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Chimerism and Tolerance in Solid Organ Transplantation

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Abstract

Transplantation has become standard of care to treat end-organ failure, replacing a failed organ with a functioning one. However, the toxicity of the immunosuppressive agents that are critical to graft maintenance is significant. Complications associated with the use of these agents include opportunistic infections, cardiovascular disorders, an increased rate of malignancy, and renal failure. As a result, approaches to induce tolerance to transplanted organs and/or minimize immunosuppression are a major priority. This review summarizes the role of chimerism in tolerance induction, presenting an historic perspective and ending on clinical protocols actively underway.

Keywords: Chimerism; Tolerance; Renal transplantation

Introduction

Donor-specific tolerance has been referred to as the "Holy Grail" of organ transplantation. It has been actively pursued for over 6 decades. Despite promising experimental success, clinical application has largely remained elusive. The recent application of the bone marrow techniques in clinical solid organ transplantation has yielded results that could fundamentally alter the role of immunosuppression in organ transplant recipients in the near future. Hematopoietic stem cell transplantation (HSCT) is established as a therapeutic option for treatment of hematological disorders. The end result of allogeneic HSCT is often the establishment of chimerism with tolerance. Conventional HSCT involves the use of aggressive myeloablative conditioning that would not be acceptable in the context of organ transplantation where the recipients have severe physiologic derangement from end stage organ failure. Recent success with 'mini bone marrow transplants' using non-myeloablative conditioning in elderly patients with hematologic malignancy [1] have opened a new avenue for the application of chimerism in solid organ transplantation. In this review, we discuss the important historic experimental data leading up to translation of chimerism to the clinic and summarize the recent clinical protocols that have achieved tolerance in renal transplant recipients.

History of Chimerism and Tolerance Data

The pioneering experimental work in tolerance began in Sir Peter Medawar's laboratory in the 1950's. Preliminary studies focused on the induction of "actively acquired tolerance" by exposing animals to donor antigens in the perinatal period. The basis for this approach was the observation that red cell chimerism in the majority of dizygotic freemartin cattle twins that shared a common placenta [2] persisted into adulthood. This suggested that presentation of non-self-antigen during fetal and early neonatal life somehow resulted in acquired tolerance [3].

Early studies by Billingham et al. demonstrated that actively acquired tolerance could be achieved by pre-conditioning of the recipient with donor cells [3]. A suspension of homogenized tissue (testis, kidney & spleen) from strain A mice was injected into fetuses of CBA strain mice. Eight weeks after delivery of the fetuses, the young CBA mice were challenged with a skin graft from the donor strain A mice. Three of five mice demonstrated prolonged graft survival for over 50 days compared to only 11 day graft survival in controls. At 50 days, one of the three mice was challenged with a second donor skin graft, which was accepted and incorporated seamlessly into the host's skin. By days 77 and 101 respectively, two of the three mice still displayed graft acceptance. When the successfully grafted mice were injected with lymphoid tissue from CBA mice that had been immunized with strain A tissue, tolerance was lost and there was rapid rejection of the grafts. It was also demonstrated that the offspring of these mice did not demonstrate the same tolerance to strain A tissue. Billingham concluded that 1) acquired tolerance is the result of the host immune system's inability to react; 2) acquired tolerance is immunologically specific; and 3) tolerance acquired in one individual is nontransferable to offspring.

Billingham further pursued the concept of fetal tolerance further to determine whether a population of genetically diverse Wistar rats could be made tolerant by inoculation of cell suspensions derived from multiple donors into newborn animals [4]. By preparing tissue suspensions derived from 10 donors selected at random from a close but non-inbred rat population, Billingham hypothesized that in theory the inoculated recipients would be exposed to the entire antigenic spectrum of the population and would therefore be tolerant to tissues from any donor selected at random. The majority of Wistar rats injected with bone marrow or splenic tissue at birth became universally tolerant of skin grafts from any random donor within the population. But tolerance was highly specific to donors within the population or highly genetically similar populations. Wistar rats rejected grafts from Brown Norway inbred rats, a genetically distinct strain, but accepted skin grafts from congenic Lewis rats. Bone marrow tissue was the most effective in inducing tolerance. Its tolerogenic properties were significantly better than the splenic preparation. This was thought to be due to the fact that bone marrow contained a lower proportion of

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immunocompetent cells. Spleen cells from newborn donors induced tolerance better than adult splenocytes. Billingham concluded from these results that acquired tolerance is highly specific and dependent on the spectrum of donor antigens that a young host is exposed to. The above studies had a profound influence on subsequent transplantation research. The inherent advantages of chimerism as a means of achieving tolerance became an established principle and ways to refine this for clinical application became an important goal of transplantation researchers in the ensuing decades.

The next important contribution came from the studies of Monaco who demonstrated that infusion of antilymphocyte serum (ALS) with large doses of donor hybrid lymph node and spleen cells into thymectomized mice resulted in tolerance to donor skin grafts. Subsequent studies focused on the optimal lymphoid cell type, cell dosage, route of administration and the timing of cell injection for tolerance induction in non-thymectomized adult ALS treated mice [5,6]. Cells from lymph nodes, spleen, thymus and bone marrow were studied using incremental doses and infused at different time points after ALS infusion. It was concluded from these studies that a) bone marrow cell infusion was consistently superior to other tissues; b) the intravenous route was the most effective route for cell infusion; c) infusion dose of 50×10^6 was most effective; and d) cell infusion between day 4 and 8 after ALS was most effective. Monaco reasoned that the superior tolerogenicity of BM cells was most likely due to the high populations of stem cells within BM. Prolongation of renal allograft survival in ALS treated dogs by post-transplant bone marrow infusion was subsequently reported by the same group [7]. In pioneering clinical translation of the model in 1985, three renal allograft subjects received BM infusions. Two of the three patients remained rejection-free at one year. The third patient lost the allograft due to non-compliance [8].

The morbidity associated with conventional allogeneic bone marrow transplantation prevented the clinical application of hematopoietic stem cell transplantation (HSCT) over the ensuing 60 years. Toxicities include graft-versus-host disease (GVHD), the need for close genetic matching, and the toxicity of ablative conditioning believed to be critical for successful donor bone marrow grafts until recently. A method of overcoming these challenges was demonstrated in the studies by Ildstad et al. [9] who compared the tolerogenicity of mixed allogeneic/syngeneic BM chimeras and complete allogeneic BM chimeras. Mixed allogeneic mice received inoculations of T cell-depleted BM cells from syngeneic (self) BM and allogeneic (donor) BM. The mixed chimeras displayed significantly superior tolerance and immunocompetence as compared to the complete allogeneic mice in both *in vitro* lymphocyte-assays and *in vivo* skin graft studies. Donor-specific skin grafts were accepted and recipients did not exhibit any GVHD. The fact that recipients with as little as 1% donor macrochimerism were tolerant opened the door for development of reduced-intensity conditioning to establish chimerism. Based on these results, Ildstad et al. concluded that the syngeneic BM components allowed hosts to overcome restriction of immune cell interactions that are seen in ablated fully allogeneic animals, while allogeneic elements promoted the conditioning of host tolerance to the donor graft. This important finding has been the basis for recent tolerance induction studies in renal transplantation.

One of the major challenges with the application of chimerism to induce tolerance is the occurrence of GVHD. While some GVHD is considered beneficial in HSCT for hematologic malignancy, it is absolutely unacceptable for tolerance-inducing strategies. Aggressive T cell-depletion of the allogeneic graft can reduce the incidence of GVHD but has drawbacks including delayed immune reconstitution and impaired donor cell engraftment [10-13]. The highest rate of graft failure occurred in MHC disparate recipients, which represent the majority of solid organ recipients. The discovery of $CD8^+/TCR^-$ facilitating cells (FC) that are distinct from T cells and promote engraftment without an increased risk of GVHD allowed strategies to promote engraftment yet avoid GVHD in mismatched recipients [14]. These cells were first phenotypically characterized as $CD8^+/TCR^-$ as well as class II^+, $Thy1^+$, $CD5^+$, $CD2^+$ cells in the marrow. FC are composed predominantly of a plasmacytoid precursor dendritic cell subpopulation (p-preDC FC) [15]. The potential of FC to promote engraftment as a means of achieving tolerance in solid organ transplantation was demonstrated in mice in 2003 [16] and has recently been translated to the clinic [17]. Preclinical and clinical studies reported by Monaco and others have demonstrated the significant advantages of bone marrow in enhancing transplant survival by promoting donor tolerance and host chimerism. However, the exact mechanistic details of tolerance induction had not been well-defined. The studies of Strober et al. [18] have been instrumental in elucidating the cellular mechanisms of BM induced tolerance.

In a mouse transplant model, Strober et al. [18,19] observed that at the cellular level, rare populations of natural killer T cells (NKT) cells in the bone marrow protected the graft recipient against lethal GVHD by modulating and suppressing conventional T cells through the secretion of specific cytokines, thereby promoting tolerance and graft survival. More specifically, a recent study has identified the iNKT subclass as the key element in prevention of GVHD [20]. The tolerogenic activity of iNKT cells has been characterized as follows: 1) iNKT cells secrete large quantities of Th1 or Th2 cytokines that can enhance or suppress conventional T cells depending on the immune environment [21]; and 2) iNKT cells also produce IL-4 that contribute to GVHD suppression [22]. The primary pathway by which IL-4 protects against GVHD is through up regulation of IL-10 production, which enhances Th2 expression by Tcells, ultimately minimizing GVHD.

In another study of combined cardiac and HSCT in a mouse model, Strober et al. demonstrated the tolerogenicity of regulatory T cells (T_{reg}), particularly $CD4^+CD25^+T_{reg}$. In the same study, it was shown that tolerance and chimerism could be restored by transferring T_{reg}cells to T_{reg}-depleted heart and BM transplant mice [23]. The tolerogenicity of T_{reg} was dependent on its production of IL-10, which in turn is dependent on iNKT secretion of IL-4.

The induction of tolerance via iNKT and T_{reg} cells has been successfully evidenced in human trials by Strober [10]. In patients with hematolymphoid malignancies that have received BM transplants, those who have received conditioning with irradiation and anti-thymocyte globulin (ATG), which alters the host and donor T cell function and balance, showed significantly lower rates of acute GVHD (<5%) compared to patients who only received irradiation therapy or chemotherapy or both (>50% in previous studies). However, despite the lowered incidence of acute GVHD events with novel T cell-modulation therapy, one-third of patients who had received ATG treatment still developed chronic GVHD. Postoperatively, levels of IL-4 production were significantly enhanced by donor $CD4^+$ T cells in the transplant patients as compared to non-transplant control group. The majority of patients who had received radiation and ATG conditioning did not require immunosuppressive agents by the end of one year.

Large Animal Chimerism and Tolerance Studies

The translation of mixed BM chimerism-induced tolerance from studies in mice has been more challenging to duplicate in large animal models. Clearly, the approach for conditioning and composition of the donor HSCT product are two important variables. One major transformational advance in allowing translation of chimerism-induced tolerance to the clinic was the recognition that non-myeloablative conditioning could be utilized to establish chimerism, replacing ablative conditioning and its associated toxicities. This has resulted in significantly reduced morbidity and mortality and is performed as an outpatient [24-28]. While 1200 cGy of TBI is ablative in humans, only 200 cGy of TBI is required for engraftment when combined with myelosuppressive agents.

Canine models

Mixed donor-host chimerism was successfully established in dogs [29]. Dog leucocyte antigen (DLA) identical dogs conditioned with a non-myeloablative dose of total body irradiation (TBI) (1-2 Gy) and immunosuppression with cyclosporine A & MMF for 4 weeks after the transplant became durable chimeras. Further, these recipients accepted kidney allografts from their marrow donors without immunosuppression. A more recent publication from the same group showed long-term acceptance of highly antigenic vascularized composite tissue allografts (VCA) using a similar protocol [30]. Elevated levels of $CD3^+$ Fox $P3^+T_{reg}$ were present in the VCA graft. Based on these results, the authors concluded that mixed allogeneic BM chimerism protocols were a viable approach to promote tolerance in the clinic.

Non-human primate (NHP) models

The translation of mixed chimerism from mice to outbred, pathogen-exposed, NHP models has been even more challenging. This is thought to be due to immunologic instability of mixed chimerism, particularly in the setting of low level of T cell chimerism. Kawai et al. [31] demonstrated acceptance of renal transplants in NHP: nearly half of highly MHC-matched cynomologous macaque recipients accepted kidney allografts following a regimen including splenectomy or anti-CD154 treatment, TBI, thymic irradiation, ATG, and donor marrow infusion.

Investigators at Emory recently described compartmentalized chimerism or split chimerism [32]. A high-level of whole blood chimerism was established in rhesus macaques conditioned with a nonmyeloablative regimen including busulfan and co-stimulation blockade/sirolimus. The chimerism was comprised of myeloid (neutrophil) chimerism with little or no T cell chimerism. A donor-specific renal allograft was rejected by these chimeric recipients. The authors concluded that the presence of transient T cell poor chimerism is not sufficient to induce tolerance to a concurrently placed renal allograft in NHP. The importance of T cell chimerism has been reported in a number of species, including humans (reviewed in Xu and Ildstad, 2012). Thus, a more consistent and stable set of protocols for tolerance induction is needed, and more mechanistically-focused conditioning strategies are required for non-human primates and human patients as opposed to small laboratory animal models [24]. A number of other groups have reported that production of donor T cells is critical for tolerance induction to occur [32-34].

In summary, the potential role of mixed chimerism as a means to induce tolerance in solid organ transplantation has a long history dating back to the elegant studies of Billingham and Medawar over 60 years ago. The exact role of FC, the requirement for donor T cell production for stable tolerance, the precise role regulatory T cells, and the biomarkers for a tolerant state remain to be fully defined. Because tolerance has remained an elusive goal, most bioassays for tolerance have been performed in operationally or functionally tolerant organ transplant recipients who have stopped their own immunosuppression and have maintained stable graft function. This cohort is comprised of a very small number of organ recipients worldwide [35]. The majority of individuals who stopped immunosuppression experienced rejection and often premature graft loss [36]. The recent clinical success in induction of tolerance in renal transplantation, in some ways, has surged ahead of mechanistic studies. Future research should further explore the mechanism of tolerance induction.

Tolerance in the Clinic

The acceptance of transplanted solid organs without immunosuppression has been sporadically recorded in the literature [36]. These reports include non-compliant patients who elected to discontinue their medications, transplantation between monozygotic twins, solid organ transplant following a prior successful bone marrow transplant from the same donor and simultaneous hematopoietic and renal transplantation for the treatment of multiple myeloma with associated renal failure.

The simplest case of donor-specific tolerance is transplantation between monozygotic genetically identical twins. A review in 2008 reported 132 such renal transplants with excellent results [37]. The experience with HLA non-identical transplants is more limited.

A small number of renal transplant recipients have been reported to develop operational immunological tolerance following non-compliance with immunosuppression. An early report [38] from Wisconsin detailed an HLA matched kidney recipient who had stable graft function 36 months after discontinuing azathioprine and prednisone. In a recent update [39], this patient had excellent graft function (creatinine 1.2 mg/dl) 30 years after stopping medications. However, only a small cohort of such subjects exists worldwide out of tens of thousands of transplants performed [35,36]. The vast majority who stopped immunosuppression experienced rejection and frequently grafts loss.

The liver is thought to be a more tolerogenic organ–a proportion of liver recipients maintain normal allograft function without immunosuppression. This has been termed operational tolerance and has been proposed to be as high as 20% [40]. In a recent prospective pilot study [41], 60% of pediatric recipients of parental living donor liver transplants remained off immunosuppressive therapy for at least 1 year with normal graft function and stable allograft histology. However, inclusion criteria required that only subjects with stable graft function on monotherapy be enrolled. Therefore, only a small proportion of liver transplant recipients would be eligible. Unfortunately, there was no reliable biomarker to predict which subjects would be successfully tapered and discontinued.

In 1989 Strober [42] reported acquired immune tolerance to deceased donor renal grafts in 3 patients conditioned with total lymphoid irradiation. Subsequent reports documented the successful transplantation of solid organs following a prior bone marrow transplant from the same donor. Sayegh [43] was the first to report immunological tolerance to renal allografts after bone marrow transplants from the same donors. The two patients described had received bone marrow transplant for acute leukemia and subsequent

renal transplantation from the same HLA identical donors at a follow-up of 1 & 2 years, respectively. The renal function was good despite lack of standard immunosuppression. There have since been other reports describing similar success in the context of HLA mismatched donors [44-47], following living donor kidney, lung and liver transplantation [48,49].

All of the above reports were sequential transplants: the bone marrow transplant was performed in the traditional way with myeloablation leading to complete chimerism followed by solid organ transplantation. The problem with this approach is that the morbidity and mortality associated with complete myeloablation is not an acceptable risk: benefit ratio for establishing donor-specific tolerance in the context of solid organ transplants. For widespread application in solid organ recipients the approach must be safe, relatively simple to perform, and successful in mismatched recipients.

The success of non-myeloablative conditioning has substantially reduced the risk of establishing chimerism. The feasibility of this approach in renal transplantation was reported in 2006 [50] when 6 patients with multiple myeloma and renal failure received simultaneous kidney and bone marrow transplantation from HLA-identical sibling donors following non-myeloablative conditioning with cyclophosphamide, anti thymocyte globulin and thymic irradiation [51]. Mixed chimerism was achieved in all subjects initially but was lost in 4 during follow-up. Despite the loss of chimerism, 3 of 4 showed renal allograft acceptance for a prolonged period (ranging from 1.3 to 7 years) without immunosuppression.

Thus, it began to emerge that mixed chimerism could be the missing link towards achieving donor-specific tolerance in renal transplantation [52]. Three recent but distinct protocols have been reported that have generated tremendous excitement in the field and promise to deliver on the much sought after clinical tolerance [17,51,53].

The Stanford protocol [53] described the successful use of total lymphoid irradiation (800 cGy) and antithymocyte globulin to achieve persistent mixed chimerism in a patient receiving combined renal and hematopoietic cell transplantation from an HLA matched living donor. The G-CSF mobilized product was apheresed and CD34-selected. A fixed number of $CD3^+$ cells were added to the final product. In a recent update of this experience, Scandling [54] reported data from 16 patients. Fifteen of them developed multilineage chimerism without the occurrence of GVHD. In 8 patients with long term chimerism (>6 months), withdrawal of immunosuppression was recorded to be successful for up to a mean of 28 months. The major limitation of this approach is that it was successful only in the setting of HLA match at A, B, C, DR, DP and DQ loci [55]. Thus, the vast majority of renal transplant recipients and virtually all recipients of deceased donor organs would not be candidates for this protocol.

The second report in the same issue of NEJM described the experience of using a different approach in HLA mismatched renal transplant recipients [51]. Patients with end-stage renal disease received combined bone marrow and kidney transplants from HLA single haplotype-mismatched living related donors. The non-myeloablative conditioning consisted of 2 doses of cyclophosphamide, 3 doses of humanized anti-CD2 monoclonal antibody, cyclosporine A and 700 cGy of thymic irradiation. Rituximab was added to the regimen after the loss of a kidney from irreversible acute humoral rejection. Transient multilineage mixed chimerism was observed in all patients but became undetectable after 2 weeks. In a recent updated report [56], 9 of 10 patients were noted to have developed transient acute kidney injury (AKI) accompanied by a capillary leak syndrome around the 10^{th} post-operative day. This poorly understood phenomenon was termed engraftment syndrome. Renal biopsies showed marked acute tubular injury with interstitial edema, hemorrhage and capillary congestion, with little or no interstitial infiltrate and marked glomerular and peritubular capillary endothelial injury and loss by electron microscopy. C4d deposition and transient arterial endothelial inflammation was noted in 2 of the patients. Six patients were treated with anti-rejection regimens including steroid pulse, ATG, plasmapheresis, IVIG and Rituximab. Overall, acute rejection was noted in 4 patients: two with humoral and two with cellular rejection. Two patients lost the renal grafts: one following acute humoral rejection and another secondary to thrombotic microangiopathy. Recovery of the acute kidney injury occurred in 8 patients who were reported to have a mean creatinine of 1.5 ± 0.3 mg/dl at 9 months to 7 years follow-up. Withdrawal of immunosuppression was completed in these 8 patients as per protocol. Only one of these 8 developed acute cellular rejection after discontinuation of medication and was reinstated on immunosuppression. It is interesting to note that 2 of the initial 5 patients developed donor-specific antibodies without graft dysfunction and remain off immunosuppression. High levels of FoxP3 mRNA were detected in some of the allograft biopsies. The chief drawbacks of this approach are the lack of durable chimerism, the near universal occurrence of engraftment syndrome with acute kidney injury and the applicability to patients receiving at least a haplomatched graft.

More recently, Leventhal and Ildstad published their experience in achieving chimerism and tolerance without GVHD in HLA-mismatched combined renal and hematopoietic stem cell transplantation [17]. The methodology included the use of a bioengineered mobilized cellular product enriched for hematopoietic stem cells and tolerogenic $CD8^+/TCR^-$ graft facilitating cells (FCRx). The non myeloablative conditioning regimen consisted of 2 doses of cyclophosphamide (days -3 and +3, 200 cGy of TBI and 3 doses of preoperative fludarabine (days-4,-3 and -2). This was followed by renal transplantation (day 0) and FCRx infusion one day after transplant. Immunosuppression after transplant consisted of MMF and tacrolimus. All patients developed a characteristic nadir of absolute neutrophil counts about a week later but showed recovery (ANC>500) by a mean of 9 days. Multilineage chimerism was achieved in all subjects at 1 month after transplant.

The first 4 patients provided important lessons for subsequent success of this protocol. The first and fourth patients lost chimerism by 5 and 3 months respectively. The first patient did not receive the second dose of cyclophosphamide due to safety concerns about the nadir in the early part of the study. Additionally, he received a lower dose of FCRx than those who developed chimerism later on. In retrospect, these 2 factors were probably responsible for the early loss of chimerism in this patient. The fourth patient received a reduced dose of FCRx due to unresolved concerns about a skin rash that had developed in the previous patient. The fourth subject had completed his conditioning before the skin biopsy report was finalized. The biopsy was consistent with sulfa drug based photosensitivity and not GVHD. Chimerism was lost at 3 months but donor-specific hyporesponsiveness was detectable at 6 months. Based on this, gradual reduction of immunosuppression was initiated. Unexpectedly, in spite of persistent donor-specific hyporesponsiveness, the protocol renal biopsy at 12 months showed subclinical Banff 1A rejection that was reversed with steroids. The patient has subsequently been maintained on tacrolimus monotherapy. The current αβ Tcell dose has been set at 3.8×10^6/kg recipient body weight. This is based on the durable donor chimerism with tolerance in the third patient. This has proven to be adequate for achieving lasting

donor chimerism without the occurrence of GVHD in 12 subsequent patients [17].

Of the initial 8 patients, the HLA match was less than haploidentical in 4 and haploidentical in 3. This is the first clinical report of achieving durable chimerism with tolerance in highly mismatched related and unrelated recipients using donor marrow infusion. This approach provides promise for the vast majority of renal transplant recipients who are HLA mismatched. The other significant finding in the study is the absence of GVHD in any of the recipients.

Future studies will need to be directed at identifying the mechanism of tolerance induction using this protocol, further characterization of the facilitating cells, long-term stability of donor chimerism & donor-specific tolerance and the immune competence of the chimeric patients. In addition, further refinement of the conditioning regimen, applicability to the deceased donor situation, delayed use of the protocol in previously transplanted patients and the adaptation to highly sensitized patients will need to be explored.

Summary

The road to tolerance has been long and arduous. The recent clinical success has placed us on the threshold of making immunosuppression free transplantation possible. However, much work remains to be performed in understanding the mechanisms of tolerance and tailoring the protocols to the complex patients with end stage organ failure. The immediate tasks are increasing the experience and longer follow-up to determine whether the tolerance is stable. If the tolerant state shows durability without compromise of immune function or occurrence of GVHD, a paradigm shift in organ transplantation would have been achieved. Cell-based therapies represent a promising new frontier that is now being translated successfully to the clinic. Successful establishment of donor chimerism in mismatched recipients could provide a promising therapy for autoimmune disorders, inherited enzyme deficiencies, and hemoglobinopathies, in addition to inducing tolerance in organ and islet recipients.

Competing Interests

S.T.I. has equity interest in Regenerex, LLC, is a start-up biotech company. The company has not been capitalized.

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