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The Use of Biomarkers in Clinical Transplant Tolerance

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Abstract

Transplant tolerance is the ultimate goal of transplant physicians and scientists alike. Here we provide a brief outline of transplant tolerance and how it can be achieved through induction protocols or immunosuppressive weaning. We also describe why research into transplant tolerance and its implementation in the clinical arena would be helped by the discovery of "tolerance biomarkers". Focusing on humans, we discuss the difficulty in identifying such biomarkers and show how, at least for the time being, there are no biomarkers of clinical transplant tolerance, but rather biomarkers of favorable or poor graft outcome. Along these lines, we describe some of the techniques that are helping to provide an indication of graft outcome, and where possible, we provide examples of their application to tolerance.

Overall, we describe how tolerance biomarkers are needed to measure susceptibility of patients to respond to tolerance-inducing regimens, to diagnose tolerance following induction or immunosuppressive weaning, to monitor the state of tolerance, and to predict its potential breakdown. The identification of such biomarkers is hampered by the fact that the mechanisms implicated in the majority of cases of tolerance observed in humans, which are probably multiple in nature, are only just beginning to be deciphered. This is further exacerbated by the difficulty in defining the ideal reference group from which to distinguish the tolerant patients. Moreover, the apparent diversity between tolerance models in rodents means that simple extrapolation across models is not likely to be possible, let alone extrapolation across species to large animal models and humans. Techniques used to search for biomarkers of graft outcome in general and their application to tolerance range from small-scale techniques such as ELISA, flow cytometry, the* trans-vivo *delayed hypersensitivity assay, quantitative PCR and TcLandscape® analysis, to wide-scale screening using DNA microarrays and proteomics. We believe that large-scale meta-analyses comparing rodent models between themselves, comparing rodents to humans, comparing different types of tolerance in humans (induced or following weaning) as well as tolerance across

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organs (e.g. kidneys and liver) could be enlightening, perhaps leading to the identification of universal biomarkers. Thus, the use of recent technological advances together with extensive collaboration should help, in the long term, to achieve this goal. **(Trends in Transplant 2007;1:46-55)**

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Key words

Biomarker. Tolerance. Clinical correlates. Immunologic monitoring.

Tolerance as a solution to the current challenges in transplantation

Modern immunosuppression has greatly improved the half-lives of organ transplants. Nevertheless, late graft loss remains a major problem. This is potentially compounded by the fact that the success of transplantation has opened up its field of application to a wider group of patients, and that the repercussion of this (i.e. the lack of adequate numbers of organ donors) means that less "desirable" donors in terms of age, cold ischemia time, etc. are now being accepted, which could adversely affect long-term results. In addition, despite having revolutionized the field of transplantation, immunosuppression is in itself detrimental to the health of transplant patients when applied lifelong, notably by increasing the risk of malignancy[1] and infection[2]. Moreover, calcineurin inhibitors are nephrotoxic[3], which in the case of kidney transplantation obviously negatively impacts graft outcome. Finally, there is the economic issue; reducing immunosuppression even in a small percentage of patients would have a considerable economic impact, reducing costs for healthcare providers and patients alike. An ideal solution to these problems would be the induction of a state of permanent graft acceptance that would ultimately require no maintenance immunosuppression and would not compromise the patients' capacity to defend themselves against infections and cancer. This sought-after goal is referred to as transplant tolerance and has been the subject of intense research for more than half a century.

The definition of tolerance and its induction

Experiments by Billingham, et al. in the 1950s showed that actively acquired immune tolerance to foreign antigens could be achieved in neonates[4]. Since then, tolerance to alloantigens has been a major goal of transplant physicians and scientists alike.

The ideal definition of transplant tolerance in rodents has several key elements: a well-functioning graft with no histologic signs of rejection, the absence of immunosuppression, immunocompetence (able to respond to other antigens such as pathogens) and donor specificity (able to accept a second graft from the same donor and reject a third-party graft).

Approaches to induce transplant tolerance fall into two major categories. The first category involves the induction of tolerance to alloantigens through central deletion and

the establishment of chimerism in the recipient[5]. The second is a more wide-ranging category encompassing one or more of several phenomena taking place in the periphery, including deletion of alloreactive cells[6], unresponsiveness or anergy[7], suppression[8], and ignorance[9].

The majority of research into transplant tolerance comes from studies in rodents where long-term graft survival can be induced by a variety of protocols from co-stimulation blockade[10] to donor-specific blood transfusion[11], or immunomodulation with alloimmune serum[12] or immunosuppressors[13]. Efforts to translate these tolerance-inducing strategies to primates have also been made[14]. More recently, certain strategies have been tested in the clinic such as peripheral depletion[15] or induction of chimerism through simultaneous bone marrow and kidney transplantation[16]. The depletion approach was unsuccessful in inducing tolerance as maintenance immunosuppression had to be introduced in all patients[15]. The bone marrow transplantation approach, on the other hand, gave promising preliminary results, with all six patients being ultimately off immunosuppression[16]. Nevertheless, this regimen is not without risks and is limited to a small number of target patients. Thus, overall, it is becoming clear that the situation prevailing in rodents in terms of tolerance induction is very different to that in humans, and transplantation tolerance is going to be difficult to deliberately induce in the clinical arena.

Tolerance following immunosuppressive drug weaning

In addition to the strategies to induce tolerance described above, there is another school of thought that immunosuppressive drug minimization may be the way to achieving tolerance in the clinic. Indeed, transplantation tolerance can occur spontaneously in certain patients following immunosuppression withdrawal. Such spontaneous operational tolerance is most common in liver transplant recipients where deliberate immunosuppressive weaning is now undertaken in some centers[17]. This phenomenon is much rarer in kidney transplant patients, although small cohorts of up to 10 patients have been reported on in the recent literature[18-21].

The fact that operational tolerance has been observed in the clinic, albeit rarely, shows that tolerance exists in humans and gives hope that one day clinical transplantation tolerance will become a more widespread phenomenon. This will depend not only on the development of tolerance-inducing strategies that actually work in humans, but also on our ability to diagnose tolerance, to monitor it, as well as to be able to provide a prognosis for patients in tolerance trials; e.g. will a given patient respond to the tolerance-induction regimen or will the tolerant state break down in the future? In order for these issues to be addressed, there will be an absolute requirement for what are becoming more and more sought-after in the field of transplantation: biomarkers.

The definition of a biomarker

A biological biomarker, as defined by the Biomarkers Definitions Working Group, is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention[22]. For a biomarker to be useful it must be able to distinguish between the disease in question from a reference state, in most cases the “normal” or healthy state.

Biomarkers are becoming more and more the subject of medical research, but the concept of biomarkers is by no means a novel one. Examples of long-standing biomarkers include glucose for diabetes and cholesterol

for heart disease. Biomarkers have multiple uses. They are often used to diagnose a disease state or to monitor the evolution of the disease or its response to treatment. Biomarkers can also be used to predict the susceptibility of patients to respond to certain treatments and thus to predict outcome. Such biomarkers can thereby provide information early on, enabling clinical intervention before it is too late.

Biomarkers may therefore help to personalize treatment strategies, and to apply treatments on an individualized basis rather than on a "one treatment suits all" basis. Biomarkers can also be used as a surrogate endpoint, where the biomarker reflects outcome and can substitute for a clinical outcome. Finally, in some circumstances, the biomarker may help to elucidate the disease pathology or the mechanism of action of the medication used to treat the disease.

In order to be useful, biomarkers need to be accurate, specific and widely adoptable. To fulfill these criteria, biomarkers need to be tested in large patient cohorts, over adequate observation periods, and over a range of disease severities and types. Ideally, they should be detectable in a noninvasive manner, for example in the blood or urine.

Biomarkers in transplantation and the choice of biological sample

For many biomarkers, the ideal biological sample is the so-called "proximal fluid", the biofluid in closest contact with the site of the disease. In the context of transplantation, the closest one can get to the graft is obviously by taking a biopsy. Indeed, histologic biomarkers of graft biopsies according to the Banff classification system[23] are currently the gold standard for diagnosing the status of organ transplants. This is all the more true given that serum creatinine and proteinuria, perhaps the most obvious biomarkers used to monitor graft function in transplantation, are not totally reliable indicators of graft injury because graft damage does not always manifest itself as a deterioration of graft function[24]. This so-called subclinical rejection[25] has been detected through the introduction of protocol biopsy programs. Biopsies are therefore vital as endpoints in clinical trials. Nevertheless, the biopsy procedure is an invasive act and accordingly carries with it some, albeit low, level of risk, as well as expense for the healthy provider and inconvenience for the transplant patient. Moreover, their invasive nature means that they are more likely to be refused by the patient.

The second-best proximal fluid for kidney transplant recipients is probably the urine. This simple, noninvasive and inexpensive way of obtaining a test sample is particularly relevant for kidney transplant patients, given the obvious contact between the urine and the kidney graft. In some circumstances, urine can almost be considered as a "biopsy surrogate". This was highlighted recently when mRNA for Granzyme B and forkhead box protein 3 (Foxp3) were shown to be biomarkers for the diagnosis and/or prediction of acute cellular graft rejection and its resolution[26-28].

For the purpose of minimally-invasive screening of transplant patients in general (kidney, heart, liver, etc.), the blood is an ideal biological sample as it carries minimum risk and is relatively inexpensive. Moreover, blood has been used for decades to monitor renal transplant patients, not only to assess renal function (serum creatinine levels) but also to detect circulating antibodies directed against human leukocyte antigen (HLA) that are now known to have a detrimental impact on late graft outcome[29,30]. Moreover, all modern hospitals are equipped with platforms to measure a plethora of blood-related parameters such as cell counts, serum enzymes, and plasma proteins, etc.

The utility of biomarkers in tolerance

Biomarkers are necessary in transplantation for diagnosis and prognosis, and are also needed to predict individual patient outcome or endpoints in clinical trials[31]. In the clinic, much effort has been made to search for biomarkers of graft rejection, given that tolerance is extremely rare. Along these lines, several groups have reported on biomarkers in humans for the diagnosis of different kinds of rejection. These include the identification of the complement split product C4d as a biomarker of chronic antibody mediated rejection[32], and the identification of biomarker gene signatures, primarily in graft biopsies, of acute rejection[33,34], and chronic rejection[33,35,36]. Biomarkers for prognosis have also been identified[37]. Nevertheless, if tolerance is to one day become a clinical reality, biomarkers are greatly needed here too.

In the context of tolerance, biomarkers will be vital for both induction using specific reagents and immunomodulating maneuvers, and for immunosuppressive weaning protocols. In both situations, biomarkers would be informative for several reasons. First, it will be necessary to diagnose the tolerant state, to determine if the induction/weaning procedure has indeed worked. Second, it would be useful to determine the susceptibility of a given individual to respond in the right way to the induction/weaning protocol. For example, it is conceivable that some patients may be refractory to tolerance, possibly those patients that have a high level of immunologic memory as a result of previous immunization through blood transfusions, pregnancies, infections and the like. This so-called heterologous immunity is thought to be a barrier to transplant tolerance[38]. Thus, biomarkers would increase the safety of tolerance trials.

Another issue that will need to be addressed is that of the stability of the tolerant state and our capacity to predict early on whether a tolerant patient is likely to stay that way or whether the tolerance is going to break down in the future. This is fundamental, given that the tolerance observed to date in large animals and humans is known to be "metastable"[39]. Examples of tolerance breaking down after long periods of time, including after several years, have been reported in nonhuman primates[40] and in humans[18,41]. This may not necessarily be a gradual process because even a case of acute rejection has been observed in a kidney transplant patient after seven years of tolerance[41]. This underlines the requirement for biomarkers to monitor tolerant patients such that any relevant medication can be introduced sufficiently early in the case of tolerance breakdown, before any permanent long-term graft damage has been inflicted.

Finally, given that some patients who stop their immunosuppression can become spontaneously tolerant to liver[17] or kidney allografts[18] following immunosuppressive weaning, it is likely that certain patients with long-term stable graft function under standard immunosuppression may also be tolerant and could also benefit from immunosuppressive weaning. Nevertheless, there is currently no means of identifying such patients, or of selecting patients for weaning protocols on a rational basis. Thus, it would be useful to identify biomarkers in operationally tolerant individuals and to use these to identify patients under immunosuppression that may benefit from immunosuppressive weaning.

The difficulty in identifying biomarkers of tolerance

Several key issues hamper the identification of biomarkers in humans. The major problem here is the very few numbers of tolerant patients that have been reported. Leading on from this, the mechanisms implicated in the majority of cases of tolerance observed in humans, which are probably multiple in nature,

have not yet been deciphered. Additionally, finding the ideal reference group from which to distinguish the tolerant patients poses a problem in that the "healthy" state is not simply a healthy individual as these have not received a transplant, and the tolerant counterparts who have stable graft function and normal kidney graft histology are under immunosuppression. Thus, the problem of confounding factors, in addition to the other issues, renders the task of identifying biomarkers even more difficult and, to date, no true biomarkers of this state have been established. What we do, however, have now is up-and-coming biomarkers of good or bad evolution of transplants. An example is the measurement of Foxp3 in the urine of renal transplant patients which can help to predict reversal of acute rejection[26]. Some of the techniques that are helping to provide an indication of diagnosis or graft outcome are described below. Where possible, examples of their application to tolerance are provided.

Current methods used in the search for biomarkers of graft outcome with examples

There is currently a long and ever-increasing list of technologies that are potentially applicable to the identification/monitoring of biomarkers for transplant tolerance. These range from more classical, small-scale techniques used to measure a defined number of characteristics, such as flow cytometry or quantitative PCR, to novel, high-throughput screening techniques such as genomics and proteomics. Whereas small-scale technology has been applied to the field of transplantation and even to tolerance, the high-throughput approaches are just beginning to be tested. Such techniques are likely to be crucial, given the growing consensus that multiple biomarkers will be required to improve specificity rather than single markers. On the whole, these different approaches do not take into account donor specificity, one of the key elements of transplant tolerance described in rodents. This may, however, not be problematic, given that it is not yet clear whether tolerance in humans is donor specific, given that this can only be tested indirectly and cannot be tested *in vivo* by subsequent donor and third-party grafts.

Two assays that are already established and widely used in transplantation are the ELISA and Luminex techniques used to measure circulating antidonor antibodies that have recently been shown to have a negative impact on graft outcome[29,30]. In nonhuman primates there is some evidence that monitoring antidonor alloantibodies could be a predictive assay for renal allograft tolerance[42]. Despite being informative in the long term, this type of biomarker may not be useful in the short term since anti-HLA antibodies can persist for years in transplant recipients before any apparent deterioration of graft function[43] and they have also been detected in a small number of operationally tolerant patients[18]. More recently, ELISA has been used to measure soluble CD30 (sCD30) in kidney transplant patients. Levels of sCD30, alone or in association with anti-HLA class II antibodies, can be used as an indicator of graft outcome, with high levels being associated with increased risk of graft failure[29].

Two other assays that also take donor specificity into account but are not very suitable for routine use are the cytotoxic T-lymphocyte (CTL) assay and the so-called *trans vivo* delayed-type hypersensitivity (DTH) assay. The former assesses CD8 T-cell-mediated activity and has so far detected hyporesponsiveness to donor antigens in a single patient[44]. The latter assay measures T-cell reactivity according to a swelling response induced following their co-injection with donor antigen into the footpads of immunodeficient mice. This assay can distinguish between allograft rejecters and acceptors in monkeys[40], and revealed donor-specific hyporesponsiveness in three kidney transplant patients that had become tolerant following

immunosuppression withdrawal[41]. Moreover, in a recent study, use of this test revealed a population of CD4+CD25low adaptive T regulatory (Treg) cells in two tolerant patients[45]. These results indicate that donor-specific hyporesponsiveness may be a biomarker of transplant tolerance. Given their application to only a limited number of patients to date, the relevance of these as biomarkers of tolerance on a wide scale needs to be determined.

Other techniques that have been used in the search for biomarkers of tolerance or could be applied in the future do not take donor specificity into consideration. In terms of small-scale screening, perhaps the most frequently used in terms of patient numbers is flow cytometry. This technique measures the number and phenotype of cell populations. In rodent models, this technique has enabled the identification of a variety of regulatory cells in recipients of allografts with long-term survival that are frequently detectable in the spleen, graft, or lymph nodes. Attention has been paid to these populations as they are in some but not all cases able to transfer tolerance. The cell types range from CD4+CD25high T-cells[46-48], CD4+ CD25- T-cells[49], CD8CD45RC T-cells[50] to B7+ non Treg cells[10] and CD103+ non T-cells[12] and certain dendritic cells[51]. However, the consideration of these populations as biomarkers of tolerance is questioned by the fact that Treg populations can coexist in animals whose grafts are functioning long term, but show signs of chronic rejection[11,50,52,53]. In addition, the fact that such populations are frequently detected in the spleen makes them inaccessible in humans. Moreover, these populations are rarely detected in increased numbers in the blood due to compartmentalization[11].

In humans, flow cytometry has been applied to small cohorts of operationally tolerant liver and kidney graft recipients. Despite an ever-increasing list of Treg markers such as Foxp3[54], glucocorticoid-induced tumor necrosis factor receptor (GITR)[55], CD103[56], chemokine receptor 5 (CCR5)[57], cytotoxic T-lymphocyte-associated antigen (CTLA)-4[58], etc., most attention has been paid to the T-cell population expressing high levels of CD25. An increase in the proportion of CD25 high Treg within the CD4+ population of T-cells has been reported in pediatric liver transplant patients[59], and an increase in proportion and total numbers of CD4+CD25 high T-cells have been reported in adult liver recipients who successfully achieved immunosuppressive weaning[60]. This was not the case in kidney transplantation, where operationally tolerant recipients showed no increase in total numbers of CD4+CD25high T-cells compared to healthy volunteers and patients with well-functioning grafts under immunosuppression[19]. Thus, peripheral blood Treg numbers/proportions may be biomarkers of liver but not kidney graft tolerance in humans. In the latter patients, however, an increase in B-cell numbers was detected compared to patients with well-functioning grafts under immunosuppression[19]. Along similar lines, an increase in the percentage of B-cells was observed in tolerant pediatric liver transplant patients[59], although no such findings were reported in adult liver allografts recipients who successfully achieved immunosuppressive weaning[60]. Together, these data indicate a potential for B-cells as biomarkers of kidney tolerance in humans with ambiguous findings in liver tolerance.

Other potential biomarkers measurable by flow cytometry include peripheral blood central memory and effector CD8 T-cells that are differentially expressed between kidney transplant patients with operational tolerance or chronic rejection[20] and peripheral blood monocyte toll-like receptor (TLR)-4 levels that can also distinguish these patients (Braudeau/Ashton-Chess, et al., submitted). As mentioned above, the difficulty in assigning these various molecules as biomarkers comes from the fact that the reference group in each case may differ.

Another technique, the so-called Tc-Landscape®, gives a global appraisal of the

T-cell repertoire, with the potential of revealing perturbations that may be specific to a particular immunologic status[61,62]. The TcLandscape® of tolerant rat-allograft recipients has been measured in various models of transplantation, including both the induction[63] and maintenance phases[11]. In humans, this technique revealed an altered blood clonal T-cell regulation with no accumulation of cytokine transcripts, suggesting a state of hyporesponsiveness of the T-cell clones in operationally tolerant kidney transplant recipients compared to patients with chronic rejection[21]. Moreover, refinement of this technique has shown that it can distinguish the T-cell repertoires in the peripheral blood of operationally tolerant patients from patients with chronic rejection on a statistical basis[64], warranting its testing on larger numbers of patients with transplant tolerance to determine its potential as a diagnostic tool.

Finally, the quantitative PCR techniques to measure a limited number of genes is now widely used and, although no quantitative PCR-identified biomarkers of tolerance have been reported to date, the technique has proven useful in identifying markers of resolution of acute rejection[26].

In addition to these small-scale assays, the advent of technological advances has driven researchers to turn towards large-scale screening systems. Such progress now makes it possible to simultaneously measure thousands of genes in a given biological sample using the strategy of genome-wide gene profiling by microarrays to identify "molecular fingerprints" of a particular biological state. In humans, a recent paper described use of microarrays in liver transplant patients to identify a gene signature in patients who underwent successful immunosuppressive weaning and became operationally tolerant[60]. In the context of kidney transplantation, this approach has been used primarily to search for biomarkers of graft rejection[33-36,65]. Our group has applied microarray profiling to the study of human operationally tolerant kidney recipients. We have been able to identify a panel of gene biomarkers in the peripheral blood of patients tolerant to kidney allografts that is different from the one of patients with chronic rejection[66]. In addition, in a separate study, a gene signature distinguishing operational tolerance in a training group of patients recruited in Europe could predict operational tolerance amongst an entirely independent cohort of patients recruited on a different continent (Basic Science Symposium, La Baule, France, 2005 and Brouard/Mansfield, et al. submitted).

Proteomics is another field of large-scale investigation that is currently opening up in transplantation. Certain peptide biomarkers (human β-defensin-1 and α-1-antichymotrypsin) for the diagnosis of acute rejection of kidney transplants were recently described in humans using this technology[67]. So far, there have been no reports concerning transplant tolerance, although this type of analysis is likely to be applied in the near future.

Finally, the implementation of protocol biopsy programs may help in the identification of key biomarkers of tolerance within the graft, such as particular histologic features, cell infiltrates, or expression of biomolecules. Such protocol biopsies will be essential for patients in whom tolerance is induced "deliberately" through specific immunosuppression interventions or immunosuppressive weaning in the context of controlled trials, as biopsies are often difficult to obtain in noncompliant patients who develop operational tolerance "unintentionally" by stopping their immunosuppressive medication of their own accord.

Conclusions – Perspectives

From the above data it is clear that much effort is being made to identify biomarkers of tolerance. Nevertheless, these studies are generally limited to particular spe-

cies, tolerance induction protocols, or cohorts of patients. Coordinated efforts are now essential to identify tolerance biomarkers across species and protocols. However, extrapolation of results across species will not be easy, given the apparent disparity between tolerance in rodents and that observed in the clinic. Even so, efforts are being made both in Europe (Indices of Tolerance and RISET) and in the USA (Immune Tolerance Network) to accumulate and simultaneously study larger cohorts of operationally tolerant patients, rather than simply performing individual analyses that are difficult to interpret on a wider scale. It would also be informative to compare patients tolerant to transplants following immunosuppression withdrawal to patients in whom tolerance has been induced deliberately by simultaneous bone marrow and kidney transplantation. Moreover, biomarkers with a wider field of application might be identified by direct comparisons between tolerant kidney and tolerant liver recipients. Complementary to these approaches in humans is the continued intensive research in rodents. Again, given the apparent diversity in the animal models, comparisons of models would be useful in the quest to identify robust biomarkers showing relevance to several models rather than just one model in particular. Finally, meta-analyses across species from rodents to nonhuman primates and humans could be enlightening, perhaps leading to the identification of universal biomarkers.

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