examined so that discussions about best care can appropriately represent the multiplicity of perspectives.

The current dialogue describing flaws in the CKD staging system and the need to modify the classification system may or may not be useful in advancing the field or caring for patients [2,3]. Increasing the precision of the predictability of progression at any given stage by adding parameters may be valuable but should not change the fundamental premise of the staging system [14]. Age-adjusted values for eGFR will not address the issues appropriately, nor will they likely lead to a change in current knowledge or outcomes.

The elderly do have CKD, to a greater proportion than do their younger counterparts. The natural history of the condition is different, not the disease itself. The care of these patients therefore is likely not the same as that of younger age groups, but we are still far from understanding how to optimize the care of these patients. Identifying progressors, irrespective of age, is a first step in a long road of discovery.

Conflict of interest statement. None declared.

(See related article by B. Conway *et al*. Predicting mortality and uptake of renal replacement therapy in patients with stage 4 chronic kidney disease. *Nephrol Dial Transplant* 2009; 24: 1930–1937.)

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Received for publication: 14.2.09; Accepted in revised form: 18.2.09

Nephrol Dial Transplant (2009) 24: 1726–1729
doi: 10.1093/ndt/gfp084
Advance Access publication 4 March 2009

Tolerance in renal transplantation: is mixed chimerism the missing link?

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Keywords: mixed chimerism; tolerance; transplantation

Three articles published in the *New England Journal of Medicine* at the beginning of 2008 demonstrated that the induction or development of mixed chimerism in kidney or liver transplant recipients can lead to long-term donor specific tolerance following transplantation, irrespective of whether the chimerism is sustained or not [1–3].

Chimerism occurs when foreign (donor) haematopoietic cells are present in an individual. Complete chimerism indicates that all haematopoietic cells (100%) are derived from a donor stem cell inoculum (for example, following myeloablation and transplantation of donor haematopoietic cells), whereas in mixed chimerism donor cells of multiple lineages constitute a varying part of the total haematopoietic pool. When the presence of donor cells occurs at levels below that detectable by flow cytometry (<1%),

and can only be detected by more sensitive methods such as polymerase chain reaction (PCR), it is referred to as microchimerism [4].

Individuals who have complete chimerism after myeloablative therapy and bone marrow transplantation subsequently accept solid organ allografts from the same donor [5]. The complete replacement of host bone marrow with that from an allogeneic donor results in the tolerance to the alloantigens of the bone marrow donor. However, this approach requires complete myeloablation that is associated with substantial morbidity and mortality. While this is essential in patients with haematological malignancy to ensure that all tumour cells are eliminated before bone marrow transplantation, it would not be justified as a treatment strategy in transplant recipients with otherwise normal bone marrow. Furthermore, bone marrow transplant (BMT) across HLA barriers carries a substantial risk of graft-versus-host disease (GVHD). In contrast, mixed chimerism can be established using non-myeloablative conditioning regimens and is associated with a reduced susceptibility to GVHD [6], whilst maintaining improved immunocompetence [7]. Consequently, there has been considerable research to develop protocols that permit mixed chimerism following transplantation.

The persistence of donor-derived haematopoietic cells has been suggested to modulate immune responsiveness to donor alloantigens facilitating the development of tolerance. Interestingly, the persistence, albeit at low levels, of maternal cells in their off-spring have been reported and implicated in the acquisition of immunological tolerance to non-inherited maternal antigens [8,9]. Microchimerism has also been detected in transplant recipients with long-term surviving allografts [4,10,11]. However, direct evidence of immunomodulation that leads to tolerance by the persistence of low levels of chimerism *in vivo* is inconsistent. One study showed that one-third of patients with long-term graft survival had microchimerism [12], whereas in others microchimerism could still be detected in patients experiencing allograft rejection [13,14] with the level of chimerism fluctuating with time [14]. Thus, there does not appear to be a clear correlation between the detection of microchimerism and clinical outcome, i.e. rejection or tolerance.

To achieve tolerance, both pre-existing mature donor-reactive T cells and developing donor-reactive T cells need to be eliminated or inactivated in a sustained manner. To achieve the latter, experimental models of mixed chimerism employ total body irradiation (TBI) [15], T cell-depleting antibodies, cytotoxic drugs, [16,17] or the induction of peripheral clonal deletion using costimulatory blockade [18]. After rendering peripheral donor-reactive T cells ineffective, central tolerance in mixed chimerism is most likely achieved when donor stem cells engraft, producing cells of multiple haematopoietic lineages including haematopoietic progenitor cells that seed the thymus. The progenitor cells that persist in the thymus can develop into specialized thymic dendritic cells [19] that can induce deletion of antigen-specific T cells (clonal deletion). Thus, in mixed chimerism, both host and donor haematopoietic cells mediate intrathymic deletion of host and donor-reactive T cells [20]. This renders the host tolerant to host and donor antigens, as long as the donor haematopoietic stem cells persist in the bone marrow.

Early work demonstrated that after TBI, mice reconstituted with a mixture of recipient and donor bone marrow develop mixed chimerism and robust tolerance to donor skin grafts [15]. Subsequent studies improved this protocol by reducing the toxicity of host conditioning, using leukocyte-depleting agents, such as anti-thymocyte or lymphocyte globulin (ATG or ALG) [21], and subsequently depleting anti-CD4 and anti-CD8 monoclonal antibodies (mAb) [16] or by co-stimulation blockade prior to a non-myeloablative dose of TBI. Immunocompetence is demonstrated by the fact that these mice are able to reject third-party grafts [16], whereas protocols that induce full chimerism also induce a degree of immuno-incompetence [23]. Interestingly, these protocols typically produce indefinite mixed chimerism in mice, whereas in non-human primates (NHP) mixed chimerism is sustained for only a few weeks [24], yet long-term graft survival is also maintained.

These promising animal results posed an interesting ethical dilemma, which delayed the translation of mixed chimerism strategies into human studies: does the potential benefit of long-term graft survival without immunosuppression outweigh the risk of bone marrow ablative therapy in patients with end-stage renal disease (ESRD) and normal bone marrow? This question remained unanswered until 1998, when a trial began in patients with renal failure due to myeloma, who therefore required both bone marrow and renal transplants. A modification of a tolerogenic NHP protocol was employed, in which patients underwent thymic irradiation together with ATG, but with cyclophosphamide (instead of TBI, which was used in NHP) prior to simultaneous HLA-matched bone marrow and renal transplantation. Six such transplants have been performed to date, with all patients accepting their grafts long-term [25]. Interestingly, three patients lost detectable chimerism but maintained graft function without immunosuppression or rejection episodes for up to 7 years [25]. This loss of chimerism was in marked contrast to what had been observed in both small and large animal studies.

Importantly, this approach has since been extended to patients with ESRD but without bone marrow disease and to HLA single haplotype-mismatched donors [2]. Protocol modifications were required to enable non-myeloablative conditioning to be used successfully in patients who were receiving transplants from HLA-mismatched donors, that included replacing ATG with anti-CD2 mAb together with the addition of anti-CD20 therapy to reduce the risk of humoral rejection, as one patient in the series of five lost their graft due to irreversible humoral rejection. The remaining four patients also suffered a rejection-like capillary-like syndrome. However, all episodes responded to corticosteroids and withdrawal of immunosuppression was still successful, demonstrated by excellent renal function for between 2 and 5 years post-transplantation to date, following withdrawal of all immunosuppressive therapy with evidence for immunologic recovery *in vivo* and *in vitro* [2].

Despite the exciting potential of these results, several important questions remain unanswered. Primarily, the underlying mechanism remains unknown, as leukocyte chimerism was only demonstrable for 3 weeks. This

does not exclude microchimerism as a possible mechanism, as the study only analysed peripheral blood samples. Furthermore, although high levels of Foxp3 messenger RNA were found in allograft biopsies, and donor-specific unresponsiveness was observed *in vitro*, no direct evidence was provided of suppressive activity attributable to regulatory T cells.

The second paper in the series reported persistent mixed chimerism in one patient, from a series with multiple myeloma and ESRD, who received a combined renal transplant and HLA-matched donor haematopoietic cells, following a conditioning regime of total lymphoid irradiation (TLI), ATG and mycophenolate mofetil for 1 month post-transplantation [3]. Allograft function has been normal in the patient with persistent mixed chimerism for more than 2 years since discontinuation of immunosuppression, with no episodes of rejection. Analysis of this patient's T cells after transplantation demonstrated that naive $CD8^+$ T cells repopulated the periphery faster than naïve $CD4^+$ T cells. This was thought to be due to peripheral expansion rather than thymic generation of new T cells. Additional analysis indicated that donor lymphocytes present in the recipient were of thymic origin, suggesting a central deletion mechanism [3]. This study supports earlier work demonstrating that pre-transplant TLI can induce mixed chimerism and immune tolerance to cadaveric renal allografts [26] whereas post-transplant TLI produces transient microchimerism and acute rejection following withdrawal of immunosuppression [27]. The authors suggest the persistent chimerism in this patient was due to engraftment of donor stem cells (as outlined earlier). This raises an interesting comparison with the previously described study from Kawai and colleagues, as stem cells were thought to contribute to chimerism in both cases, but chimerism was not maintained in the former study, yet both studies demonstrated excellent graft function after withdrawal of immunosuppression. Clearly, there is a strong need for more basic scientific research to dissect the differences in these protocols, and determine the role of chimerism in tolerance induction.

Interestingly, in a third article in the same issue of the *New England Journal of Medicine* [22], a unique report demonstrates how the evolution of chimerism may be affected by immunosuppression: a 9-year-old girl developed acute fulminant hepatitis after a nonspecific viral illness, and underwent liver transplantation from a deceased male donor. Nine months after transplantation, her peripheral blood had converted to the HLA of the donor, indicating the development of chimerism by engraftment of recipient marrow by passenger haematopoietic stem cells from the transplanted liver. One month later, the patient developed steroid-resistant severe intravascular haemolysis with haemoglobinuria and transient renal insufficiency. Haemolysis was thought to be due to residual recipient peripheral B cells (of which 98% were donor & 2% were host derived), as marrow analysis indicated that all B cells were of donor origin (XY). Immunosuppression was withdrawn, which permitted completion of engraftment and resolution of the anaemia. In this patient, the profound lymphopenia at presentation and in the subsequent months after transplantation, plus the immunosuppressive effects of drugs such as tacrolimus, azathioprine and ganciclovir, may have contributed to the engraftment of donor haematopoietic stem cells. The cause of hepatitis and profound lymphopenia remains unknown, although various hepatotrophic viruses can cause both these symptoms [28]. Despite the fortuitous nature of the outcome, this study provides exciting evidence linking preoperative lymphodepletion with a successful long-term graft outcome. Whether accidental or intentional, preoperative lymphodepletion links all three articles described above. Starzl has suggested that this process permits reciprocal clonal deletion of donor and recipient immunocompetent cells after engraftment, which facilitates a tolerant state [29]. Any imbalance in this process will produce either graft versus host disease, or host versus graft effects (rejection). However, the significance of this mechanism is not fully understood, because current immunosuppressive regimens are likely to interfere with the process of clonal deletion.

Taken together, these three reports show that protocols which induce mixed chimerism can lead to long-term donor-specific tolerance following renal transplantation, whether chimerism is sustained or not. Although these studies provide a fascinating insight into the potential role of chimerism in humans, data remain sparse in comparison to animal studies. Notably, recent animal data indicate that donor tissue rejection by chimeras can still occur, presumably because some tissue antigens are not expressed on donor haematopoietic cells [30] or that other mechanisms associated with the function of the adaptive immune system may play a role [31]. Such processes must be borne in mind when we consider the application of strategies to induce mixed chimerism in humans. However, although the mechanisms remain incompletely understood, the potential therapeutic application of these approaches is promising. The experience learned from the adverse events in the human studies we described should enable the development of more robust protocols, which is vital before such protocols are more widely employed.

Conflict of interest statement. None declared.

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Received for publication: 10.2.09; Accepted in revised form: 10.2.09