Considerations Regarding the Strengths and Weaknesses of Experimental Approaches Employed in Basic Transplant Research: TGFß1 Gene Transfer in Transplantation

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Graft 2001; 4; 508

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Gene Transfer in Transplantation

The transfer of genes encoding immunomodulatory agents into allografts holds promise as an inductive therapy in transplantation (reviewed in refs. 1–3). This approach is clinically applicable, in that vascularized transplants are routinely perfused at the time of organ harvest and, therefore, may be transfected by perfusion. However, many fundamental aspects of this technology must be addressed before it may be optimally applied to clinical transplantation. For example, it has been suggested that immunosuppressive gene therapy may provide advantages over conventional immunosuppression.[1-3] Notably, gene transfer should allow for the persistent, local release of the agent within the microenvironment of the graft, thereby negating the deleterious side effects of systemic immunosuppression. Although this feature of immunosuppressive gene transfer is attractive, it has not been validated. Indeed, adenoviral-mediated transfer of CTLA4Ig in liver allografts results in readily detectable levels of the transgene product in the sera.[4] Hence, local secretion of the transgene product may result in systemic immunosuppression and increased susceptibility to infections and neoplasia. This fundamental aspect of immunosuppressive gene therapy has not been fully addressed and should be rigorously investigated.

Questions regarding the duration of immunosuppressive gene expression in transfected allografts have not been adequately addressed. Observations made in other gene transfer systems[5-13] suggest that transgene expression might be transient in transfected allografts. Although this would limit the use of gene transfer as a gene replacement therapy or in the treatment of chronic diseases, transient gene expression may be beneficial in the context of transplantation. For example, transient production of an immunosuppressive agent may provide an inductive therapy aimed at inhibiting alloreactive T-cell priming, altering the function of graft-reactive cells and/or inducing regulatory cells. In addition, transient expression may avoid complications associated with prolonged exposure to the immunosuppressive agent. However, the silencing of transgene expression reported in other systems is generally associated with the development of an immune response to the transgene product and/or the DNA delivery vector.[5-13] The transfer of an immunosuppressive gene would likely mute the vector-induced immune response,[14-18] thereby allowing transgene expression to persist within the graft. Indeed, we have reported that adenoviral-mediated transfection of cardiac allografts with TGFβ1 results in transgene expression for at least 60 days.[19] Clearly, a better understanding of the mechanisms that regulate the duration of immunosuppressive gene expression is warranted, and systems that allow controlled transgene expression must be developed.

Reporter gene studies have validated the ability to transfect grafts either by direct injection or by

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perfusion of the transplant.[20-27] In addition, cellular grafts have been successfully transfected in vitro by a variety of techniques.[28-33] Furthermore, intrathymic gene transfer of allogeneic major histocompatibility complex (MHC) molecules has proved successful as a preconditioning regimen aimed at decreasing the frequency of donor-reactive T cells.[34] Hence, immunosuppressive gene therapy is applicable to the transplant setting. The majority of studies that have transferred immunosuppressive genes into allografts have focused on transgene expression and graft survival and have not addressed the mechanisms by which this therapy alters the immune system. Reports that have examined immune function indicate that immunosuppressive gene therapy may decrease TNFα and IFNγ expression,[35] induce Th2 responses,[4] inhibit Th1 responses,[19] decrease the frequency of donor-specific T cells,[34,36,37] and/or inhibit alloantibody responses,[19,37] depending on the transgene. Whether these alterations in immune function require continued exposure to the transgene product or reflect the induction of regulatory cells is not fully understood. Therefore, further studies are warranted to assess interactions between immune effector mechanisms and transfected allografts. Information gained from such studies will provide insight for optimizing the suppressive effects of gene therapy in transplantation.

DNA Delivery Vectors for Use in Transplantation

DNA delivery systems may be grouped into two categories: viral and nonviral vectors. In general, the use of viral vectors in transplantation is limited by their immunogenicity, target cell specificity, and/or biologic safety concerns.[38-40] For example, *retroviral vectors* represent an attractive vehicle for gene delivery because they integrate into the host genome, thereby ensuring sustained gene expression.[41] However, this may lead to insertional mutagenesis. The major disadvantage of retroviral vectors is that they only transfect dividing cells and therefore are of limited use in adult solid organ transplantation. *Lentiviral vectors* are appealing in that, unlike current retroviral vectors, they are capable of transfecting nondividing cells in vivo.[42] Because lentiviral vectors have been developed relatively recently, very little is known about the immune response to these vectors. *Other viral vectors* have been developed based on herpes simplex virus (HSV),[43] vaccinia virus,[44] SV40,[45] and adeno-associated virus (AAV).[46,47] Each of these vectors will likely prove to be associated with unique advantages and disadvantages in the context of transplantation.

Adenoviral vectors have proven to be the most effective of viral vectors for use in transplantation.[4,17,20-23,26,28,30,32,35] Adenoviral vectors are capable of transfecting a wide variety of cell types and may be produced inexpensively at high titers.[48,49] These features make adenoviral vectors attractive for in vivo gene transfer strategies aimed at transfecting quiescent cells. However, in vivo administration of adenoviral vectors induces an immune response in naive animals, and the use of these vectors in humans is further complicated by the fact that most individuals have a preexisting immunity to adenovirus. Although first-generation adenoviral vectors are rendered replication-deficient due to deletion of E1 region genes, these vectors can still express low levels of both early and late viral genes. Therefore, cytoxic T lymphocyte (CTL) epitopes that are present on viral structural proteins, including the hexon and fiber proteins, may be processed and presented by transfected cells via the Class I MHC pathway.[50] In addition, input viral particles may be processed and presented by host antigen presenting cells (APC) via the Class II MHC pathway, leading to CD4+ helper T lymphocyte development and antiadenoviral antibody production.[11] The antiadenoviral immune response has been implicated in inflammation and loss of transgene expression in a variety of transfected tissues including lung,[11] liver,[12] and muscle.[13] Several approaches have been reported to decrease the immunogenicity of adenoviral vectors. These include construction of vectors that have a temperature-sensitive mutation within the E2A-encoded DNA binding protein[51] and vectors that are deleted of all viral genes.[52,53] However, the immune response to this newer generation of adenoviral vectors has not been fully explored.

Despite the numerous reports that emphasize the limitations of adenoviral vectors, we have found that the degree of inflammation and tissue damage and the duration of transgene expression depends upon which tissue is transfected with adenovirus. Specifically, adenoviral-mediated gene transfer of

β-galactosidase into cardiac isografts does not result in overt inflammation within the graft, despite the fact that a vigorous antiadenoviral immune response is induced.[54] This contrasts the inflammatory response that occurs in the liver when adenovirus is administered intravenously. Furthermore, adenoviral-mediated transfer of TGFβ1 into vascularized cardiac allografts is not protective unless recipients are depleted of CD8+ T cells.[19] In these studies, adenoviral-mediated transfer of TGFβ1 ablated CD4+ Th1 development, yet had no effect on CD8+ Th1 function. These observations emphasize how little we know about the suitability of adenoviral vectors in immunosuppressive gene therapy.

Cationic liposomes provide a nonimmunogenic DNA delivery vehicle for in vivo gene transfer.[55-57] Cationic lipids bind and condense plasmid DNA spontaneously to form complexes that have a high affinity for cell membranes. These *DNA-liposome complexes* are readily endocytosed, and the endosomal membrane is subsequently disrupted, releasing the plasmid into the cytoplasm.[55-57] However, the transfection efficiency of DNA-liposome complexes is relatively low compared with that of adenoviral vectors, and it is estimated that only 1 per 1000 plasmids reaches the nucleus and is expressed.[56] Nonetheless, several reports have documented the ability of DNA-liposome complexes to transfect vascularized cardiac transplants.[19,24,25,27,37] With improvements in lipid formulations, it is now possible to increase transfection efficiency without lipid toxicity.[58] Indeed, we have used the experimental lipid GAP DLRIE/DOPE to effectively transfect vascularized cardiac allografts with either TGFβ1[19] or viral IL-10[37] and have demonstrated the suppressive effects of the transgene product in vivo.

Immunosuppressive Transgenes for Use in Transplantation

Immunosuppressive genes of interest for transfecting allografts may be grouped into 2 broad categories. One category is composed of *genes that encode molecules that render the graft less susceptible to immune damage.* This class of immunosuppressive genes is aimed at modifying the graft and is best exemplified by Fas ligand. Grafts expressing Fas ligand would theoretically induce apoptosis of infiltrating cells, thereby rendering the graft an immune privileged site, analogous to the eye[59] or testis.[60] However, results obtained from transfecting allografts with Fas ligand are controversial to date. In some systems, Fas ligand–expressing allografts enjoy prolonged survival,[29,61] whereas in other systems they do not.[32,62] However, we have shown that Fas ligand expression by allogeneic tumor cells inhibits alloantibody production and donor-reactive T-cell expansion.[63] These observations indicate that Fas ligand expression by allogeneic cells alters systemic, alloantigen-specific responses.

The second category includes *genes that encode soluble cytokines, cytokine receptors, or other soluble suppressive agents*. Transfer of these genes is aimed at generating high concentrations of the transgene product within the microenvironment of the allograft, thus providing local immunosuppression.[1-3] However, systemic effects of these soluble transgene products have not been ruled out and should be investigated. Prolonged allograft survival has been achieved with gene transfer of TGFβ1,[19,26,35,64] viral IL-10,[17,26,36,37] soluble tumor necrosis factor (TNF) receptors,[65] and CTLA4Ig.[4,33,66] Due to the controversy surrounding the protective effects of Th2 cytokines in transplantation,[67] the suppressive activities of IL-4 or mammalian IL-10 gene transfer remain in question.

TGFβ1 as an Immunosuppressive Transgene

We have selected the active form of TGFβ1 for use in immunosuppressive gene therapy due to its broad range of immunosuppressive activities.[68] For example, TGFβ1 inhibits Th1 responses,[69] E-selectin expression by endothelial cells,[70] and CTL development.[71] In addition, TGFβ1 inhibits B cell proliferation and induces apoptosis in B cells[72,73] and fully differentiated plasma cells.[68] Paradoxically, TGFβ1 may have stimulatory or suppressive activities on macrophages, depending on their state of differentiation and activation.[68,74] In resting blood monocytes, TGFβ1 induces expression of IL-1, TNFα, platelet derived growth factor (PDGF), and basic fibroblast growth factor (FGF), which play a role in wound healing and angiogenesis. TGFβ1 also up-regulates adhesion molecule expression on blood monocytes, which facilitates their entry into inflammatory tissues. However, TGFβ1 inhibits interferon gamma (IFNγ and inducible

nitric oxide synthase (iNOS) production and increases expression of IL-1 receptor antagonist by activated macrophages. These anti-inflammatory activities of TGFβ1 are believed to favor the wound-healing functions of macrophages once they enter inflammatory tissues.[68,75,76]

Many activities of TGFβ1 are antagonized by either IFNγ or IL-12, and TGFβ1 inhibits the production of these proinflammatory cytokines.[68,77] A similar antagonistic relationship has been established for TNFα and TGFβ1.[78,79] Hence, a balance exists between TGFβ1 and proinflammatory cytokines that may influence the efficacy of TGFβ1 gene transfer. Indeed, we have shown that IL-12 overrides the suppressive activities of TGFβ1 gene transfer.[19] It should be noted that TGFβ1 induces its own production from B cells,[80] as well as IL-10 production from APC.[81] These activities may amplify the suppressive effects of TGFβ1 gene transfer. Similarly, TGFβ1 promotes the development of IL-4-producing Th2.[68,77,82] In the absence of IL-12 (which is inhibited by TGFβ1),[83] IL-4 promotes the development of TGFβ1-producing "Th3".[68] This represents an additional pathway by which TGFβ1 gene transfer may amplify its suppressive activity. Hence, studies aimed at elucidating cytokine interactions that amplify or negate the suppressive activity of TGFβ1 gene transfer are clearly warranted.

Finally, it should be noted that TGFβ1 is secreted as a latent complex that remains associated with its propeptide.[84] To elicit a biologic response, TGFβ1 must be released from the latent complex. This is a critical step in the control of TGFβ1 activity, as enhanced TGFβ1 expression does not correlate with increased levels of active TGFβ1.[85] Latent TGFβ1 may be activated by heat, acidic pH, or the serine proteases plasmin and cathepsin D.[84] Two interchain disulfide bonds at cysteines 223 and 225 are critical for TGFβ1 binding to the latent complex. Indeed, site-directed mutagenesis converting these cysteines to serines generates a cDNA that encodes active TGFβ1.[86,87] Hence, gene transfer with this mutated cDNA results in the expression of biologically active TGFβ1.

Our initial studies compared the efficacy of adenoviral versus DNA-liposome-mediated gene transfer of the active form of human TGFβ1 in vascularized cardiac allografts.[19] Although both forms of TGFβ1 gene delivery markedly prolonged allograft survival in mice depleted of CD8+ cells, prolonged transgene expression was associated with advanced fibrosis of the graft.

Hence, TGFβ1 gene transfer represents a double-edged sword, thereby allowing for the study of both the pros and cons of immunosuppressive gene therapy in transplantation. Ongoing studies in this system are aimed at addressing the following questions:

1. What are the effects of TGFβ1 gene therapy on T cell subsets, as well as immunologic memory?
2. What are the benefits and drawbacks of viral versus nonviral DNA delivery vectors?
3. Does gene transfer truly provide local immunosuppression, or are the effects systemic?
4. What is the duration of immunosuppressive transgene expression, and can the transgene be silenced after an appropriate period of inductive immunosuppression?

The answers to these questions will increase our understanding of the immunobiology of immunosuppressive gene therapy and should facilitate the development of this technology for use in clinical transplantation.

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