Microchimerism and Renal Transplantation: Doubt Still Persists

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ABSTRACT

Background: The Presence of donor leukocytes in recipients of organ allograft has been shown even several years after transplantation. However, it remains unclear whether this donor cell microchimerism plays an effective role in allograft acceptance or is simply a consequence of immunosuppressive conditions in recipients. **Objective:** To study microchimerism in a group of kidney transplant recipients. **Methods:** In this study, the Peripheral Blood Microchimerism (PBM) after renal transplantation was retrospectively evaluated in 32 male-to-female recipients of living (unrelated) and cadaveric donor renal transplants. Using a Nested Polymerase Chain Reaction (Nested-PCR) amplification specific for SRY region of the Y chromosome, microchimerism was detected with a sensitivity of 1:1000000. Recipients were classified and compared according to the presence of PBM, acute and chronic rejection episodes, type of allotransplant, recipient and donor age at transplantation, previous male labor or blood transfusion, allograft function (serum creatinine level), and body mass index. **Results:** Among 32 recipients, 7 (21.9) were positive for PBM in multiple testing at different post-transplantation times. All microchimeric recipients had received kidney from living-unrelated donors. No significant difference was observed with regard to other parameters mentioned above. In addition, acute rejection rate in the microchimeric group was 3 (42%) versus 4 (16%) in the nonmicrochimeric recipients (not significant). **Conclusion:** Our results demonstrate better establishment of microchimerism after living donor kidney transplantation. However, concerning the true effect of microchimerism after renal transplantation doubt still persists; and it seems that microchimerism alone has no major protective role in renal allograft survival.

Key words: Microchimerism, PCR, Transplantation

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INTRODUCTION

The persistence of donor leukocytes in recipients of organ allograft has been shown even in long-term allograft recipients. However, it remains unclear whether this donor cell microchimerism plays an effective role in allograft acceptance or is simply a consequence of sufficient immunosuppression to avoid destruction of microchimeric donor cells. During the last decade, there have been many reports concerning the detection of a small population of donor bone marrow-derived cells in various tissues like kidney (1), liver (1,2,3), and heart recipients (3). A novel theory to elucidate graft tolerance has been proposed. This theory contended that long-lived donor-derived hematopoietic cells play an important role in generating long-term allograft acceptance (4,5). However, consensus has not emerged concerning the causal relationship between such chimerism and tolerance. Current limitations in clinical transplantation technology necessitate the use of continuous post-transplant immunosuppression in recipients to prevent allograft rejection. Thus, the persistence of donor hematopoietic cells in recipient tissues may be caused by the inability of an immunosuppressed host to effectively eliminate these cells. Indeed, rather than inducing tolerance itself, donor chimerism may be an epiphenomenon accompanying immunosuppression-induced allograft acceptance. In this study, we retrospectively evaluated the Peripheral Blood Microchimerism (PBM) after renal transplantation in 32 male-to-female recipients of living-unrelated and cadaveric donor renal transplants. Nested Polymerase Chain Reaction (Nested-PCR) amplification of SRY region of the Y chromosome was used as a hypersensitive method for detection of microchimerism. Then, clinical parameters were compared between microchimeric and nonmicrochimeric recipients.

SUBJECTS AND METHODS

This study included 32 female kidney allograft recipients who underwent living-unrelated and cadaveric renal transplantation from male donors at Sina Hospital, Kidney Transplantation Research Center, Tehran, Iran, between November 1993 and August 2002. All transplantations were done by the same team and all recipients followed up in the same nephrology ward at regular intervals. The immunosuppressive protocol of recipients included: Prednisolone, Cyclosporine, and Azathioprine or Cellcept. Based on the presence of PBM, recipients were classified and compared according to: microchimeric and nonmicrochimeric groups, acute and chronic rejection episodes, type of allotransplant (living or cadaveric donor), recipient and donor age at transplantation, previous male labor or blood transfusion, allograft function (serum creatinine level), and body mass index. Genomic DNA was isolated from recipients' peripheral blood samples. Nested-PCR was used to determine PBM in female kidney allograft recipients by looking for a Y-chromosome specific DNA sequence of the SRY-region.

Nested-PCR reaction. In this study we used nested PCR to determine PBM in female kidney allograft recipients by looking for a Y-chromosome specific DNA sequence, the SRY region. PCR was performed in a 50 μL reaction mixture contain-

ing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3 at 25ºC), 0.2 mM dNTPs, 0.2 each of the SRY-specific forward and reverse primers, 1 unit of AmpliTaq® DNA polymerase (Perkin-Elmer, Branchburg, NJ, USA) and template DNA (500ng for female and 5 ng or 0.5 ng for male subjects). Forty cycles of Amplification were done with a first set (external) of SRY primers: SRY-1F 5'CAG TGT GAA ACG GGA GAA AAC AGT3' and SRY-2R 5'CCT CCG ACG AGG TCG ATA CTT ATA3' with denaturation, 30 s at 94ºC; annealing, 30s at 58ºC; and extention 1 min at 72ºC, in GeneAmp® 9700 PCR system (PE Applied Biosystems, Foster city, CA, USA). One microliter of the above PCR product was then entered into a second PCR reaction containing the following nested (internal) primers: N-1F 5'CGC ATT CAT CGT GTG GTC TC3' and N-2R 5'TCC GGT ATT TCT CTC TGT3'. The nested PCR was done for 30 cycles using the same reagent concentrations and temperature profile as the original PCR. The PCR products were then visualized by running 10 µL of reaction mix on a 2% agarose gel. (GIBCO-BRL Life Technologies, paisley, UK), prestained with ethidium bromide (0.5 µg/ml gel), in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM ethylene-diamine tetra-acetic acid [EDTA] pH = 8.0). Gels were viewed and photographed under ultra

Table 1. Comparison of clinical parameters between microchimeric and non-microchimeric recipients

Variables	Mic.	Non-Mic.	Totals	P-value†
age (years)	36.3 ± 10	36.3 ± 11.9	36.3 ± 11.3	0.99
Creatinine (mg/dl)	1.07 ± 0.4	1.07 ± 0.38	1.07 ± 0.38	0.98
BMI (kg/m2)	24.06 ± 5	23.1 ± 3.8	23.3 ± 4	0.58
Tx. Type				
Living	7 (100%)	20 (80%)	27(84.4%)	0.198
cadaveric	0 (0.0%)	5 (20%)	5(15.6%)	
Rejection Episodes				
No	4 (57.1%)	20 (80%)	24 (75%)	0.292
Acute	3 (42.9%)	4 (16%)	7 (21.9%)	
Chronic	0 (0.0%)	1 (4 %)	1 (3.1%)	
Blood TR				
No	2(28.6%)	6 (24%)	8 (25%)	0.81
Yes	5 (71.4%)	19 (76%)	24 (75%)	
M. Labor				
No	1 (14.3%)	13 (52%)	14 (43.8%)	0.075
Yes	6 (85.7%)	12 (48%)	18 (56.2%)	

† P-values are for comparisons of frequencies between Microchimeric and Non-Microchimeric Groups, using the Fisher exact tests and were calculated by the statistical software SAS system version 8.00; Mic. = Microchimeric; Non-Mic.= non-microchimeric; Tx = transplantation; TR = transfusion; M = mal

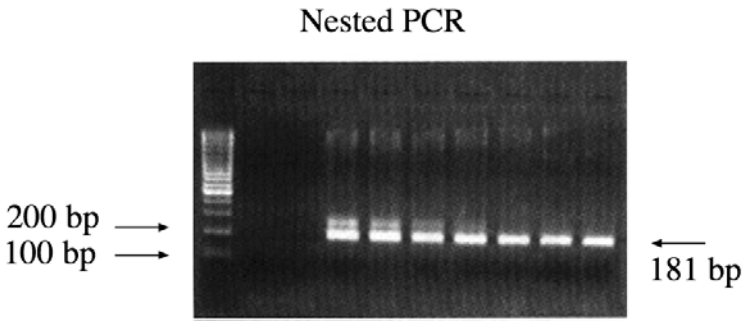

Figure 1. Results of nested PCR. Samples of female DNA (500 ng) artificially spiked with decreasing quantities (50-0.0005 ng) of male DNA; lane 1, molecular weight marker; lane 2, no template (contamination control); lane 3, female DNA negative control; lanes 4-10, (1:10), (1:100), (1:1000), (1:10000), (1:50000), (1:100000), (1:1000000) of spiked male: female DNA. Nested PCR was found to be 1000 times more sensitive than standard PCR

violet (UV) transillumination. With the first set of primers, SRY-1F and SRY-2R, PCR product of 270bp and with nested primers, N-1F and N-2R PCR product of 181bp were demonstrated using DNA of male donors as well as female DNA samples containing male DNA. Because the sequence of these nested PCR product and its identity with the unique sequence within the SRY regions had already been determined, we relied on those data and abandoned additional sequencing of PCR products. Multiple experiments with two separate DNA samples with each healthy female negative control showed no cross-reactivity and proved that this system has enough specificity to detect SRY gene-specific DNA sequence. If simultaneously analyzed negative and positive controls demonstrated a reliable pattern, presence of a clearly visible SRY-specific PCR product was a positive result for microchimerism. All microchimeric bands were detected through nested (second) PCR.

The assay sensitivity for detection of chimerism was determined by two approaches: 1) serial dilution of male DNA in female (background) DNA; and 2) spiking experiments, in which male DNA was serially diluted in water and added to a fixed amount of female DNA. Comparison between the two approaches demonstrated that spiking experiments had more precise and reproducible results than serial dilutions. This does not mean that the results of the two approaches were not comparable; but, as in serial dilution, we added male DNA to the first tube and then diluted it serially with female DNA in another tube. It is likely that the transfer of male DNA did not occur well. This may be a reason for fluctuating results in the serial dilution approach. With spiking experiments, the sensitivity of standard and nested PCR to detect the specific DNA sequence of the SRY region 1:1000 and 1:1000000, respectively; i.e. nested PCR was about 1000 times more sensitive than standard (first) PCR (Fig. 1).

RESULTS

Among 32 recipients, 7 were positive for PBM in multiple testing at different post-transplantation times. All microchimeric recipients had received kidney from living-unrelated donors. Recipients of cadaveric kidneys were negative throughout the study. Acute rejection rate in the microchimeric group was 3(42%) versus 4(16%) in nonmicrochimeric recipients (not significant). Regarding all clinical parameters mentioned above statistical analyses showed no significant difference (Table 1).

DISCUSSION

It has been shown that there is an association between graft survival and the presence of microchimeism after solid organ transplantation. Although there were multiple reports showing obvious low incidence of acute rejection in microchimeric recipients (6,7,8), but it is still not clear whether graft stability is the result of the presence of microchimerism or the result of successful immunosuppression. Also there are reports that induction of the microchimeric state, by using perioperative Donor Bone Marrow Transplantation (DBMT), improved graft survival and decreased acute rejection episodes (6,9,10,11-14,15). On the other hand, there were reports of acute or chronic transplant rejections in natural and induced microchimeric recipients (8,15). According to these findings, the role of microchimerism in graft survival is still under question.

Previously, we demonstrated that successful immunosuppression and HLA class-II matching can improve microchimerism, and there were obvious associations among microchimerism and graft stability. However, the presence of chronic rejection in one microchimeric patient encouraged us to extend our study in a new series of patients, to observe whether there is any significant association between the presence of microchimerism and long term graft survival.

In this study, we used Nested PCR amplification for SRY region of Y-chromosome for detection of donors' cells in peripheral circulation of female recipients. Due to the fluctuation of micochimerism (3,8,16), and for maximum accuracy, we tested multiple recipient samples at different intervals.

Many of non-microchimeric recipients had pretransplantation male labor or allogenic blood transfusion, while repeated testing revealed no evidence of microchimerism in these patients. This could be due either to minute number of transplacental or blood transfusion (19) or destruction of hemopoietic cells by refrigeration and storage (8). On the other hand, because immunosupression is not normally administered post transfusion or post labor, hemopoietic cells were destroyed and cleared from the transfused donors' blood before a microchimeric state could be established (18).

In our study, there was no evidence of microchimerism among recipients who were transplanted by cadaveric kidney. This could be due to super activation of immune system in all cadaveric tissues secondary to post-trauma stress, which leads to super-allogenic reaction of the recipient immune system against donors' hemopoietic cells and ultimately early post transplantation clearance from recipient peripheral circulation.

The presence of acute rejection episodes in microchimeric recipients could be due to HLA class-II (DR, DQ) mismatching between donors and recipients, while graft survival had been kept by successful immunosuppression (8).
Finally long term graft stability, in microchimeric and non microchimeric recipients, revealed that presence or absence of microchimerism is not essential for long term graft survival.
Other studies have revealed similar results. Strober tested one case of cadaveric renal transplant recipient given pretransplant total lymphoid irradiation and withdrawn from immunosuppressive drugs for more than 12 years. He concluded that there was good graft tolerance and function in spite of donor and recipient HLA-A, B, C and DR mismatching with absence of microchimerism in the presence of anti-donor reactivity in the mixed lymphocytes reaction. This suggests that neither chimerism nor clonal deletion or anergy of the recipient T-cells to allogens presented by donor class-II HLA molecules is required for the persistence of the tolerant state (19). Mc Daniel et al. studied the presence of microchimerism among 48 consecutive solid organs transplants. He found 10 microchimeric cases, with fluctuating microchimeric states. The presence of acute rejection episodes in 3 microchimeric recipients highlighted the difficulty in establishment of the correlation between microchimerism and transplant tolerance (18). Sahota showed that microchimerism was generally associated with higher incidence of acute rejection for heart, lung and kidney transplants (9).
Schlitt mentioned that there was no relation between acute or chronic rejections and microchimerism after heart transplantation (16). Caillat-Zucman discovered that among 12 long term graft survivals there was just one case of microchimerism. This result raised doubts about the major role of chimerism in development of long lasting specific tolerance, following kidney allografting (20). Ishida studied 13 recipients 15 years after transplantation. In this study, microchimerism was detected in just one recipient who was associated with high responesiveness against donor antigen. In contrast there were donor specific antigen hyporesponsiveness in some patients with no microchimerism, indicating that microchimerism is not highly frequent in long term survivors of kidney allograft transplantation, and this is irrelevant to donor-specific unresponsiveness (21).
Hisanaga showed that peripheral microchimerism frequently develops after different types of transplantation and represents a dynamic process but without diagnostic value to predict the immunological risk for patients (3). Reinsmoen mentioned that not all hyporesponsive kidney recipients had peripheral microchimerism (22). Kim demonstrated that there was no statistical difference between the presence of microchimerism and clinical findings such as: type of donors, type of immunosuppressions, episodes of rejection and age of recipients. Their study did not show any clinical microchimeric relevance (23). Although it was proven that feto-maternal hemopoietic cell transfusion could be involved in certain maternal autoimmune diseases (such as sclerodermia) (24,25,26); however, there is still no evidence proving graft survival enhancement by feto-maternal microchimerism. Mahanty concluded that it is possible that persistent microchimerism of fetal cells in maternal circulation may, for some mothers, cause a detectable improvement in graft survival, if mothers are tolerant to their offspring. Our results; however, indicate that

microchimerism may not improve allograft survival in offspring donor to maternal recipient combination (27). In addition, a better establishment of microchimerism after living donor kidney transplantation is suggested. But, concerning the true effect of microchimerism after renal transplantation, doubt still persists; and it seems that microchimerism alone has no major protective role in renal allograft survival.

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