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ESTABLISHING IMMUNOLOGICAL TOLERANCE THROUGH THE INDUCTION OF MOLECULAR CHIMERISM

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1. ABSTRACT

One of the major goals of transplantation biology is to overcome transplant rejection without the need for life-long immunosuppression. Over the last several years, fundamental advances in our understanding of the immune response to allogeneic and xenogeneic antigens have stimulated a great deal of interest in the possibility of using gene therapy approaches to overcome the host response leading to transplant rejection while alleviating the need for non-specific immunosuppression. Here, we review recent progress in the field on the use of gene therapy to induce transplantation tolerance to donor organs and tissues.

2. INTRODUCTION

Since 1988 the total number of organ transplants performed world-wide has greatly increased, primarily due to the development of powerful non-specific immunosuppressive agents, such as cyclosporine A, rapamycin and FK506, that have allowed for one year graft survival rates as high as 94% in living-related kidney transplants. The increased success of transplantation has opened up the possibility that a wider variety of disorders may be effectively treated using organ or tissue transplants. Indeed, transplantation is regarded as a modern medical success story, which has in turn led to an increased demand for transplantable human organs. This increase in demand has exacerbated shortages of suitable organs. As of March, 2002, the United Network for Organ Sharing (http://www.unos.org) reported that in the United States alone, while over 79,000 patients were on the national waiting list for organ transplants, only 22,000 transplants were performed in 2000, using organs recovered from approximately 11,000 donors.

The use of powerful immunosuppressive drugs, which patients must take for the rest of their lives to prevent organ rejection, is not without adverse consequences. These drugs predispose patients to an increased susceptibility to infectious disease, lymphoproliferative disorders and cancer, and can also cause diabetes, as well as liver, kidney and neurological toxicity (1-3). Although current immunosuppressive regimens are effective at preventing acute rejection episodes in the first few years after transplantation, they are less effective at preventing chronic rejection, which is currently the major factor limiting long-term survival of transplants (4,5).

Because of problems associated with long-term immunosuppression, the induction of immunological tolerance to transplantation antigens has become a major goal in the field because of its promise to allow replacement of host organs without the need for immunosuppression. The development of strategies to induce specific tolerance to allogeneic organs may also make it possible to attain acceptable graft survival rates using partially- or fully-

mismatched organs, or perhaps even xenogeneic organs, for life-saving transplants. Thus, the development of therapies that would result in specific tolerance to transplanted organs would be of great clinical benefit. The strategies used in order to induce specific tolerance to organ grafts fall into two categories: modification of the donor organ, or modification of the host immune response. In this review we shall focus on recent advances in the field designed to allow for modification of the host immune response resulting in tolerance using gene therapy to induce molecular chimerism.

3. MODIFICATION OF HOST IMMUNE SYSTEM: BUILDING ON THE CONCEPT OF MIXED HEMATOPOIETIC CHIMERISM

It has been known for many years that a state of mixed donor-host hematopoietic chimerism leads to immunological tolerance to major and minor donor transplantation antigens (6,7). The intentional creation of mixed allogeneic bone marrow chimerism as a strategy to induce tolerance to donor transplantation antigens was first demonstrated by Ildstad and Sachs (8). This approach involves the use of allogeneic bone marrow transplantation to establish host-donor mixed hematopoietic chimerism in conditioned recipients prior to donor organ transplantation (reviewed in (9)). The development of donor-type bone marrow-derived cells leads to negative selection of donor-reactive lymphocytes, essentially establishing donor as part of "self". The establishment of mixed chimerism through bone marrow transplantation in adult animals leads to specific tolerance, permitting transplantation of donor-type organs without any additional immunosuppression, in an otherwise fully immunocompetent host (8,10-13). The effectiveness of this approach has been demonstrated in non-human primates (14), and it has been shown that mixed chimerism can be achieved in humans receiving non-myeloablative conditioning prior to bone marrow transplantation (15). Furthermore, mixed chimerism can be used to induce tolerance to concordant xenografts in rodent models (16).

While it has been known for some time that inducing mixed chimerism by bone marrow transplantation can be used to induce tolerance, bone marrow transplantation across major histocompatibility (MHC) barriers is risky for a variety of reasons, including the severity of the preparative regimen required to establish donor bone marrow engraftment, the potential for inducing severe graft-versus-host disease (GVHD), and engraftment failure. The risk associated with bone marrow transplantation has therefore limited the use of mixed chimerism in the clinical transplantation setting. However, if one could overcome the risk of GVHD and the use of toxic host conditioning to achieve engraftment of donor-type cells, the use of mixed chimerism to induce tolerance to foreign organs could become the method of choice to allow for permanent allo or even xenograft acceptance without immunosuppression.

4. INDUCTION OF MOLECULAR CHIMERISM BY GENE THERAPY

Mixed chimerism induced by bone marrow transplantation demonstrates that expression of allo or xenogeneic antigens on bone marrow-derived cells is sufficient to induce tolerance. Thus, genetic engineering of hematopoietic stem cells to allow expression of allogeneic antigens on bone marrow-derived cells may be an attractive alternative to mixed bone marrow chimerism. Such an approach could potentially accomplish the goal of inducing functional tolerance by transferring allo or even xenogeneic genes rather than immunocompetent cells into the host, effectively establishing molecular rather than cellular chimerism. A gene therapy-based approach designed to achieve molecular chimerism may theoretically carry with it a lower level of risk than allogeneic bone marrow transplantation, because the modified bone marrow would be autologous. The use of genes rather than cells eliminates the possibility of GVHD and the need to deplete T cells from the bone marrow, which may result in improved engraftment. In the case of xenotransplants, because it has proved difficult to achieve engraftment of bone marrow across species barriers, modification of autologous cells with genes encoding xenogeneic antigens may make it possible to induce tolerance in cases where bone marrow transplants are ineffectual.

5. TESTING THE FEASIBILITY OF USING MOLECULAR CHIMERISM TO INDUCE TOLERANCE

To explore the feasibility of using genetic engineering to induce transplantation tolerance, a murine model that utilizes retroviral-mediated gene transfer of allogeneic MHC genes into host bone marrow was developed (17-19). This model uses two congenic strains of mice B10.AKM ($H\text{-}2K^k,I^k,D^q$) and B10.MBR ($H\text{-}2K^b,I^k,D^q$). The B10.MBR strain was derived from a recombination event that occurred during the back-crossing of B10.AKM to C57BL/10, and differs from the B10.AKM strain in only the MHC class I *H-2K* region (20). Because of this MHC class I disparity, B10.AKM CD8 and CD4 T cells are able to mediate rejection of B10.MBR skin grafts through the so-called direct and indirect pathways of antigen presentation (21-24). It was hypothesized that introduction of the $H\text{-}2K^b$ (K^b) gene into B10.AKM bone marrow by retroviral-mediated transduction should theoretically give rise to a mouse that expresses all cell surface MHC antigens of the B10.MBR strain on its bone marrow-derived hematopoietic cells, a molecular chimera. If expression of the transduced K^b gene in bone-marrow derived cells is sufficient to induce transplantation tolerance, through central or peripheral mechanisms, one would predict that mice receiving the K^b gene should become tolerant to the K^b alloantigen exactly as is seen in cellular bone marrow chimeras, and be able to accept K^b-disparate skin grafts from B10.MBR mice.

To test this hypothesis, bone marrow cells from B10.AKM mice were infected with retroviruses carrying the gene encoding K^b or the neomycin resistance gene (Neo^r) as a control. Infected bone marrow was then used to reconstitute B10.AKM mice that had been conditioned using lethal irradiation. B10.AKM mice reconstituted with K^b-transduced bone marrow showed significantly prolonged survival of K^b-disparate skin grafts from

B10.MBR mice, but were able to promptly reject third party skin grafts from B10.BR mice (*H-2K*^{k},*I*^{k},*D*^{k}). In contrast, B10.AKM mice reconstituted with Neo^r-transduced bone marrow rejected both B10.MBR and B10.BR grafts in a similar time-frame (17-19). Prolongation of allogeneic skin graft survival is considered to be the ultimate test of tolerance in rodents (25,26), therefore the prolonged survival of K^b-expressing B10.MBR skin grafts in this model is powerful evidence that alloreactive CD4 and CD8 T cells have been rendered hyporesponsive.

The ability of molecular chimerism to induce T cell hyporesponsiveness has now been observed by several groups. The basic finding that expression of the K^b gene in bone marrow-derived cells can induce hyporesponsiveness was extended by demonstrating acceptance of K^b-expressing cardiac allografts after infusion of genetically-modified tumor cells expressing an allogeneic MHC class I antigen (27). However, in studies using cardiac transplantation as a model system, it is important to point out that while administration of genetically-modified tumor cells was necessary to induce tolerance, it was probably not sufficient, because the heart transplants themselves probably contributed to establishing graft acceptance, consistent with studies showing that the presence of the cardiac allografts participates in maintenance of tolerance in these models (28). Others have reported that a retroviral gene therapy approach similar to those outlined above can be used to suppress antigen-specific immune responses in mice to an HLA class I antigen (29). However, in this study it was unclear whether the suppression achieved would allow acceptance of allografts expressing HLA class I gene products. Adenovirus-mediated transfer of a single MHC class I gene to bone marrow cells has been shown to induce a similar hyporesponsiveness to fully-allogeneic cardiac grafts, suggesting that induction of hyporesponsiveness to one MHC class I antigen may induce infectious tolerance to other MHC class I and class II alloantigens, although it is not clear whether these observations can be extended to the more rigorous prolongation of skin graft survival (30,31).

Molecular chimerism has also been used in large animal studies. Hyporesponsiveness to an MHC class II-mismatched kidney graft has been induced in pigs, although it is not clear to what extent the transplanted organ itself contributed to establishing allograft acceptance, as mentioned above (32,33). The induction of hyporesponsiveness to marker genes expressed in bone marrow derived cells has also been achieved in rhesus macaques (34). Some have also suggested that gene therapy approaches may induce hyporesponsiveness across to xenogeneic antigens in baboons (35). Retroviral-mediated gene transfer has also been used to tolerize pathogenic autoimmune T cells in a mouse model of diabetes (36). Similarly, the introduction of auto-antigens linked to an immunoglobulin region on the surface of relatively tolerogenic unactivated B cells has been suggested to induce tolerance in a number of autoimmune models (reviewed in (37)). Thus, the induction of molecular chimerism has been shown to be effective at the induction of hyporesponsiveness in multiple animal models, and using multiple therapeutic genes, and may have indications in the induction of tolerance in autoimmunity.

6. MECHANISMS LEADING TO HYPORESPONSIVENESS INDUCED BY GENE THERAPY

The studies described above clearly demonstrated in several model systems that molecular chimerism can be used to induced hyporesponsiveness to antigens expressed in bone marrow derived cells. However, not one of the studies was able to conclusively demonstrate stable long-term tolerance. For example, although prolongation of K^b-expressing B10.MBR skin grafts on mice reconstituted with K^b-transduced bone marrow was significant and reproducible, all K^b-disparate grafts were eventually rejected. Thus, in contrast to mixed cellular chimerism, gene therapy resulted in hyporesponsiveness to K^b, rather than true tolerance.

Upon further investigation, it became clear that K^b-reactive cytotoxic T lymphocytes did not appear to be deleted, but rather a deficit in T cell help induced following expression of K^b in bone marrow-derived cells led to prolonged survival of skin grafts. The presence of additional allogeneic MHC class I or II determinants expressed on skin together with K^b restored rapid rejection (18,19). Furthermore, alloreactive cytotoxic T cells specific for K^b could be detected *in vitro* when IL-2 was added to the cultures, supporting the hypothesis that a deficit in T cell help was induced rather that true tolerance (19). Detailed analysis of mutant K^b genes designed to encode MHC class I molecules that can enter the indirect pathway of antigen presentation through distinct cellular pathways showed that both CD4 and CD8 T cells which respond to K^b through the indirect pathway were capable of mediating graft rejection. In order to prevent rejection of K^b-disparate grafts, CD8 T cells that recognize K^b via the direct pathway, as well as CD4 and CD8 T cells that recognize K^b through the indirect pathways of antigen presentation, had to be tolerized in order to achieve prolonged skin graft survival (38). Interestingly, it appeared that CD4 T cells capable of recognizing K^b through the indirect pathway were most affected in this system, supporting the notion that prolongation of skin graft survival induced by gene therapy involved a peripheral mechanism controlled at the level of T cell help.

7. MOVING MOLECULAR CHIMERISM FROM HYPORESPONSIVENESS TO TOLERANCE

We speculated that the failure to induce stable long-term tolerance to alloantigens after antigen challenge by gene engineering of bone marrow could have been due to a failure to efficiently express the retrovirally-encoded alloantigen on cell types capable of inducing deletional tolerance, loss of gene expression, or to an extremely low level of initial expression. It was previously suggested that multi-lineage long-term chimerism is required to maintain stable tolerance induced by allogeneic bone marrow transplantation (39). We therefore hypothesized that to

achieve stable T cell tolerance, rather than hyporesponsiveness, by gene therapy, it would be necessary to achieve long-term expression of the retrovirally-transduced alloantigen on multiple hematopoietic cell lineages at relatively high levels. Indeed, it is clear that in the models described above in which gene therapy resulted in hyporesponsiveness, expression levels achieved were low, in many cases detectable only using PCR-based methods, and long-term expression was not demonstrated.

To establish tolerance rather than hyporesponsiveness, we set out to improve expression of K^b and achieve long-term expression. Improvements in vector construction and bone marrow transduction conditions allowed us to significantly improve transduction efficiency and achieve multi-lineage long-term expression of K^b on approximately 20% of bone marrow-derived cells readily detectable by cell surface staining and flow cytometry (40). Efficient expression of K^b on bone marrow-derived cells was sufficient to allow for permanent survival of K^b disparate B10.MBR skin grafts on 40% of B10.AKM mice reconstituted with K^b-transduced bone marrow; in all cases, third party control grafts were rapidly rejected. Importantly, in all mice, cytotoxic T cells capable of lysing K^b-expressing targets were undetectable in vitro even after in vivo sensitization by skin grafting, and subsequent re-exposure to antigen by immunization followed by re-stimulation in vitro in the presence of IL-2. This result was in sharp contrast to previous studies in which T cell hyporesponsiveness to K^b induced by genetic engineering of bone marrow could be broken by provision of T cell help (18,19). We suggest that improved expression of K^b on the surface of bone marrow-derived cells induced stable T cell tolerance, rather than hyporesponsiveness. Indeed, recent data we have acquired suggest that expression levels of K^b directly affect the ability to achieve tolerance versus hyporesponsiveness, and that central deletional mechanisms, rather than an effect on T cell help, lead to tolerance when efficient long-term expression of K^b is achieved (In preparation). These data strongly suggest that gene therapy can be used to reshape the T cell repertoire, leading to negative selection of alloreactive T cells in the thymus.

7. INDUCTION OF B CELL TOLERANCE THROUGH MOLECULAR CHIMERISM

In addition to inducing T cell tolerance, molecular chimerism could also be used to induce B cell tolerance. The ability to induce B cell tolerance may be particularly desirable in the case of discordant xenotransplantation. Shortages of human organs for transplantation have led to research into the possibility of using non-human species as organ donors. Pigs are now regarded as the most likely species to serve as donors for clinical xenotransplantation (41,42). However, rejection of pig tissues and organs, mediated by antibodies in the host that recognize a single carbohydrate antigen present on pig tissue, Galalpha1-3Galß1-4GlcNAc-R, hereafter referred to as alphaGal (43, 44-48) remain a significant barrier to successful pig-to-primate xenotransplantation (reviewed in (49)).

Mutant mice, generated by gene targeting in embryonic stem cells, which lack the alpha(1,3)galactosyltransferase (alphaGT) that synthesizes alphaGal, produce alphaGal reactive natural antibodies as do humans, without the need for immunization (50,51). These mice have therefore been used as a model to examine methods that will prevent production of alphaGal-reactive natural antibodies. While mixed chimerism induced by bone marrow transplantation has been shown to induce tolerance to the alphaGal epitope in alphaGT homozygous knockout mice (GT^0 mice) (52), as mentioned above, it seems unlikely that a mixed cellular chimerism approach will be useful in the setting of xenotransplantation because of the difficulty in establishing porcine bone marrow engraftment and long-term lymphohematopoeisis in primates (53,54). Using GT^0 mice , we therefore examined whether expression of alphaGT in bone marrow-derived cells could be used to induce tolerance to alphaGal, preventing the production of alphaGal-reactive natural antibodies as well as antibodies with the same specificity produced following rigorous antigen challenge. We demonstrated that expression of alphaGT in bone marrow-derived cells was able to completely inhibit production of alphaGal-reactive natural antibodies (55), and resulted in stable long-term tolerance to alphaGal even after rigorous antigen challenge (56). Importantly, tolerance remained intact even after mice received cardiac transplants from wild-type mice that expressed alphaGal, and prevented antibody-mediated rejection (57). Analysis of B cells from mice reconstituted with alphaGT-transduced bone marrow revealed that B cells which produce alphaGal-reactive antibodies were eliminated from the immunological repertoire following gene therapy. Collectively, these data suggest that gene therapy approaches may be used to induce B cell tolerance. Importantly, tolerance could be induced even in presensitized animals (Submitted). Therefore, gene therapy can be used to reshape the B cell repertoire.

To our knowledge, this is the first example in which gene therapy has been successfully applied to induce stable B cell tolerance to pre-existing natural antibodies without any additional immunosuppression. While others have shown that gene therapy can be used to induce B cell hyporesponsiveness (29,58,59), these studies failed to demonstrate tolerance. While we are not certain why in other models hyporesponsiveness rather than tolerance was achieved, we would suggest that the level of transduction and expression as well as the duration of expression could play a role. In our model, achieving efficient bone marrow transduction and long-term expression on bone marrow-derived cells was required in order to induce tolerance (56). Schumacher *et al*. reported very low transduction efficiencies (29). In their study it was not possible to determine if full B cell tolerance was achieved because only production of complement-fixing antibodies was examined at relatively early time points after reconstitution with genetically-modified cells. It is also possible that differences in the cell types expressing the retrovirally-transduced genes could have played a role. Bone marrow transduction conditions used by Kang *et al.* (59) included the growth factor IL-7, which is known to favor survival

and proliferation of B cell lineages. Interestingly, it has been reported that B cells can only tolerize virgin T cells (60). Since the antibody response studied by Kang *et al.* is clearly T cell-dependent, it is possible that because sublethally-irradiated mice were used as recipients of genetically-modified cells, memory T cells were not effectively tolerized, resulting in hyporesponsiveness.

9. MOVING MOLECULAR CHIMERISM TO PRECLINICAL NON-HUMAN PRIMATE MODELS

Using molecular chimerism to induce tolerance in preclinical non-human primate models represents the next logical step in determining whether any of the approaches outlined above will have potential for clinical application. Preliminary results obtained so far using gene-marking studies suggest that the induction of molecular chimerism may indeed be able to induce tolerance in primates (34). However, it is important to extend these findings to the transplantation setting in which the immune response to either allo or xenogeneic tissues is relatively potent and pre-existing.

Several issues must be considered when choosing an appropriate non-human primate model to examine the ability of gene therapy to induce tolerance. While most investigators would like to use a species that is most similar to humans, for ethical and practical reasons we are left with rhesus macaques, cynomologus monkeys and baboons as viable models. In xenotransplantation, most investigators regard the baboon as most similar to humans in terms of alphaGal antibody levels. However, of the three species mentioned, it appears that baboon CD34+ cells are the most difficult to infect with retroviruses utilizing various viral envelopes (our unpublished data and (61)). While a full evaluation of the ability of different envelopes to allow efficient transduction of various CD34+ cells from different species awaits, issues of infectability of different species with various viruses will influence which primate model is most appropriate. Clearly, in order to initiate proof of principle experiments in non-human primates several factors must be considered. We suggest that species should be selected for experimentation that allow for efficient isolation and infection of hematopoietic progenitors, have the ability to withstand bone marrow transplants, and have a relatively low level of pre-existing immunity to the product of transduced genes of interest. These species should be used first, and fully studied in order to further to optimize protocols and procedures. Only after it can be clearly demonstrated in primates that molecular chimerism can be used consistently to induce tolerance should one consider moving into a primate model that is a closer representative of humans or into the clinic.

10. CONCLUSIONS

Provided that appropriate levels of gene expression can be achieved on bone marrow-derived cells, the induction of molecular chimerism is capable of inducing immunological tolerance. Importantly, molecular chimerism can result in the same life-long, specific tolerance observed using mixed chimerism. However, several obstacles to clinical application remain. For example, it is important to determine if gene therapy can be effective in inducing tolerance using non-toxic host conditioning regimens. It will also be important to examine how far this technology can be extended. Can one use gene therapy to induce tolerance to multiple antigens, essentially resulting in matching of host and donor? It will also be important to follow gene therapy-treated animals long-term to examine whether chronic rejection can be prevented by gene therapy. Lastly, it will be important to investigate whether similar approaches can be used to treat other disorders, such as autoimmunity.

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