have already suggested that microparticles play a part in coagulation, as these particles can express phosphatidylserine and bind active coagulation factors [24]. Moreover, they express tissue factor, which initiates the coagulation cascade [24,31]. Recent studies from our laboratory demonstrated that extracellular vesicles produced by human liver-derived MSCs also contain mRNA coding for FVIII (G. Rommelaere, S. Eeckhoudt, C. Lombard, E. Sokal, unpublished data). Future investigation is now required to demonstrate whether infused MSCs could influence host cells, including recipient liver endothelial cells, and promote their secretion of FVIII through extracellular vesicle-mediated horizontal transfer of FVIII mRNA.

What we have learned from mature liver cell transplantation in humans?

Mature liver cell transplantation has been successfully performed in a variety of inborn errors of metabolism cases [32,33]. Mature hepatocytes are not considered FVIII-producing cells and therefore not candidates for treating hemophilia A [12,13]. Liver cell transplantation

has significantly increased our clinical experience and skill in terms of using a portal vein approach for delivering cells into the liver, via either the percutaneous route or an implanted catheter system [23,34]. The liver is the ideal organ for cell delivery and homing, given that the portal system is a low-pressure, terminal circulation system.

Allogeneic liver cell transplantation in two siblings with FVII deficiency induced a decrease in FVII requirement to 20% of the pretreatment doses at 8–10 weeks post-therapy [35]. The result was transient, however, and both patients later required full liver transplantation, at 7 and 8 months following the liver cell transplantation, respectively [35]. This constituted the first evidence that cell transplantation in humans can correct clotting factor deficiencies, although hepatocytes would probably not be a suitable cell choice for hemophilia A for the aforementioned reasons. Clinical liver cell transplantation experiments in various conditions have demonstrated that, while it is feasible to mitigate the underlying metabolic defect, the durability of response is limited to a maximum of 12–18 months, and any cure of the disease is only partial [17,32,33,36,37]. Partial activity or protein replacement may, however, be sufficient to transform severe disease into that with a mild phenotype. To overcome the problem of liver donor shortages, it is thus feasible to use liver cells from one metabolic patient to treat another suffering from a different metabolic disease. This is known as the 'domino' concept and is already exploited in orthotopic liver transplantation [38].

Despite these findings, hepatocyte technology has not emerged as a widely applicable technique given the following obstacles to its use: the requirement of one liver for only a few recipients, the limited durability, the poor resistance of hepatocytes in surviving cryopreservation and storage [39], the risk of pathogen transmission comparable to the risk in full-organ transplants, and the lack of adequate infrastructure in most hospitals to enable cell preparation for infusion.

MSCs in human clinical applications

The human clinical use of MSCs is based on the different properties of these cells, including replacing a patient's deficient cells in the target tissue [40], promoting endogenous tissue repair from the patient's own pluripotent stem cells [41], and providing immunomodulatory treatment in conditions like graft versus host diseases or other immune-related diseases [42]. When MSCs are used to treat genetic disorders, they must be of allogeneic origin, while autologous cells can be used for tissue repair in acquired diseases. In 2012, over 2000 patients underwent cell-based therapies in Europe, half of which consisted of the transplantation of MSCs primarily originating from the bone marrow or adipose tissue [43]. The cells were autologous in 60% of patients, who mostly suffered from rheumatological, musculoskeletal, or cardiovascular disorders. Allo-

genic cells were primarily used in hematology–oncology disorders. The MSCs transplanted in these cases were obtained from the bone marrow or adipose tissue, yet these cells can also be harvested from Wharton’s jelly in the umbilical cord or from other tissues, including the liver [19,44]. Adult human liver-derived MSCs can, when submitted to specific hepatocyte differentiation protocols, acquire functional capacities, such as urea synthesis, bilirubin conjugation, *de novo* glucose synthesis, and cytochrome P450 activities. These cells express and produce FVIII in undifferentiated state, yet lose this capacity following hepatocyte differentiation, thus providing further evidence of the absence of FVIII expression by hepatocytes [12,13]. Adult human liver-derived MSCs have been demonstrated capable of engrafting and repopulating rodent livers following infusion in the portal system [19,45].

Before exploring human applications of liver-derived MSCs, preclinical safety studies demonstrated that these cells in culture do not proliferate indefinitely, but reach normal senescence. In culture conditions, they do not express oncogenes, exhibit a normal shortening of telomerase following culture passages, and do not induce tumors when injected into immunodeficient mice [46]. In humans, MSCs have proven safe in numerous different clinical applications and have never been reported to cause malignancies in humans in the numerous ongoing trials [43].

It was demonstrated that, in one patient, indium-labeled liver-derived MSCs distribute uniformly and persist in human liver tissue following portal infusion, with no extra-hepatic distribution [47]. Liver-derived MSCs were successfully administered in a young infant suffering from urea cycle disease, and transplanted cells could be identified in the liver 3 months following infusion in two different biopsy samples [48].

A Louvain University spin-off biotech company, Promethera Biosciences, successfully completed phase I/II multicentre clinical trials using liver-derived MSCs under European Medicine Agency (EMA) regulations, following a Pediatric Investigation Plan. The trial included 20 pediatric patients suffering from urea cycle disorder or Crigler–Najjar syndrome. Safety and preliminary efficacy data were presented at the International Society for Inborn Errors of Metabolism (SSIEM) in 2014 [49]. The patients had received cell quantities corresponding to between 0.3 and 4% of their theoretical liver mass, that is, 12.5–200 million cells per kg. The preliminary efficacy data demonstrated improvement in urea synthesis in several patients with urea cycle disorder, as assessed by the measurement of C13 incorporation into the urea [49].

Other stem cells have now undergone full clinical development planning. As an example, in 2014, the Committee for Medicinal Products for Human Use (CHMP) at the European Medicines Agency (EMA) recommended Holoclar^R (Chiesi Farmaceutici, Parma, Italy), the first advanced therapy medicinal product (ATMP) containing *ex vivo* expanded human epithelial cells, for approval in

the European Union (EU). Holoclar^R is a treatment for moderate to severe limbal stem cell deficiency caused by physical or chemical burns to the eyes in adults.

In conclusion, stem cell technology is an attractive approach for treating congenital genetic diseases, such as hemophilia. This concept has been proven valid for inherited metabolic disorders, with hepatocyte transplantation demonstrating that metabolic activities can be transferred to recipients of allogeneic cells, and this approach is now in clinical development using stem cell technology. This technique ensures additional safety, as the stem cells are produced in accordance with GMP under strict control. Different types of stem cells can produce coagulation factors in culture conditions, including liver-derived MSCs. When such cells are transplanted *in vivo*, the mechanism of action can be related not only to the direct engraftment of stem cells producing FVIII or FIX, but possibly also via the transfer of genetic material incorporated into extracellular vesicles and exosomes.

Disclosure of Conflict of Interests

E. Sokal is a full-time employee of the Université Catholique de Louvain. He is founder of the spin-off biotech issued from his laboratory at the university (Promethera Biosciences), and he is scientific consultant for the company and receives honoraria. He is entitled to founding shares from the university. The other authors state that they have no conflict of interest.

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