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REVIEW

Cells as vehicles for therapeutic genes to treat liver diseases

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Gene therapy involves the transfer of genetic sequences to tissues to obtain a curative effect. Effective gene transfer can be achieved by introducing the therapeutic gene into virus-like particles that facilitate the penetration of the transgene into the cells. However, direct injection of viral vectors may activate innate immunity leading to toxic effects. On the other hand, viral vectors frequently induce neutralizing antibodies, which limit the efficacy of repeated vector administration. Moreover, targeting of the transgene to the desired tissue is a goal that not always can be attained with current vectors. The use of cells as vehicles for therapeutic genes may offer solutions for these issues. Ex vivo *transduction of specific cells with vectors encoding therapeutic genes followed by injection of the engineered cells to the patient will reduce the inherent toxicity of the vector while preventing the development of neutralizing antibodies. At the same time, this therapeutic approach can take advantage of the homing properties of the transduced cells to target transgene expression to the sites of interest. Thus, it has been shown that administration of dendritic cells engineered* ex vivo *with vectors encoding selected antigenic determinants or immunostimulatory molecules is an efficient means to elicit protective immune responses. Similarly, since endothelial progenitor cells (EPC) move to inflammed, ischemic or neoplastic tissues, the injection of EPC transduced* ex vivo *with appropriate therapeutic genes is an effective method to direct transgene expression to the lesions to be treated. Promising data in animal models of disease point to a future clinical application of this therapeutic strategy.*
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Introduction

Gene therapy has emerged as an efficient procedure to treat human diseases. This method is based on the transfer of genetic material to tissues to induce a curative effect. Gene therapy vectors are molecular constructs used to facilitate the penetration of the therapeutic genetic material inside the cells. Vectors are frequently generated from viral genomes, which are modified to eliminate their pathogenic properties and adapted to transport the therapeutic gene(s). Non-viral vectors have, in general, lower transduction efficiency than virus-based vectors. Viral vectors have, however, several limitations when administered directly to the patient. They may cause significant toxicity by activating innate immunity or by eliciting an adaptive immune response against viral proteins. In the former case, a systemic inflammatory reaction (which may be lethal) can occur and in the latter case, the transduced cells can be eliminated by activated T cells leading to tissue damage and abrogation of transgene expression.[1] On the other side, when the vector is injected directly into the subject, it can stimulate the production of neutralizing antibodies making re-treatment ineffective.[1] In addition, targeting the vector to the desired site is an issue when given systemically.

In recent years, the use of specific cell types transduced *ex vivo* with gene therapy vectors and then injected into the patient has generated a great deal of interest. This combination of cell and gene therapy has several appealing aspects. One of the advantages is that specific cell types serving as vehicles for therapeutic genes can be attracted to the desired locations by chemotactic factors produced at the site of tissue injury or inflammation. Neutrophils, for instance, express the chemokine receptors CXCR1 and CXCR2 and are directed to tissues with acute or chronic inflammation by chemokines CXCL8/interleukin (IL)-8 (which interacts with both CXCR1 and CXCR2) and CXCL1 (selective for CXCR2) produced in the inflammatory milieu. It is conceivable that *ex vivo* arming of neutrophils with cytoprotective or antifibrogenic genes followed by systemic administration of these engineered cells may be a useful approach to reduce inflammatory organ damage or excessive fibrogenesis in inflammed organs (Pereboeva L, personal communication).

The use of cells as vehicles for therapeutic genes has other additional advantages. The administration of gene-modified cells instead of viral particles to the patient prevents the direct interaction of the vector with Toll-like

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receptors, thus avoiding activation of innate immunity. This would reduce or abolish the inflammatory reaction, which frequently follows treatment with recombinant viruses contributing to better tolerance of the therapy. Similarly, it is expected that neutralizing antibodies will be a less problem when using transduced cells as therapy.

Combining cell and gene therapy demands the development of methodologies for efficient gene transfer to the purified cell population, which is to be employed as gene carrier. In a pioneer work, Pereboev *et al*.[2] were able to increase considerably the transduction efficiency of dendritic cells (DCs) with adenoviral vectors using an 'adapter' to enhance the interaction of the virus with the cell membrane. This adapter protein consists of the ectodomain of CAR (the cell receptor for adenovirus) fused to mouse CD40 ligand through a trimerization motif—fibritin. This adapter was able to bind both human and mouse CD40 and adenoviral fiber knob. Incubation of adenoviral vector with the adapter not only dramatically increased the transduction efficiency of DCs, but also induced their maturation as reflected by a marked increase in the synthesis of IL-12 upon incubation of the cells with adapter-coated adenovirus.[2] As in the case of DCs and adenoviruses, adapters can also be designed for other cell types (neutrophils, endothelial progenitor cells (EPCs), and so on) and for other viral vectors.

In this short review, we will focus mainly in the use of two cell types as vehicles for therapeutic genes to treat liver diseases: DCs and EPCs.

Induction of antitumor and antiviral immunity using engineered DCs

DCs have been widely employed as cell vehicles for genes subservient immunotherapeutic purposes in patients and animal models of liver diseases. These cells are professional antigen-presenting cells having important functions: sensing the presence of pathogens and linking innate and acquired immunity by presenting antigens to T cells.[3] DCs monitor their environment becoming activated upon antigen encounter or during inflammation caused by pathogens. This process, also known as maturation, leads to upregulation of MHC, adhesion and co-stimulatory molecules, as well as to expression of cytokines, chemokines and chemokine receptors, which increase the ability of DCs to migrate to lymph nodes and to attract and activate T-cells. Due to these properties, DCs have been considered for the formulation of cell vaccines potentially useful for the treatment of liver tumors and chronic hepatitis caused by hepatitis B (HBV) and C viruses (HCV). For these applications, DCs can be genetically modified to enhance their immunostimulatory potential.

Concerning genetic modification of DCs for antitumor therapy, two main approaches have been used: expression of genes of tumor antigens which can serve as potential targets for T cells, or expression of genes of cytokines and molecules enhancing T-cell functions. In the former case, DCs are transfected with previously well-characterized tumor antigens before administration to animals or patients. Antigens expressed by tumor cells or by antigen-presenting cells in the tumor environment are poorly immunogenic. Consequently, administration of *ex vivo*-activated DCs can potentially overcome the lack of immunogenicity of tumor antigens. The most widely used antigen for inmmunotherapy of hepatocellular carcinoma is alpha-fetoprotein,[4–6] which is produced by many liver tumors. Tumor antigens commonly expressed by gastrointestinal malignancies, such as carcinoembryonic antigen and mucin 1,[7] have also been used to treat metastatic liver cancer. Different type of vectors have been employed to transduce DCs, including lentivirus,[8] vaccinia virus[7] and adenovirus.[4,5,9] Adenoviruses have frequently been employed, as they not only infect DCs but also induce DCs maturation.[10] In some cases, DCs have been transfected using mRNA from the tumor extract,[6,11–13] a procedure useful when the relevant tumor antigen is unknown. Besides tumor antigens, genes encoding cytokines and/or costimulatory and adhesion molecules have also been transferred to DCs. IL-12 and IL-18[14,15] are the main cytokines in the first group, due to their ability to shift T-cell responses towards a Th1 profile, whereas CD80, ICAM-1 or LFA-1 belongs to the second group.[16] In some cases, to enhance both antigen expression and DCs function, the cells have been simultaneously transduced with genes encoding tumor antigens, cytokines and/or costimulatory molecules, resulting in higher DC activation and more intense T-cell responses than when using single-gene transduction.[7,17]

In a recent clinical trial conducted in patients with advanced primary or metastatic liver cancer, monocyte-derived DCs engineered with adenoviral vector encoding IL-12 were injected directly into the tumor.[18] In this trial, 3 monthly doses of the transduced cells were administered, and it was expected that, after intratumoral injection, DCs would take up tumor antigens and migrate to regional lymph nodes to stimulate an antitumor T-cell response that would be facilitated by the *in situ* production of IL-12. In this trial, the observed antitumor effect was very poor. By radioactive labeling of the cells and single photon emission computed tomography imaging, it was demonstrated that DCs did not migrate to lymph nodes, but were retained inside the tumor mass. The authors showed that tumor nodules produced high levels of IL-8, which possibly forced DCs expressing CXCR1 and CXCR2, to remain at the place of the injection, failing to orchestrate antitumor immunity. These results indicate that engineered DCs should be rather pulsed with tumor extracts *ex vivo* and then injected inside or in the vicinity of lymph nodes.

In chronic viral infections caused by HBV and HCV, different reports have proposed the use of DCs transduced with genes encoding viral antigens as a possible therapeutic vaccination. As in the case of tumors, the expression of genes of viral antigens by DCs transduced and activated *ex vivo*, can result in fully functional antigen presentation, which may overcome the tolerance to viral proteins found in patients with chronic infection. Due to the ability of some viral genes to interfere with the cell machinery responsible for DC function, an important issue in this approach is the choice of the appropriate antigen to elicit effective immune responses. In fact, it has been demonstrated that under certain circumstances, expression of viral genes, such as core and E1 from HCV, may impair DC functions, and as a consequence, induction of T-cell responses.[19,20] Thus, besides a good

immunogenicity, viral antigens to be produced in DC should lack these immunosuppressive properties. In the case of HBV, surface antigen is usually the preferred antigen,[21–23] although core and X[24,25] have also been used. Similarly, for HCV, non-structural 3 (NS3) protein,[26–30] and to a lesser extent core protein,[26,27,31] have been employed. In all cases, the selected antigens should be those that in the course of natural infection provoke immune responses capable of clearing the pathogen. Again, adenoviruses have been the vectors of choice for gene transfer to DCs,[26–31] although other systems have been utilized including adeno-associated viruses,[25] retroviruses[24] and mRNA transfection.[31] By employing this strategy, DCs transfected with viral genes have demonstrated a remarkable ability to induce antiviral T-cell responses both *in vitro* and *in vivo*, being in some cases stronger than those obtained by direct administration of the vector encoding the viral antigen.[30] There is, therefore, a need for pilot clinical trials to determine whether the injection of transduced DCs could be an effective procedure for therapeutic vaccination against HCV and HBV virus infections. This vaccination modality would allow repeated doses of the vaccine without the limitation imposed by the development of neutralizing antibodies.

Bone marrow-derived endothelial progenitor cells as carriers of therapeutic genes

In 1997, Asahara and cols[32] described the existence of EPC in peripheral blood from normal adult individuals. This cell population represents less than 1/10 000 circulating mononuclear cells and less than 1% of the cells from the bone marrow (BM). EPC derived from hemangioblast, which is a common precursor of both hematopoietic cells and endothelial cells. Abundant evidence has been provided to show that EPCs contribute to neovascularization of adult tissues in pathological conditions such as tumors and ischemic lesions of the heart or extremities and also in situations of tissue damage and repair (Figure 1).[33] It is now accepted that the neovascularization process in the adult can take place not only by activation and sprouting of pre-existing endothelium, a process known as angiogenesis, but also by recruitment to the new vessels of circulating EPC, a process named vasculogenesis. This latter process occurs not only in the embryonic period of life but also in pathological conditions in the adult.

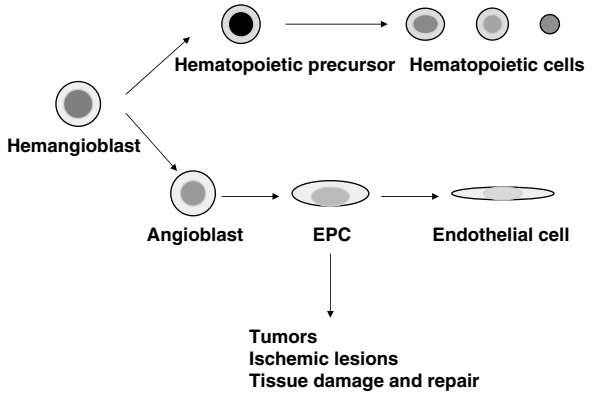

Figure 1 Differentiation and function of endothelial progenitor cell (EPC). BM-derived EPCs are of hematopoietic origin and possibly derive from the hemangioblast, which is also hematopoietic precursor to generate hematopoietic cells. The growth factors such as vascular endothelium growth factor differentiate hemangioblast to angioblast, EPC and finally to endothelia cells. These cells are involved in the tumor angiogenesis, ischemic lesions and tissue damage and repair.

EPCs are recruited to the sites of ischemia or tissue injury by the local production of chemotactic molecules such as vascular endothelium growth factor (VEGF) and stem-cell-derived factor-1. The synthesis of these factors is stimulated by the expression in ischemic tissues and damaged organs of hypoxia-inducible factor-1. EPCs express receptors for VEGF, especially VGFER2 (or KDR), and for stem-cell-derived factor-1, namely CXCR4. The ligand–receptor interaction promotes EPC migration to injured organs where they actively participate in tissue repair.[33–37] At the site of tissue damage, EPCs secrete a variety of cytoprotective and angiogenic factors including insulin-like growth factor 1, VEGF, hepatocyte growth factor and stem-cell-derived factor-1.[38] As mentioned below, EPC have been considered as a potentially useful therapy to treat acute and chronic liver conditions. For this purpose, the production of insulin-like growth factor 1 and hepatocyte growth factor is of great interest as these factors exert both hepatoprotective and antifibrogenic effects.[39]

EPCs are characterized by the expression of cell surface markers characteristic of hematopoietic cells (CD133), stem cells (CD34) and endothelial cells (VEGFR2). By culturing BM-derived mononuclear cells in medium containing specific factors, two types of EPCs are generated: early and late EPCs. The former appear after 7 days of culture and produce growth factors abundantly, while the latter appear after 4–6 weeks of culture, have high clonogenic capability and produce scarcity of growth factors.[40] The former have been most frequently used to stimulate tissue repair. Different molecules, some of them generated at the site of injury, facilitate the mobilization of EPC from BM to peripheral circulation.[41,42] Stem-cell-derived factor-1, VEGF, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor and erythropoietin have significant EPC-mobilizing activity.

For therapeutic purposes, EPC can be obtained from BM or from peripheral blood,[2–4] either by selection of CD133+ cells using immunomagnetic procedures or by culturing mononuclear cells in specific culture medium.[33–35] The therapeutic potential of EPC in stimulating liver regeneration has recently been demonstrated in a clinical trial performed at the University of Dusseldorf. The authors showed that administration of $2–10 \times 10^7$ CD133+ cells obtained from BM aspirate after selection with immunomagnetic procedure and given by intraportal administration (through catheter placed in the veins of segment II and III) was able to significantly accelerate the regeneration of the left liver lobe following right portal embolization in patients that were to undergo right hepatectomy.[43]

In animals, many studies have shown that EPC (both of human and rodent origin) can be used with success to treat ischemic lesions of the heart and limbs.[44,45] Suh and co-workers reported that human EPC obtained by

culturing in specific medium peripheral blood mononuclear cells and given to nude mice induced an acceleration of wound healing by local production of growth factors, stimulation of tissue repair and neovascularization.[46] In 2006, Taniguchi *et al*.[47] showed that EPC obtained from human peripheral blood mononuclear cells or from murine BM after culture in specific medium for 10 days and given intrasplenically were able to ameliorate acute carbon tetrachloride liver damage in nude mice (human EPC) or in BALB/c mice (murine EPC) and to improve survival. They demonstrated that EPC generated high levels of hepatocyte growth factor, VEGF and heparin-binding EGF-like growth factor and stimulated the endogenous production of VEGF.[47] The same group reported in 2007 that EPC (characterized by the expression of CD133, VEGFR2, Tie-2 and CD31) given intravenously were able to reduce significantly liver damage and fibrogenesis in a model of liver cirrhosis induced by chronic administration of carbon tetrachloride.[48] In our group,[49] we observed that intrasplenic injection of EPC resulted in marked improvement and long-term survival of mice injected with an adenovirus encoding CD40L, a model of fulminant hepatitis with 100% mortality.[50]

Neovascularization plays a critical role in the growth and metastatic spread of tumors and involves recruitment of circulating EPC from BM as well as sprouting of preexisting endothelial cells. EPCs have been detected at increased frequency in the circulation of cancer patients and lymphoma-bearing mice, and tumor volume and tumor production of VEGF were found to correlate with EPC mobilization.[51,52] A recent study has shown that mobilized EPCs participate in tumor vasculogenesis of hepatocellular carcinoma. AC133 gene or antigen (marker of EPC) in peripheral blood and liver tissue could be used as a biomarker for predicting the progression of hepatocellular carcinoma.[53] Moreover, when EPCs are administered to immunocompromized mice, they incorporate into the vasculature of xenotransplanted tumors.[54,55] Our recent data showed that EPCs can be transduced by lentiviral and adenoviral vectors. Immunohistochemical and immunofluorescence analysis showed that EPCs were incorporated in the tumor tissues in animal model with orthotopic tumor models (Figure 2). The *in vivo* imaging for luciferase expression demonstrated that EPCs were recruited preferentially by tumor nodules and that the maximal recruitment occurred at days 2–5 day after EPC administration (Figure 2).[56] These data show that EPC are attracted to tumors and could serve as vehicles for genes encoding antitumor factors to be expressed inside the tumor mass.

From what has been exposed, EPCs are recruited both to sites of tissue injury and to neoplastic lesions. This property makes EPCs useful tools to convey therapeutic genes to diseased organs in patients with tissue damage or neoplastic conditions.[57] However, the number of EPC that can be obtained for clinical use is scarce and may be insufficient for specific applications. Moreover, in some circumstances such as diabetes or obesity EPCs may be dysfunctional. It is therefore of great interest to develop means to enhance the therapeutic potential of a limited number of EPC or to revert EPC dysfunction. Recently, it has been proposed that genetic engineering of EPC aimed at increasing the production of growth factors, promoting their cytoprotective properties or extending their life span

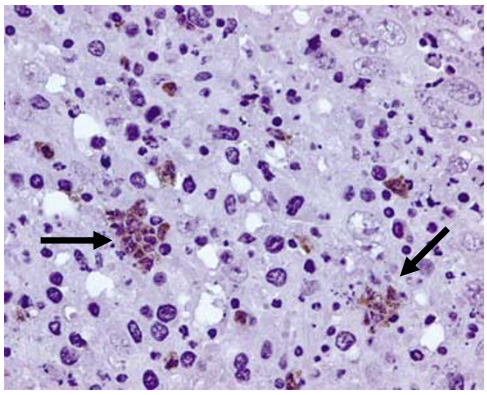

Figure 2 Recruitment of endothelial progenitor cell (EPC) transduced by adenoviral vector encoding firefly luciferase reporter gene in orthotopic liver cancer. C57BL/6 mice received MC38 colon carcinoma cells to induce a liver tumor. After 10 days, 10^6 of EPC transduced with adenovirus expressing luciferase were intravenously injected into tumor-bearing animals. Luciferase-positive EPC are recruited by the tumor being detected by immunohistochemistry using antibody against luciferase. Arrows indicate luciferase positive cells.

is an effective procedure to improve the curative effects of EPC.[58] Choi *et al*.[59] showed that inhibition of glycogen synthase kinase-3β (GSK3β) signaling pathway led to increased nuclear translocation of β-catenin and increased secretion of angiogenic cytokines (VEGF and IL-8). It enhanced the survival and proliferation of early EPC, whereas it promoted the survival and differentiation of late EPC. Transplantation of either of these genetically modified EPC into the ischemic hindlimb model of athymic nude mouse significantly improved blood flow, limb salvage and tissue capillary density compared with nontransduced EPC.[59] In an animal model of experimentally induced limb ischemia, Iwaguro *el al*.[60] showed that administration of EPC transduced with adenovirus encoding VEGF improved neovascularization and blood flow recovery and reduced amputation rate. In this study, EPC-mediated adenoviral VEGF gene therapy was able to achieve curative effects despite the use of subtherapeutical EPC doses.[60] Thus, combined EPC and gene therapy is an option to address the problem of limitations in the number of EPC that can be obtained for clinical applications.

Genetic engineering of EPCs may also be beneficial in aged individuals where EPC function is impaired due to senescence. In these cases, cell therapy may be inefficient despite adequate EPC mobilization from BM. It should be noted that EPC are not true pluripotent self-renewing stem cells, but a committed lineage and therefore are subjected, as other somatic cells, to limited number of cell divisions. Only true stem cells express telomerase reverse transcriptase (TERT) and are able to replicate indefinitely as they maintain telomeric DNA replication. These cells can divide beyond Hayflick limit without experiencing senescence.[61] Gene transfer of TERT to differentiated cells such as fibroblast has been shown to confer unlimited replicative potential without inducing malignant transformation.[62] It has been shown that TERT gene transfer to endothelial cell lines was able to confer resistance to programmed cell death and senescence, and to result in increased functional competence.[58] Murasawa *et al*.[63] have demonstrated that telomerase

activity contributes to EPC angiogenic properties: mitogenic activity, migratory activity and cell survival. This enhanced regenerative activity of EPCs by hTERT transfer will provide a novel therapeutic strategy for postnatal neovascularization in patients with severe ischemic disease and to improve the biological performance of EPC in aged individuals.

Prospective

Although combination of cell and gene therapy shows great promise for therapy of different types of diseases, several issues should be considered for future clinical applications. The first is the choice of vectors for cell transduction. The selected vector should enable high transduction efficiency and should be devoid of any damaging effect on cell viability. If the cells divide actively an integrative vector should be considered. In some cases, according to the type of the transgene, vectors with inducible promoters will be necessary. Second, for a diversity of applications, the cell fate after transplantation should be monitored using adequate *in vivo* imaging systems such as positron emission tomography or single photon emission computed tomography. Third, future development of combined cell and gene therapy implies a full understanding of the impact of the cell vehicle and transgene in the pathogenesis of the specific disease we wish to treat.

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