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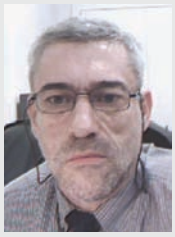

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"There is much reason for optimism, but caution is imperative in order not to raise false expectations in our patients."

Future therapeutic products based on advanced therapies such as gene and cell therapy and tissue engineering or induced pluripotent stem cell technology may offer innumerable potential clinical applications for the treatment of several monogenic disorders including hemophilia. Although hemophilia is particularly amenable to treatment with these techniques given its monogenic nature and the lower levels of deficient coagulation factor required to achieve a moderate phenotype, research in the hemophilia field is still at a teething stage and further work must be undertaken to determine whether advanced therapies can be safely applied to this patient population, which presents specific clinical characteristics. There is much reason for optimism, but caution is imperative in order not to raise false expectations in our patients.

The development of biotechnology has resulted in the emergence of new therapies that are bound to change medical practice. Advanced gene-, cell- and tissue-based therapies (gene therapy, cell therapy and regenerative medicine [101]) constitute new strategies for the treatment of some diseases [1]. Their purpose is either the regeneration of tissues or the restoration of function. Cell therapy consists of the transplantation of living cells into an organism in order to repair tissue or restore an absent or deficient function. Gene therapy, in turn, consists of transplantation of genetically modified cells so that they may produce a deficient protein.

Cells are useful in these therapies because of their ability to differentiate into the specific cell lines required for restoring a given type of tissue. Nonetheless, only 20% of stem cell-related studies in the literature constitute a genuine advancement in scientific knowledge. This can be attributed to the high cost of this kind of research and to the multiplicity of yet-to-be-resolved issues such as the need to develop new guidelines on best practices in cell culture and cell transplantation procedures, and guarantee the genetic stability of stem cells before and after transplantation, their quantity and quality when used therapeutically and their safety, specifically with respect to the absence of teratogenicity [2].

Current treatment of hemophilia is based on the replacement of deficient coagulation factors by prophylactic or on-demand intravenous administration. Given the monogenic nature of hemophilia and the low coagulation factor levels required to convert a severe into a mild or moderate phenotype, it is thought that the disease may in future be particularly amenable to treatment with advanced therapies [3–5]. Most clinical and preclinical studies conducted to date on the effects of cell therapy and gene therapy on hemophilia, using both viral and nonviral vectors, have shown no adverse effects, although the immune response against the vectors' viral envelopes and the transgenes encoded constitute the limit to the clinical application of these therapies.

Gene therapy strategies for hemophilia have been based on the use of lentiviral and adeno-associated virus (AAV) vectors, adult stem cells and autologous fibroblasts, platelets and hematopoietic stem cells.

Keywords: advanced therapies • cell therapy • gene therapy • hemophilia

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Nonviral gene transfer and chimerical oligonucleotide-based mutation repair have also been used. Cell therapy for hemophilia is based, mainly, on the transplantation of healthy cells to repair or replace deficient functions, for example the transplantation of liver sinusoidal endothelial cells or endothelial progenitor cells derived from induced pluripotent stem cells. Of particular interest in the field of advanced therapies are the results obtained by High *et al.* who used zinc finger nucleases and adeno-associated vectors to correct hemophilia B mutations through the 'editing' of DNA-mutated sequences [6]. Although in this case factor IX (FIX) expression is only 5% of normal levels, the advantage of this strategy is that it allows strict control of the integration of normal sequences into DNA, thus preventing the development of insertional mutagenesis-induced tumors.

In our laboratory, we have used nucleofection-based nonviral gene transfer to promote the expression of human FIX in adult adipose tissue-derived mesenchymal stem cells [7]. Despite the fact that the expression efficacy of the nonviral method is lower than that obtained with viral vectors, the former provides higher safety levels, with the additional advantage that 5% of the coagulation factor level is enough to transform a severe hemophilia phenotype into a moderate one. The most significant problems that remain to be addressed are related to increasing the efficacy of factor expression levels and maintaining them at a constant level in the long term, and control of the immune response to vectors and transgenes.

> **"...research in the hemophilia field is still at a teething stage and further work must be undertaken to determine whether advanced therapies can be safely applied to this patient population, which presents specific clinical characteristics."**

Nathwani *et al.* have recently completed a clinical trial in patients with hemophilia B. The trial included patients with severe hemophilia B (<1% FIX) who were injected with a serotype-8-pseudotyped, self-complementary AAV, that expresses FIX and can efficiently transduce hepatocytes [8]. This is a more efficient vector as it obviates the need for second-strand synthesis or re-annealing of positive and negative AAV strands to generate transcription-competent dsDNA templates. The results showed that patients expressed between 3 and 11% of normal FIX levels. Another encouraging finding was that no inhibitors (anti-FIX antibodies) were detected. These results must be considered taking into account, first of all, that the expression of FIX corresponds to a mild-to-moderate phenotype of the disease and, second, that concomitant glucocorticoid treatment is required in order to prevent immune rejection and an elevation of liver transaminases.

The results obtained to date constitute the beginning of the future application of advanced therapies to the treatment of hemophilia. The number of patients included in the clinical trials on advanced therapies conducted so far, including the one by Nathwani *et al.*, has been very low. In addition, results have been highly variable. Although the clinical trial by Nathwani *et al.* is the first study to show a substantial expression of FIX in humans, immune-mediated clearance of AAV-transduced hepatocytes remains a concern. Specifically, a patient who had received a high dose of the vector developed Grade III liver toxicity, which was related to the vector itself. The same patient also showed an increase in plasma transaminase levels and a concomitant decrease in FIX levels. All of these findings were related to the presence of AAV8 caspid-specific T cells. An important question that remains to be resolved is that of the potential relationship between liver toxicity and the immune response it generates. Additionally, there are still a few things to be clarified about the study by Nathwani *et al.*, such as the case of two patients who were administered a mild dose of the vector and, although they did not present with liver toxicity, they did exhibit an immune response to the vector capsid. In order to resolve this, future clinical trials must include a larger number of patients. Another important consideration is that even if the expression levels obtained are substantial, they are not sufficient to consider healing of hemophilia and to assume that bleeding episodes further to trauma or injury will be prevented. Future efforts must be aimed at improving the design of the vectors as the truncated liver-specific promoter used in this clinical trial to accommodate the *FIX* gene in the self-complementary AAV backbone to make it more efficient. This could allow the use of lower vector doses and the reduction of potential vector-dependent liver toxicity. Another drawback is the problem of transient transgene expression.

There is much reason for optimism, but we need to act cautiously so as not to create premature expectations in our patients. It is almost two decades since the first reports on the benefits of gene therapy for the treatment of hemophilia came to light. In those days, prominent experts envisioned that the cure of this disease could become an achievable goal by the first decade of the 21st century [9]. These predictions fueled the hopes of both hemophilic patients and the physicians treating them, but unfortunately they were in for a disappointment. Problems started to emerge, especially in the field of biosafety, as early as the 1990s when the first clinical trials on gene therapy were launched. Although significant progress has been made since then in terms of the design of transfer vectors, some of the drawbacks associated with the technique have not as yet been fully overcome (host immunity, insertional mutagenesis, efficacy and expression time, clinical trial recruitment and large-scale vector production).

The first question that must be asked is whether the time and financial investment required to establish advanced therapy protocols, that may in future be applied to the treatment of this pathology is justified. Although it must be admitted that current treatment of hemophilia is optimal, the answer to the question is in the affirmative since hemophilia is a chronic condition and current high-frequency – especially in prophylaxis – treatment protocols apart from being extremely costly, could result in devastating pathogen-induced infections. The second question is whether advanced therapies are at all feasible. In this regard, hemophilia

is considered an optimal candidate for such treatments as it is a monogenic disease; the expression of low levels of the coagulation factor (1–5%) can achieve a moderate phenotype; a large variety of applicable target cells exist; there is no need to regulate factor expression; and a large amount of animal models are available for experimentation.

Other more general questions that may be raised include whether it will be possible to extrapolate the safety- and expression level-related outcomes obtained in animals to human beings, whether the combination of cell therapy/gene therapy with the use of mesenchymal stem cells will be the most efficient tool and whether protocols will have to be restricted to AAV and nonviral vectors.

The problems to be addressed in the future are the immunogenicity and biosafety of the therapies as well as the maintenance of the levels and time of factor expression. Moreover, although most research is currently focused on viral vectors, nonviral vectors should also be taken into consideration.

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At any event, the main criterion to be considered should be the ratio between efficacy and safety, taking into account that this is a highly sensitive issue for both patients and physicians given the special immunologic situation of the patients and the lethal consequences that viral infections (HIV/HCV) have had on the hemophilic population in the past. Would it be wise to forego a set of stringent expression requirements in return for greater safety [10]?

Furthermore, caution must be exercised when bringing to light the results of any studies that may be conducted in the field. With regard to the clinical trial by Nathwani *et al*., in which severe hemophilia B patients were subjected to perfusion of a serotype-8-pseudotyped, self-complementary AAV that expresses FIX, its results must be examined taking into account first and foremost that the expression levels obtained correspond to a moderate-to-mild phenotype of the disease. Moreover, it must be remembered that concomitant gluco-corticoid therapy is indispensable to prevent immunological rejection and normalize liver transaminase levels. In addition, the virus is hepatotoxic, the number of patients is limited and results are highly variable.

Although the results reported by Nathwani *et al.* are a breakthrough, the significant difficulties posed by the different strategies of gene and cell therapy used so far require extreme prudence and objectivity to be exercised before any conclusions are drawn. Statements in the study such as "If further studies determine that this approach is safe, it may replace the expensive protein therapy currently used for patients with hemophilia B," or in the accompanying editorial [11], are rather unwarranted, because it may be years or even decades before this technology can be used in clinical practice.

False expectations with respect to advanced therapies that are only in the initial phases as potentially highly promising therapeutic strategies should not be raised. In the longer term, after overcoming the challenges mentioned above, advanced gene- and cell-based therapies might become a plausible alternative for patients with hemophilia. Optimism is in order, but fantasy is best avoided.

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