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Review

Gene transfer of cytoprotective and immunomodulatory molecules for prevention of cardiac allograft rejection

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Summary

Current treatments of heart transplantation are limited by incomplete effectiveness, significant toxicity, and failure to prevent chronic rejection. Genetic manipulation of the donor heart at the time of removal offers the unique opportunity to produce a therapeutic molecule within the graft itself, while minimizing systemic effects. Cytoprotective approaches including gene transfer of heme oxygenase (HO)-1, endothelial nitric oxide synthase, and antisense oligodeoxynucleotides specific for nuclear factor (NF)-κB or intercellular adhesion molecule (ICAM)-1 reduced ischaemia–reperfusion injury and delayed cardiac allograft rejection in small animals. Exogenous overexpression of immunomodulatory cytokines such as interleukin (IL)-4, IL-10 and transforming growth factor-β, as well as gene transfer of inhibitors of pro-inflammatory cytokines also delayed graft rejection. Gene transfer-based blockade of T-cell costimulatory activation with CTLA4-Ig or CD40-Ig resulted in long-lasting graft survival and donor-specific unresponsiveness, as manifested by acceptance of a second graft from the original donor strain but rejection of third-party grafts. Similar results were obtained with donor major histocompatibility complex class I gene transfer into bone marrow cells. Gene therapy approaches to chronic rejection included gene transfer of HO-1, soluble Fas, tissue plasminogen activator and antisense oligodeoxynucleotides specific for the anti-apoptotic mediator Bcl-x or the E2F transcription factor. Despite major experimental advances, however, gene therapy for heart transplantation has not entered the clinical arena yet. Fundamental questions regarding the most suitable vector, the best gene, and safety issues remain unanswered. Well-controlled studies that compare gene therapy with established treatments in non-human primates are needed before clinical trials can be started.

Keywords: Transplantation; Heart; Gene therapy; Gene transfer

1. Introduction

Transplantation is the treatment of choice for end-stage organ failure [1]. However, maintenance of a functional allograft requires life-long immunosuppression to prevent rejection by the immune system. Current immunosuppressive drugs such as cyclosporine and corticoids act by indiscriminately blocking T-cell activation, the primary mechanism of graft rejection. Unfortunately, these drugs are associated with significant side effects including renal toxicity, dyslipidaemia, diabetes, and increased risk of infections and malignancies. Moreover, current treatments have failed to prevent chronic rejection, or graft arteriopathy [2]. As a result, although 1-year survival rates for transplanted organs now exceed 90%, overall 10-year graft survival rates remain below 50% [3]. Clearly, novel approaches to organ transplantation are needed.

New immunosuppressive drugs include humanized anti-interleukin (IL)-2 receptor monoclonal antibody (daclizumab) [4]; tacrolimus, which blocks IL-2-dependent T-cell activation; mycophenolate mofetil, which blocks lymphocyte purine biosynthesis; and sirolimus (rapamycin), which inhibits multiple cell cycle regulators. Initial clinical trials have shown that the new drugs improve the short-term outcome after organ transplantation [4,5]. However, they are associated with significant toxicity, and their long-term effects are unknown because the clinical follow-up is still too short.

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Gene therapy is defined as the introduction and expression of recombinant DNA in order to ameliorate or cure a disease condition. The easy access to the donor organ for genetic manipulation at the time of removal and the need for a localized biological effect make organ transplantation particularly well-suited to gene therapy approaches. Indeed, ex vivo gene transfer into the donor organ can be performed under controlled, optimized conditions. Because the foreign gene is not directly administered to the patient, its systemic dissemination is minimized. Most importantly, the protective factor can be produced for extended periods of time [6,7], potentially for a lifetime, after a single gene administration. Obviously, sustained production of the therapeutic molecule is of major relevance for organ transplantation, which requires lifelong immunosuppression.

Despite the extensive publicity devoted to gene therapy, this field is still in its infancy. Recently, cardiovascular gene therapy has entered the clinical arena, and promising results have been reported in initial trials for coronary artery disease [8]. By contrast, no clinical applications in gene therapy for heart transplantation have been reported so far. Nevertheless, increasing experimental evidence suggests that this approach may be feasible. This paper is devoted principally to a review of the theoretical basis of gene therapy for heart transplantation, as established by experimental studies in animal models.

2. Routes of gene administration

General requirements for a successful gene therapy strategy include: (1) a suitable route of gene administration; (2) an efficient gene transfer vector; (3) a gene product that mediates a strong biological effect; (4) a sufficient duration of gene expression; and (5) an acceptable risk profile.

Both systemic and localized approaches have been used to deliver a gene of interest to the transplanted heart. Systemic gene delivery may be suitable in the case when the delivered gene encodes a secreted factor that acts on neighbouring or remote cells via a paracrine mechanism. This approach involves systemic dissemination of the foreign gene, of course. However, targeted vectors that bind to tissue-specific surface markers or contain tissue-specific promoters have been developed [9]. After systemic administration, these vectors mediate gene transfer selectively to target tissues. However, targeted vectors may not be required for gene therapy for heart transplantation because transgene expression after ex vivo gene transfer into the donor heart is largely confined to the graft itself.

Various routes of administration including intracoronary infusion, intramyocardial and endomyocardial injection, and pericardial instillation have been used to deliver a gene of interest to the donor heart [7,10]. Using a Langendorff in vitro perfusion system, adenovirus-mediated gene transfer into the isolated rat heart varied as a function of vector concentration and perfusion time [11]. Pre-treatment with hypocalcaemic solutions, serotonin or vascular endothelial growth factor (VEGF; originally termed vascular permeability factor) increases vascular permeability, potentially enhancing myocardial gene transfer. Ex vivo gene transfer by intracoronary vector infusion into the isolated donor heart is more efficient than in vivo gene transfer by vector instillation into the coronary circulation. This difference is due to the long dwelling time of the vector within the isolated donor heart, which can be equivalent to the organ preservation time. In contrast, the transit time of vector particles through the coronary circulation in vivo is short, ranging from a few seconds to a few minutes during blood flow arrest. As a result, cardiac uptake of vector particles instilled into coronary arteries in vivo is relatively low. As an example, the number of adenovirus genomes after intracoronary infusion of an adenoviral vector was 33-fold lower than after intramyocardial injection in pigs [12]. In the clinical setting, the isolated donor organ is routinely perfused with a tissue preserving solution. This procedure could be combined with the administration of a therapeutic gene.

3. Gene transfer systems

Several vectors including recombinant adenovirus, plasmid DNA, liposome–DNA and hemagglutinating virus of Japan (HVJ)–liposome–DNA complexes have been used to deliver a gene of interest to the donor heart [10,13,14]. Each vector has distinct advantages and disadvantages, and hence, a perfect vector for all applications does not exist. Instead, the vector used should be tailored to any given application, taking into account the cellular target, the predicted levels of transgene expression, and the duration of expression. Accordingly, gene transfer-based prevention of acute and chronic rejection may require different vectors because long-term expression of the protective gene is highly desirable in many approaches to chronic rejection. However, short-lived transgene expression can also mediate long-lasting effects, especially in the case when immunological tolerance toward donor antigens can be induced (see below). The cellular target should also be taken into account when choosing the vector. While cardiomyocytes are the primary target of most gene therapy approaches to acute rejection, endothelial cells and other vascular cells are important targets for the prevention of graft arteriopathy.

The number of cells that need to express the transgene in order to achieve a biological effect depends on the delivered gene itself. In the case when the gene encodes a secreted peptide that acts on neighbouring cells via a paracrine mechanism, limited numbers of gene-transduced cells may be sufficient to elicit a therapeutic effect. What really matters is the concentration of the protective gene product within the graft, or in the plasma, depending on

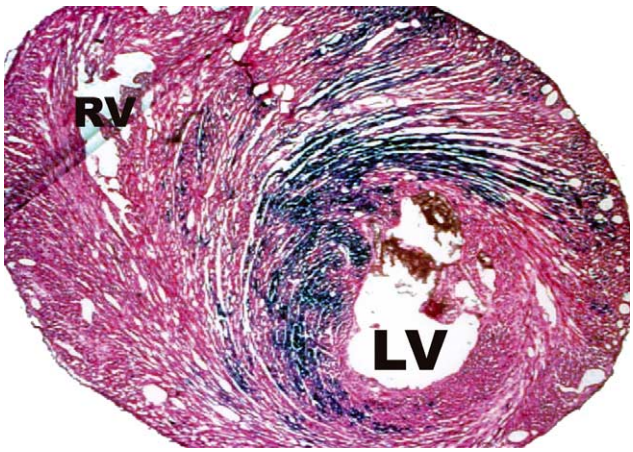

Fig. 1. Adenovirus-mediated transfer of a *lac* Z reporter gene into a transplanted rat heart. Cryosection through the heart shows patchy yet widespread β-galactosidase expression (blue areas after X-gal staining), most abundantly in the interventricular septum. It should be considered that X-gal staining underestimates gene transfer efficiency [15]. Empty spaces within myocardium are cyrosectioning artefacts. The apparent thickening of the RV wall is an artefact due to the slightly oblique sectioning axis; LV/RV, left/right ventricular cavities.

the mechanism of action. Conversely, in the case when the therapeutic gene encodes an intracellular factor, as many cardiac cells as possible should express the cytoprotective molecule.

Replication-deficient, recombinant adenoviral vectors have been used in the vast majority of gene therapy studies for heart transplantation. These vectors efficiently transduce genes into both cardiomyocytes and endothelial cells in vivo [10–13]. Using a *lac* Z reporter gene, the β-galactosidase gene product is readily visualized by histochemical reaction with the chromogenic substrate X-gal (Fig. 1). It should be noted, however, that the efficiency of gene transfer is underestimated by X-gal staining due to *lac* Z expression below the detection threshold in a proportion of cells [15]. Conversely, false-positive X-gal staining due to micro-infarctions, rather than effective *lac* Z gene transfer, was reported after intramyocardial injection [16].

Limitations of adenoviral vectors include tissue inflammation and short-lived transgene expression (≈2–4 weeks) [12,17,18]. The absence of vector integration into the cell genome, as well as immune responses to viral proteins are responsible for the short duration of gene expression with adenoviral vectors. Non-integrated DNA is inherently unstable due to the presence of DNA digesting enzymes within the cell. Immune responses include both cytotoxic T cells that eliminate cells that express adenoviral antigens and neutralizing antibody that preclude successful readministration of the adenoviral vector [17]. By analogy, pre-existing antibody as a result of previous infection with wild-type adenovirus may preclude adenoviral gene transfer in humans. In a cohort of healthy adult individuals, we found a 57%-prevalence of neutralizing antibody to adenovirus [17]. Thus, many candidate patients may be refractory to adenovirus-based gene therapy. In a clinical trial of gene therapy for coronary artery disease, the extent of anti-adenovirus antibody formation in patients who received intramyocardial adenoviral vectors was strongly correlated with pre-existing antibodies [18].

Adenovirus-induced inflammation has been studied in donor hearts transplanted into genetically identical hosts, thus avoiding confounding alloimmune responses. Adenovirus-mediated *lac* Z gene transfer into rat cardiac isografts caused significant myocardial inflammation that was associated with rapid extinction of *lac* Z expression [19]. In contrast, negligible inflammation and long-lasting *lac* Z expression were observed in adenovirally transduced mouse cardiac isografts, despite the fact that the same vector induced marked hepatic inflammation when administered to the liver [20]. Although the differences between the two organs have not been fully explained, the lower antigen-presenting cell (APC) content of cardiac tissue, as compared to the liver, may play a part. Dendritic cells as professional APCs have been shown to migrate from the donor heart and localize in the host's spleen, where they generate productive cellular and humoral immune responses [21].

Plasmid DNA, liposome–DNA and HVJ–liposome–DNA vectors have also been used to deliver genes of interest to the donor heart [13,14,22,23]. Although these vectors are intrinsically less efficient than adenoviral vectors, they also induce less tissue inflammation. Interestingly, those rare studies that directly compared different vectors to each other showed that the most efficient vector does not always mediate the best therapeutic effect. For instance, liposome–DNA transfection of the active form of transforming growth factor (TGF)-β1 was more effective than adenovirus-mediated

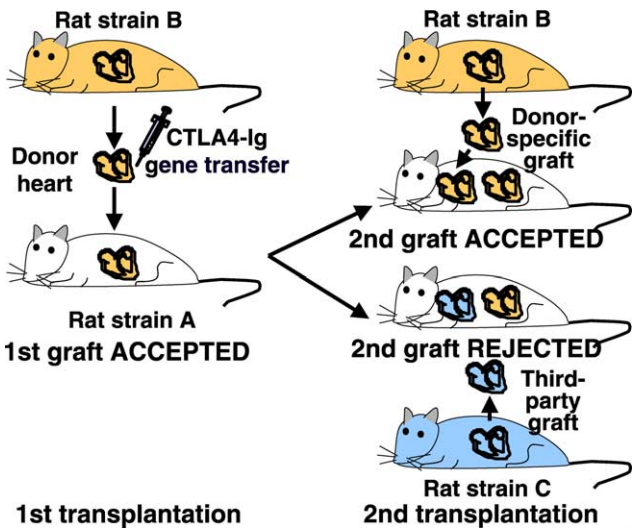

Fig. 2. Schematic of donor-specific hyporesponsiveness induced by CTLA4-Ig gene transfer. A donor heart of the strain B transduced ex vivo with the CTLA4-Ig gene and transplanted into the recipient strain A survives indefinitely (>100 days). A second cardiac graft from the original donor strain B is accepted in the absence of new treatment. In contrast, a third-party graft from the strain C is rejected in the absence of treatment. Modified from Ref. [7].

gene transduction with respect to the prolongation of cardiac allograft survival in mice [22]. HVJ–liposome vectors are more efficient than most liposome and plasmid DNA vectors. In addition, repeated gene transfer with these vectors is feasible, and their safety profile is relatively good [23]. Thus, HVJ–liposome–DNA complexes provide a useful alternative to adenoviral vectors for gene therapy applications in heart transplantation.

Recombinant adeno-associated virus (AAV) vectors can integrate into the host cell genome, thus providing a potential for permanent genetic modifications of target cells. We have shown that AAV-mediated expression of a green fluorescence protein (GFP) reporter gene lasts for extended periods of time (>1 year) in mouse myocardium [6]. Attractive features of AAV vectors also include negligible tissue inflammation and a good safety profile because wild-type AAV is not a human pathogen. However, delayed onset of expression (by ~1–2 weeks, as compared to a few hours with adenoviral vectors [6]) limits the usefulness of AAV vectors for applications in heart transplantation. Nevertheless, AAV vectors could be used in combination with immunosuppressive drugs to bridge the gap from transplantation to onset of expression.

Retroviral vectors have been used only in a few studies of heart transplantation because they do not efficiently transduce genes into non-dividing cells such as cardiomyocytes and the large majority of endothelial cells in normal vessels. On the other hand, retroviral vectors have been used to express donor major histocompatibility complex (MHC) molecules in bone marrow cells to induce donor-specific tolerance in the host [24]. Among retroviral vectors, lentiviral vectors are unique in that they transduce genes into both dividing and non-dividing cells. We have shown that lentiviral vectors efficiently transduce and express genes for extended periods of time (>10 weeks) in adult rat myocardium [25]. The safety of the last generation of lentiviral vectors is believed to be similar, if not superior, to that of retroviral vectors used in clinical trials of gene therapy. However, both vectors integrate at non-specific sites in the cell genome. This raises the concern of insertional mutagenesis, as discussed in the final section of this review.

4. Gene transfer of cytoprotective factors

Several factors including brain death of the donor [26], organ preservation, surgical stress, and ischaemia–reperfusion injury [27] activate inflammatory cascades within the graft in the first few hours and days after transplantation. These alloantigen-independent insults to the graft up-regulate adhesion molecules that mediate leucocyte adhesion to the endothelium. During ischaemia–reperfusion injury, oxidative stress, apoptosis (i.e. programmed cell death) and pro-inflammatory cytokines cause early cell damage which enhances subsequent alloresponses.

Gene transfer of cytoprotective, anti-inflammatory and immunomodulatory molecules has been evaluated in heart transplantation models in small animals (Table 1). Among cytoprotective approaches, double-stranded oligodeoxynucleotides with specific affinity for nuclear factor-κB (NF-κB decoy), a transcriptional activator for adhesion molecule genes, were tested in rat cardiac transplants [28]. After 16 h of donor heart preservation at 4 °C in Euro-Collins solution and 1 h of reperfusion, the NF-κB decoy significantly reduced myocardial damage, as manifested by decreases in serum creatine phosphokinase, tissue IL-8 and neutrophil infiltration.

Table 1
Gene therapy for acute cardiac allograft rejection (non-exhaustive list)

Protective effect	Therapeutic gene	Biological activity	Vector	Model	Ref.
Cardioprotection	NFκB asDNA	NFκB inhibition	HVJ	Rat	[28]
	eNOS	NFκB inhibition	Liposomes	rabbit	[29]
	B2702, RDP1257	HO-1 activation	Liposomes	Mouse	[33]
Anti-inflammatory	TNFRp55-Ig	TNFR inhibition	Ad	Rat	[42]
	IL-4	Th2 responses	Ad	Rat	[52]
	IL-10	T-cell apoptosis	Ad, liposomes	Rat, rabbit	[48,49,51]
	vIL-10	APC inhibition	Liposomes retrovirus	Rat, mouse	[50,53]
	IL-13	HO-1 activation	Ad	Rat	[54]
	TGF-β	T-, B-cell inhibition	Ad	Rabbit	[48]
	IL-1RII-Ig	IL-1 signaling inhibition	Ad	Rat	[44]
	vMIP-II, MC148	Chemokine blockade	Liposomes	Mouse	[56]
	8ND-RANTES	Chemokine blockade	Ad	Rat	[44]
Cell adhesion inhibition	ICAM-1 asDNA	ICAM-1 inhibition	AS-ODN	Rat	[60]
T-cell suicide	HSV-TK (+gancyclovir)	Death of dividing T cells	–	Mouse	[65]
Tolerance induction	Donor MHC-I (+donor cells)	Donor-specific unresponsiveness	Liposomes Ad	Rat, mouse	[68,69]
Inhibition of T-cell costimulation	CTLA4-Ig	B7 blockade	Ad	Rat	[7,62,63]
	CD40-Ig	CD40 blockade	Ad	Rat	[64]

Therapeutic genes and their mechanisms of action are shown. HVJ, hemagglutinating virus of Japan-liposome vector; Ad, adenoviral vector; AS-ODN, anti-sense oligodeoxynucleotides. Other abbreviations: see text.

An alternate approach involved endothelial nitric oxide synthase (eNOS) gene transfer. eNOS catalyses the synthesis of NO, a vasodilator molecule that plays key roles in endothelial integrity and function, including inhibition of neutrophil adhesion, platelet aggregation, and vascular smooth muscle cell proliferation. Liposome-mediated eNOS transfection was shown to reduce NFκB activation and to delay cardiac allograft rejection in rabbits [29].

Heat shock proteins (HSPs) are physiologically up-regulated as an adaptive response to ischaemia and reperfusion. In a kidney transplantation model, heat shock and recovery conferred protection to the donor organ against ischaemia–reperfusion injury [30]. In native rat hearts, HSP-70 gene transfection was associated with attenuated ischaemia–reperfusion injury, as manifested by decreased creatine phosphokinase release, increased mitochondrial respiratory indices, and improved ventricular function [31]. By analogy, HSP gene transfer may be beneficial in heart transplantation as well, although this needs to be directly established.

Heme oxygenase (HO)-1 catalyses the rate-limiting step in the degradation of heme to bilirubin. The enzyme has potent anti-oxidant and anti-apoptotic effects. Enhanced HO-1 activity after stimulation with cobalt protoporphyrin prevented acute rejection and attenuated chronic rejection of mouse cardiac allografts [32]. Consistently, gene transfer of B2702 or RDP1257, two decapeptides that stimulate HO-1 activity, delayed cardiac allograft rejection in another study [33]. Together, these results suggest that endogenous up-regulation or exogenous overexpression of cytoprotective genes mitigates ischaemia–reperfusion injury and acute rejection.

5. Gene transfer of inhibitors of pro-inflammatory cytokines

Alloimmune responses involve T-cell activation and proliferation, cytokine production, natural killer (NK) cell and B-cell activation, and antibody formation. T-helper (Th) responses to antigen can be divided into type 1 (Th1) and type 2 (Th2) [34]. Th1 responses include secretion of IL-2, IL-12, interferon (IFN)-γ, and generation of cytotoxic T cells that recognize specific antigen. Th1 responses are stimulated by IL-12 and IFN-γ, and they are inhibited by IL-4, IL-10 and TGF-β [35]. Th2 responses include IL-4 secretion and production of specific antibody to the antigen. In long-term surviving grafts, decreases in Th1 cytokines with concomitant increases in Th2 cytokines have suggested the hypothesis that Th1 responses mediate acute rejection, whereas Th2 responses may promote allograft acceptance [36,37]. Data in mice deficient in the IFN-γ, IL-4, IL-10 or TGF-β genes lend support to this hypothesis [38–41].

Tumor necrosis factor (TNF)-α and IL-1 act in concert to activate T cells and vascular cells during ischaemia–reperfusion injury. Targeting of the TNF-α receptor by adenovirus-mediated TNFRp55-Ig gene transfer resulted in decreased inflammation in rat cardiac allografts [42]. Similarly, functional neutralization of the IL-1 type I receptor by exogenous overexpression of IL-1 receptor antagonist (IL-1Ra) protected native rat hearts against ischaemia–reperfusion injury [43]. We took an alternate approach to inhibit IL-1 signalling, namely gene transfer of a soluble IL-1 type II receptor fused to human IgG1 heavy chain (IL-1RII-Ig). The rationale for this approach is that the non-signalling IL-1 type II receptor has a higher affinity for IL-1β than the signalling type I receptor. Hence, soluble IL-1RII-Ig acts as a scavenger for IL-1β. Adenovirus-mediated IL-1RII-Ig gene transfer moderately prolonged cardiac allograft survival in rats [44].

IL-17 and IL-18, originally termed IFN-γ-inducing factor, are pro-inflammatory cytokines involved in Th1 responses [45–47]. We have shown that adenovirus-mediated gene transfer of either soluble IL-17 receptor-IgG (IL-17R-Ig) or IL-18 binding protein (IL-18BP), the naturally occurring inhibitor of IL-18 [47], delays cardiac allograft rejection in rats (unpublished data). These observations are consistent with data showing that IL-17R-Ig protein treatment prolongs cardiac allograft survival in mice [46]. The protective effect was associated with impaired functional differentiation of dendritic cell progenitors. Together, these results suggest that gene transfer-based inhibition of pro-inflammatory cytokines may slow down acute rejection, although it does not fully prevent it. Incomplete protection presumably relates to the redundancy of cytokine signalling pathways, whereby multiple cytokines can activate the same inflammatory cascades. This consideration implies that inhibition of an individual cytokine may not be sufficient to suppress alloimmune responses.

6. Gene transfer of immunomodulatory cytokines

Immunomodulatory cytokines such as IL-4, IL-10, IL-13 and TGF-β down-regulate Th1, while up-regulating Th2 responses [35]. Consistently, adenoviral transduction or liposomal transfection of IL-4, IL-10, IL-13, or TGF-β genes prolonged cardiac allograft survival in small animals [22,48–53]. Exogenous IL-4 or IL-10 overexpression was associated with Th2-dependent expression of protective molecules [52,53]. Moreover, IL-10 gene transfer induced apoptosis of alloreactive T cells via the Fas/Fas ligand pathway and caused APC dysfunction within the allograft [51,53]. IL-13 mediated immunomodulatory and anti-apoptotic effects that were associated with HO-1 up-regulation [54]. These results suggest that exogenous overexpression of immunomodulatory cytokines may confer partial protection against acute rejection.

7. Gene transfer of chemokine inhibitors

Chemokines are a family of chemoattractant cytokines that regulate leucocyte trafficking in inflammatory processes. As such, chemokines play a key role in the recruitment of lymphocytes and monocytes in the allograft [55]. Monocyte chemoattractant protein (MCP)-1, IL-8 and RANTES, among other chemokines, have been implicated in allograft rejection. Interestingly, some viruses produce chemokine homologues that inhibit leucocyte recruitment, thereby allowing viruses to escape cellular immune defences. Adenovirus-mediated gene transfer of the virally encoded chemokine homologues vMIP-II or MC148 significantly delayed cardiac allograft rejection in mice [56]. We obtained similar results with adenoviral vectors expressing N-terminally deleted analogues of RANTES [44] or MCP-1 (unpublished data). These truncated analogues antagonize the respective full-length chemokines for binding to their receptors [57]. Similarly to inhibitors of pro-inflammatory cytokines, however, inhibitors of chemokines delayed graft rejection only for limited periods of time. Again, the incomplete effectiveness of these inhibitors may be due to the redundancy of chemokine signalling pathways. This concept refers to the fact that a leucocyte population can be attracted by multiple chemokines, each of which may bind to multiple receptors, and vice-versa [55]. Consequently, chemokine inhibition could be expected to slow down leucocyte recruitment but not to suppress rejection. In partial contrast to these considerations, however, mice deficient in the CCR1 or CCR5 chemokine receptors (which bind RANTES and other chemokines) did not effectively reject cardiac allografts [58,59]. These data suggest that chemokine inhibition may be of clinical significance. On the other hand, the modest protection conferred by adenovirus-mediated gene transfer of chemokine inhibitors [44,56] may be due to suboptimal transgene expression, rapid degradation of the gene product, and tissue inflammation due to the adenoviral vector, which may counteract the anti-inflammatory effects of chemokine inhibition.

8. Gene transfer of adhesion molecule inhibitors

Adhesion molecules are up-regulated after heart transplantation and mediate leucocyte adhesion to the luminal surface of vascular endothelium. Hyperbaric transfection of the donor heart with antisense oligodeoxynucleotides specific for intercellular adhesion molecule (ICAM)-1, in combination with a neutralizing antibody against leucocyte function-associated antigen-1 (LFA-1), effectively prevented cardiac allograft rejection in rodents [60]. These results suggest that vascular adhesion molecules are major targets of gene therapy for heart transplantation.

9. Gene transfer-based blockade of T-cell costimulatory activation

Current immunosuppressive drugs block T-cell activation but do not eliminate alloreactive T cells. As a result, alloimmune activity resumes if treatment is withdrawn. By contrast, induction of immunological unresponsiveness, or tolerance, toward donor antigens prevents generation of alloreactive T cells. The physiological mechanisms underlying tolerance include central and peripheral T-cell deletion and suppression, as well as anergy, which is defined as unresponsiveness following restimulation with the same antigen.

The concept of T-cell costimulatory activation is important to understand anergy. This concept dictates that two distinct signals are required for efficient T-cell activation [61]. The first signal is mediated by the T-cell receptor (TCR) occupied by antigenic peptides that are presented on MHC molecules by APCs. The second signal arises from interactions between costimulatory molecules expressed on T cells and APCs. Costimulatory interactions between CD28 and members of the B7 family, and between CD40 ligand (CD154) and CD40 have been extensively characterized. Antigenic peptide that occupies the TCR in the absence of costimulatory activation induces antigen-specific T-cell anergy. The T cell does not react to the peptide it originally encountered in the absence of costimulatory signals. However, the cell can react to other antigens presented to it at earlier or later time points in the presence of costimulatory activation.

CTLA-4 is up-regulated on activated T cells and competes with CD28 for B7-1 and B7-2 binding, thereby inhibiting costimulatory activation. A soluble CTLA-4 immunoglobulin fusion molecule (CTLA4-Ig) has been evaluated as a treatment for autoimmune diseases and graft rejection. Systemic CTLA4-Ig gene transfer or protein treatment around the time of transplantation delayed cardiac allograft rejection for limited periods of time ($\approx 20-30$ days) in rodents [7,62,63]. In contrast, direct adenovirus-mediated CTLA4-Ig gene transfer into the donor heart resulted in indefinite cardiac allograft survival (>100 days) in rats [7]. Intragraft and serum CTLA4-Ig levels were detectable for more than 1 year after direct gene delivery to the donor heart, whereas serum CTLA4-Ig levels rapidly declined after systemic protein or gene administration. The long-term persistence of CTLA4-Ig expression after localized gene transfer might be due to inhibition of anti-adenoviral immune responses in the presence of high CTLA4-Ig concentrations. This highlights the possibility that an immunomodulatory gene product promotes both the persistence of transduced cells the gene product itself and graft survival at the same time. Remarkably, recipients of long-surviving grafts accepted a second cardiac allograft from the original donor strain but rejected third-party grafts, demonstrating donor-specific unresponsiveness (Fig. 2).

Localized CTLA4-Ig gene transfer and systemic CTLA4-Ig treatment had different impacts on the immune system. Intragraft CTLA4-Ig expression reduced IL-2 receptor and MHC antigen expression, as well as humoral and cellular immune responses against donor antigen and cognate antigens in total splenocytes. Antibody production against donor alloantigen and cognate antigens was suppressed for more than 120 days. Both methods reduced proliferative responses of graft-infiltrating cells and total splenocytes to alloantigenic and mitogenic stimuli. However, localized CTLA4-Ig gene transfer did not affect responses of lymph node cells and T cells purified from splenocytes, whereas systemic CTLA4-Ig treatment did. Thus, generalized immunocompetence was essentially preserved with localized CTLA4-Ig gene transfer.

Adenovirus-mediated CD40-Ig gene transfer was used to block the CD40/CD154 costimulatory pathway. Exogenous overexpression of soluble CD40-Ig mediated indefinite (>200 days) cardiac allograft survival in rats [64]. These results underline the remarkable potential of gene therapy strategies that block T-cell costimulatory activation.

10. Gene transfer of T-cell suicide molecules

Gene transfer of suicide genes such as herpes simplex virus thymidine kinase (TK) has been used to kill proliferating cells selectively. TK converts the nucleoside analogue gancyclovir into gancyclovir-monophosphate, which is then converted into gancyclovir-triphosphate and incorporated into elongating DNA, causing death of dividing cells. Transgenic mice that express TK in their T cells have been used as an experimental model to evaluate the usefulness of suicide gene-approaches for heart transplantation. In TK-transgenic mice, a 7-day gancyclovir course around the time of transplantation resulted in donor-specific unresponsiveness and long-lasting allograft survival, in the absence of generalized immunosuppression [65]. Similar approaches have been used in clinical trials of hematopoietic stem cell transplantation, where gancyclovir controlled graft-versus-host disease caused by TK transgenic T cells [66]. Of course, suicide gene-approaches to heart transplantation would require complex protocols including administration of autologous genetically modified T cells after a T cell-depleting immunosuppressive regimen. An attractive aspect, however, is that gancyclovir treatment would be needed only in the first few days after transplantation.

11. Gene transfer of donor-specific MHC class I molecules

Donor-specific MHC class I gene transfer to the host has also been used to induce immunological unresponsiveness to the allograft [24,67]. Retrovirus-mediated gene transfer of soluble donor MHC class I molecules into the thymus or bone marrow cells resulted in prolonged allograft survival in a high-responder heart transplantation model [68]. Similarly, adenovirus-mediated gene transfer of a single donor-specific MHC class I molecule into bone marrow cells, in combination with transient depletion of $CD4^+$ cells, mediated long-lasting survival of fully allogeneic cardiac grafts, in the absence of detectable microchimerism [69].

12. Gene therapy for chronic rejection (graft arteriosclerosis)

Accelerated graft arteriopathy is one of the most discouraging aspects of clinical transplantation [2,3]. Coronary artery lesions in transplanted hearts develop at a ~20-fold higher pace compared to naturally occurring arteriosclerosis. Vascular endothelium is the first donor-derived tissue encountered by the host's circulating lymphocytes, and alloresponses to donor antigens expressed on endothelial cells have been implicated in the pathogenesis of graft arteriopathy. Anti-endothelial antibodies are detectable in the sera of a significant proportion of patients with the disease [70]. An important role for humoral immune mechanisms is also supported by data in B cell-deficient mice, which show attenuated graft arteriopathy [71]. However, data in mice with different forms of profound immune deficiencies suggest that both antigen-dependent and independent (innate) cellular responses are also involved [71–73]. These responses differ between acute and chronic rejection. A clinical association between acute and chronic rejection has been established in kidney transplantation but is still controversial in heart transplantation [74,75]. A non-exhaustive list of gene therapy approaches to chronic rejection is shown in Table 2.

Gene transfer-based blockade of T-cell costimulatory activation was evaluated both in aortic and in cardiac models of graft arteriopathy. CD40-Ig gene transfer reduced alloreactive antibody formation, leucocyte infiltrates in the arterial wall, and intimal thickening in rat aortic allografts [76]. In contrast, CD40-Ig gene transfer did not prevent graft arteriopathy in rat cardiac allografts, despite preserving them from acute rejection [64]. These results are consistent with data in mice deficient in CD40 ligand. These mice do not acutely reject cardiac allografts but develop arteriopathic lesions in their coronary arteries [77].

An alternate approach to graft arteriopathy involves gene transfer of soluble Fas. The rationale for this approach is that activation of the death receptor Fas by Fas ligand, which is triggered by activated macrophages, mediates vascular cell death in graft coronary arteries. Soluble Fas sequesters Fas ligand, thereby inhibiting Fas activation on vascular cells. Gene transfer of soluble Fas significantly reduced arteriopathic lesions in rat aortic allografts [78]. Similar results were obtained by either endogenous or exogenous overexpression of HO-1, an enzyme with

Table 2
Gene therapy for graft arteriosclerosis (non-exhaustive list)

Protective effect	Therapeutic gene	Biological activity	Vector	Model	Ref.
Anti-apoptosis	Soluble Fas	Fas blockade	Ad	Rat aorta	[78]
	HO-1	Anti-oxidant	Ad	Rat aorta	[79]
	Bcl-x	Anti-apoptotic	AS-ODN	Mouse heart	[80]
Cell cycle regulation	E2F AS-DNA	E2F inhibition	HVJ	Mouse, monkey hearts	[82]
Inhibition of T-cell costimulation	CD40-Ig	CD40 blockade	Ad	Rat aorta	[76]
Anti-thrombotic (?)	tPA	Fibrinolysis (?)	Liposomes	Rabbit heart	[81]

Successfully tested protective genes acting by different mechanisms are shown. HVJ, hemagglutinating virus of Japan-liposome vector; Ad, adenoviral vector; AS-ODN DNA, anti-sense oligodeoxynucleotides. Other abbreviations: see text.

anti-oxidant and anti-apoptotic activities, in cardiac and aortic allografts, respectively [32,79].

Antisense oligonucleotides specific for the Bcl-x gene were tested in mouse cardiac allografts. Bcl-x inhibits apoptotic cell death of proliferating vascular smooth muscle cells that are responsible for intimal thickening. Targeting of Bcl-x reduced coronary artery lesions in mouse allografts [80].

Tissue plasminogen activator (tPA), a fibrinolytic factor also involved in wound healing, is down-regulated in transplant arteriosclerosis. Intracoronary tPA gene transfection with cationic liposomes attenuated arteriopathic lesions in rabbit cardiac allografts [81].

Finally, promising results were obtained by targeting the transcription factor E2F, which plays a central role in the transcription of multiple cell-cycle regulatory genes. Up-regulation of these genes in vascular smooth muscle cells promotes cell proliferation and intimal thickening in graft coronary arteries. Ex vivo transfection of the donor heart with double-stranded DNA with specific affinity for E2F (E2F decoy) prevented graft neointima formation for up to 8 weeks both in mice and in non-human primates [82].

13. Gene therapy for tolerance induction

Tolerance was first described 50 years ago as a state of immunological unresponsiveness toward antigens to which the immune system was exposed during the embryonic or neonatal period, which was maintained by the continuous presence of the antigens [83]. In transplantation immunology, tolerance refers to specific non-reactivity to graft alloantigens in the absence of ongoing therapy. In the clinical setting, however, tolerance does not mean complete unresponsiveness to the graft, but rather a lack of destructive alloresponses in an immunocompetent host [84]. Sporadic clinical cases of spontaneous allograft tolerance have been reported. A few patients stopped immunosuppression without losing their graft, and occasional patients treated by total body irradiation as induction therapy for transplantation maintained their graft in the absence of immunosuppression [85,86]. However, some of these reports were subsequently revised due to graft loss at late follow-up [85].

Tolerogenic protocols include bone marrow chimerism [87], in vitro manipulated or immature donor dendritic cells [88], T-cell costimulatory blockade [7,64,89], and T-cell depleting agents [90]. However, development of clinically suitable non-myeloablative regimens that allow bone marrow transplantation and long-lasting chimerism in HLA-mismatched patients is a difficult task [84]. Blockade of T-cell costimulatory activation may represent a more practical option. Recently, this approach has been tested in preclinical studies of organ transplantation and in clinical trials of bone marrow transplantation and autoimmune diseases. Short-term treatment with a humanized anti-CD154 antibody prevented acute renal allograft rejection in non-human primates [89]. CTLA4-Ig protein treatment showed some beneficial effects in patients with autoimmune psoriasis vulgaris or bone marrow transplantation [91,92] but not in those with systemic lupus erythematosus [93].

Several gene therapy studies claimed tolerance induction based on long-lasting cardiac allograft survival (>100 days) with acceptance of a second graft from the original donor strain in rodents [7,64,65]. However, these results should be cautiously considered as evidence for prolonged donor-specific unresponsiveness, rather than true tolerance. It is still unclear whether or not blockade of T-cell costimulatory activation can establish tolerance on its own. This approach is likely most effective when the size of the T-cell population that needs to be tolerized is small, which is not the case in organ transplantation [84]. Concomitant use of adjunct strategies involving donor antigens or dendritic cells may be required to induce sustained tolerance to cardiac allografts [94]. For instance, the combination of an anti-CD154 monoclonal antibody plus donor cells suppressed graft arteriopathy in mouse cardiac allografts [32]. Recently, novel T-cell costimulatory pathways involving ICOS/B-7h, CD134/CD134L, CD27/CD70, and PD-1/PD-L1 interactions have been identified. The complexity of these pathways suggests that treatment with multiple costimulatory inhibitors may be more effective than individual inhibitors.

An additional issue that is still unresolved is the relationship between tolerance and graft arteriopathy. As a manifestation of chronic rejection, graft arteriopathy would

not be expected to occur in tolerant hosts. However, a recent study by Russell et al. questioned this assumption [73]. In this study, two methods were used to tolerize recipient mice: 'classical' tolerance was induced by neonatal administration of allogeneic spleen cells, whereas bone marrow was infused to suitably prepared adult recipients to produce 'mixed chimerism'. Both methods induced states of profound tolerance, as manifested by donor-specific acceptance of cardiac and skin grafts, and undetectable specific antibody in all recipients. In both groups, however, cardiac grafts developed striking proliferative lesions in their coronary arteries. These results came as a surprise. The authors concluded that incompatibilities, at least of MHC-determined antigens, are sufficient to trigger graft vasculopathy in the absence of any demonstrable immune activity, either cellular or humoral. They speculated that the innate or primitive pathway of responsiveness that involves cytotoxicity and cytokine release by NK cells could be responsible for graft arteriopathy in tolerant animals. In another study, tolerance induction by infusion of donor bone marrow cells, in combination with a short cyclosporine course, prevented graft arteriopathy in rat cardiac transplants [95]. Similarly, tolerance induction by donor-specific kidney transplants plus a short cyclosporine course prevented arterial lesion formation in cardiac allografts in mini-swine [96]. These inconsistencies in results are difficult to explain but the variability in innate responsiveness among species and differences between the tolerogenic protocols could play a part. To sum up, induction of clinical tolerance remains a major challenge. Even if this goal can be achieved, this may not necessarily translate into complete protection against graft arteriopathy. Safety issues regarding the risk of tolerance induction toward infectious agents present at the time of treatment and the long-term risk of malignancies also need to be addressed.

14. Limitations of gene therapy studies

Despite significant advances in transplantation immunology, the clinical reality is still characterized by ineffective treatments for chronic rejection. The failure of gene therapy for heart transplantation to give rise to clinical applications may be accounted for by several factors. First, most studies have been devoted to acute rejection, for which effective treatments already exist, whereas fewer investigations have focused on graft vasculopathy, which is the more relevant clinical target. Second, most studies did not compare gene therapy approaches and established treatments. Third, the vast majority of gene therapy studies were performed in rodents; however, results in rodents are often not applicable to humans [84]. For instance, the absence of MHC class II molecules on mouse endothelium may ease allograft acceptance in this species. By contrast, genes encoding TCR and MHC proteins are well conserved between rhesus monkeys and humans. Both species express MHC class I and II molecules on endothelial cells and reject vascularized grafts in a similar manner [88,97]. Unfortunately, only few data regarding gene therapy for heart transplantation in non-human primates are available [82].

15. Future perspectives

It is difficult to forecast when clinical applications in gene therapy for heart transplantation can be initiated. Preclinical studies that compare gene therapy approaches with established treatments in non-human primates are needed before clinical trials can be started. Because of the lack of reliable clinical predictors of graft arteriosclerosis, all transplanted patients would need to be treated in order to prevent the disease in a subgroup of them. This implies that new treatments should be highly effective and safe. With respect to gene therapy, fundamental questions regarding the most efficient vector system, the best therapeutic gene, and safety issues remain unanswered. While constitutively active promoters have been used to drive transgene expression in experimental studies, regulatable promoters that permit to adjust gene expression to the clinical needs offer major advantages for clinical applications. These promoters make 'gene dosage' possible, for instance by oral administration of a drug (e.g. tetracycline) that modulates promoter activity [98]. Should side effects occur, regulatable vectors could be 'switched off'. In principle, physiological regulation of the therapeutic effect by an endogenous marker of the disease is also feasible. An example was provided by gene transfer of the V2 vasopressin receptor, which stimulates myocardial contractility, into failing rabbit hearts [99]. Because the endogenous ligand, arginine vasopressin, is increased in heart failure, activation of the transgenic receptor correlates with the severity of the disease. However, further improvements in regulated gene transfer systems are needed before they can be considered for gene therapy applications.

The gene therapy field has been criticized for promising too much and yielding too little. Most recently, the first severe adverse events in clinical trials of gene therapy came like dark clouds in a blue sky. A teenager died in 1999 soon after receiving adenovirus-based gene therapy for treatment of partial ornithine transcarbamylase (OTC) deficiency [100]. The patient suffered from a mild form of the disease, an X-linked defect of the urea cycle in which nitrogen metabolism is affected, resulting in neurological symptoms. After receiving the highest dose of the vector in the trial, the patient became comatose, developed acute respiratory distress syndrome and died 2 days later of multiple organ failure. Although the precise cause of the death remained unclear, an unusually strong inflammatory reaction to the adenoviral vector was involved. It should be emphasized, however, that early-generation adenoviral vectors like the one administered in this trial are now likely to be phased out for most diseases [100]. Novel adenoviral vectors deleted in

most or all viral genes have been developed [101]. We have shown that these so-called gutless vectors efficiently transduce genes into rat myocardium, while causing decreased inflammation compared to conventional adenoviral vectors (unpublished data).

Two serious adverse events occurred in a clinical trial of gene therapy for inherited, X chromosome-linked, severe combined immune deficiency (X-SCID) caused by common gamma (gamma-c) gene mutations. This trial involved retrovirus-mediated gamma-c gene transfer into marrow $CD34^+$ cells [102]. In the summer of 2002, one of the patients treated with this vector developed a lymphocytosis during a viral infection, followed by a lymphoproliferative syndrome. In December 2002, a second case of leukaemia-like illness was detected [103]. In both cases, hyperproliferating cells were found to bear an insertion of the vector near the proto-oncogene Lmo2. The emergence of a second case of vector insertion at the same location near the proto-oncogene raised further concerns about insertional mutagenesis as a result of random vector integration into the cell genome [104]. However, the absence of this problem in previous studies and trials suggests that peculiar factors including the gene and vector used and the immunodeficient state of the patients may have favoured these adverse events. As a result of these adverse events, 27 gene therapy trials were put on hold in the United States. Researchers are now going back to the bench to further investigate the risks associated with these approaches. Meanwhile, novel vectors that integrate at specific chromosomal locations have been developed to minimize the risk of insertional mutagenesis [105].

In conclusion, like any other new treatment, gene therapy was not expected to advance without adverse events. Notwithstanding, the potential of this approach to organ transplantation appears to be essentially intact, as genetic modification of the donor organ remains an appealing strategy. Recent advances in transplantation immunology, cardiac biology and gene delivery technology have improved our perspectives for clinical applications in this field.

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