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A Positive Pre-Transplant Endothelial Precursor Cell Crossmatch does not Imply Reduced Long-Term Kidney Graft Function

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Absract

A flow cytometric crossmatch test detecting antibodies specific for donor Endothelial Precursor Cells (EPC) was evaluated in a multicenter study in 2005-06. A Positive Pre-Transplant EPC Crossmatch (EPCXM) was associated with a higher frequency of early rejections and reduced renal function at three and six months. The long-term follow-up of all patients (n = 53/147) recruited at our center is reported. Patients were retrospectively evaluated regarding rejections, patient/ graft survival and renal function over a four-year follow-up.

As for the whole multicenter study patient population, significantly more early rejections occurred in EPCXM positive compared to EPCXM negative patients (5/7 vs. 5/46, $p = 0.002$). The EPCXM positive group had higher SCr at three (183 vs. 118 µmol/l, $p = 0.01$) and six (172 vs. 124 µmol/l, $p = 0.02$) months compared to the EPCXM negative group, and measured Glomerular Filtration Rate (mGFR) was decreased in the EPCXM positive group at 6 months (50 vs. 29 ml/min, $p = 0.01$). SCr decreased and mGFR increased over time in the EPCXM positive group, while SCr increased slightly and mGFR decreased slightly in the EPCXM negative group eliminating the difference in renal function between the groups.

A positive EPCXM pre-transplantation is associated with higher frequency of early graft rejections, but does not influence long (4 year) term renal function.

Keywords: Antibody-mediated rejection; Anti-endothelial cell antibodies; Crossmatch; Kidney graft function; Non-HLA

Abbreviations

ABS: Antibodies; ACR: Acute Cellular Rejection; AMR: Antibody Mediated Rejection; CAN: Chronic Allograft Nephropathy; CDC: Complement Dependent Cytotoxicity; EPC: Endothelial Precursor Cell; EPCXM: Endothelial Precursor Cell Crossmatch; LXM: Lymphocyte Crossmatch; mGFR: measured Glomerular Filtration Rate; PRA: Panel Reactive Antibodies; SCr: Serum Creatinine; XM: Crossmatch

Introduction

With the introduction of solid-phase techniques for detection and specificity-determination of HLA Antibodies (Abs) [1,2], their importance for both acute and more chronic forms of Antibody-Mediated Rejection (AMR) has been established (reviewed in [3-5]). It is also clear that non-HLA-specific Abs contribute to AMR [6-9]. This notion is reinforced by the fact that patients receiving HLA identical grafts may be lost in AMR [10]. Graft losses due to AMR may also be seen in patients having negative Lymphocyte Crossmatch (LXM) tests and no donor-specific HLA Abs [11,12]. The degree of sensitization has been shown to reduce long-term graft survival in recipients of HLA identical grafts, suggesting that Abs against other targets than HLA may contribute to poor graft survival [13].

Despite the fact that there are several reports on the significance of non-HLA Abs for graft survival, this clinical problem remains poorly defined. In large, this can be explained by lack of suitable assays for detection of this population of Abs, which in addition can be expected to be heterogeneous with regard to the antigens recognized. Thus finding an assay detecting all of the potential specificities may be difficult. Since Endothelial Cells (EC) are likely to be the most prominent target cells for non-HLA Abs causing AMR, many tests used in the past have utilized various cultured EC [12,14,15]. Problems with this strategy include that it is difficult for a clinical routine laboratory to keep cells in culture for EC Crossmatch (XM) testing and that cultured cell lines usually prohibit donor-specific XM testing.

Recently, a novel flow cytometric XM test was evaluated in a multicenter kidney transplantation trial [16]. This XM test utilizes as target cells donor-derived Endothelial Precursor Cells (EPC) defined by expression of the angiopoietin receptor, Tie-2 [16,17]. Patients with a positive EPCXM had a significantly increased frequency of rejections as well as higher Serum Creatinine (SCr) levels at three and six months post-transplantation [16]. This communication reports the four-year follow-up of all patients

recruited at our center (n = 53) and reveals that long term graft survival and renal function are not significantly different between the anti-EPC positive and negative patient groups beyond six months and during the four-year follow-up.

Materials and Methods

Patients

Fifty-three patients at our institution previously reported in the Multicenter Trial (MCT) [16] were retrospectively reviewed in this study (Table 1). Patients were accepted for transplantation based on negative T- and B-cell Complement-Dependent Cytotoxic XM (CDCXM) tests. Sixty-one patients were originally recruited between November 2005 and October 2006. Eight patients were excluded; two because they were not transplanted, three because there were not enough cells to perform an EPCXM, two patients lost their grafts early from surgical complications and one patient had a negative control outside the specified range in the EPCXM. Twenty-eight patients received kidneys from living donors and 25 received kidneys from deceased donors. Two patients received ABO incompatible grafts from living donors, one had a positive EPCXM and one had a negative EPCXM.

All patients were retrospectively reviewed up to four years post-Tx and no patient was lost to follow up. Relevant clinical data including SCr, measured Glomerular Filtration Rate (mGFR) and rejection episodes were recorded. GFR was measured by the ^{51}Cr -EDTA or Inulin clearance techniques depending on the local hospital practice. Three patients died during the follow-up period; one patient died of septicemia nine months after transplantation and two patients died of cardiac failure 14 months after transplantation. All three patients had negative EPCXM and died with functioning grafts. Three grafts were lost during the follow-up period. One graft in an EPCXM positive patient was lost due to hepatorenal syndrome and two grafts were lost in EPCXM negative patients, one to Chronic Allograft Nephropathy (CAN) and one to recurrence of IgA nephropathy. The MCT was approved by the Stockholm regional human ethics committee (docket no. 2005/222-31/1).

Table 1: Patient Demographics.

	All patients (n = 53)	**EPCXM positive patients (n = 7)**	**EPCXM negative patients (n = 46)**	**MCT (n = 147)**
Age (years)	48 ± 13	51 ± 12	47 ± 13	46 ± 14.5
Male	36	4	32	87
Female	17	3	14	60
Living donors	28	3	25	122
Deceased donors	25	4	21	25
HLA-sensitization				
NS (PRA > 10%)	40(76%)	3(43%)	37(80%)	113(77.5%)
S (PRA 10 - 80%)	8(15%)	2(28.5%)	6(13%)	25(17%)
HS(PR>80%)	5(9%)	2(28.5%)	3(7%)	8(5.5%)
LXM[a]				
T-cell	32(4+)	5(3+)	27(1+)	
B-cell	32(3+)	5(2+)	27(1+)	

[a]Number of B- and T-cell flow cytometric lymphocyte crossmatch-tests performed and number of tests with Positive results in parenthesis.
EPCXM: Endothelial Precursor Cell Crossmatch; LXM: Lymphocyte Crossmatch; NS: Non-Sensitized; S: Sensitized;
HS: Highly Sensitized; MCT: Multicenter Trial

Table 2: Rejections.

	< 3 Months after transplantation		**> 3 Months after transplantation**	
Rejection type[a]	EPCXM positive patients	EPCXM negative patients	EPCXM positive patients	EPCXM negative patients
Antibody mediated	0	0	0	0
Borderline	1	1	0	0
Type IA	0	2	0	2
Type IB	0	0	0	0
Type IIA	4	1	0	1
Type IIB	0	1	0	0
Time to rejection (mean)	6 days	20 days	-	27 months

[a]Biopsy-proven rejections according to the 2003 upgraded Banff 97 classification of renal allograft rejection.
EPCXM: Endothelial Precursor Cell Crossmatch

Immunosuppression

Induction therapy was given in 22/53 patients and included Anti-Thymocyte Globulin (ATG) (n = 7), rituximab (n = 10) and IL-2 receptor antagonists (n = 6). One patient received both rituximab and an IL-2 receptor antagonist. Initial maintenance immunosuppression included tacrolimus and Mycophenolate Mofetil (MMF; n = 26), cyclosporine and MMF (n = 21), rapamycin and MMF (n = 3), tacrolimus and rapamycin (n = 1), tacrolimus, rapamycin and MMF (n = 1) and tacrolimus and azathioprine (n = 1). All patients received steroids.

Often acute rejection episodes occurring < 3 months after transplantation, five episodes were treated with methylprednisolone and five with Anti-Thymocyte Globulin (ATG). One patient who received an ABO-incompatible graft underwent plasmapheresis in addition to ATG. In three acute rejections that occurred late, > 3 months after transplantation, two patients were treated with methylprednisolone and one patient was not treated at all because the kidney was considered too marginal. The two patients that received ABO-incompatible grafts were pretreated with blood group-specific immunoadsorption (GlycoSorb-ABO®, Glycorex Transplantation AB, Lund, Sweden) and anti-CD20 (rituximab) as induction.

HLA typing and antibody analysis

The HLA-A, -B, and -DRβ1 loci of patients and donors were typed by serology or Site-Specific Primer (SSP)-PCR using Olerup SSP® kits (Olerup SSP AB, Salts jöbaden, Sweden) as described by the manufacturer.

The levels of Panel-Reactive HLA class I and II Abs (PRAs) in the pre-transplant sera of patients were determined by Flow Cytometric (FC) analysis using the Flow PRA® test according to the manufacturer's instructions (One Lambda, Inc.). The samples were acquired on a FACScan flow cytometer and analyzed using the Cell Quest Pro software (BD Biosciences, San Jose, CA, USA). In addition, the PRA values were determined by Complement-Dependent Cytotoxicity (CDC) using T- and B-lymphocytes from a panel of 30 donors typed with regard to HLA-A, -B and -DR1.

Crossmatch testing

Before transplantation all patients had a CDC XM performed as previously described [18]. Thirty-two patients also had a T- and B-cell flow cytometric XM performed pre-Tx (Table 1). EPCXM tests were performed on the day of transplantation using Tie-2 (angiopoietin receptor) positive EPC isolated from donor blood with the XM-ONE® kit according to the manufacturer's instructions (AbSorber AB, Stockholm, Sweden) and as previously described [16]. The EPCXM results were not routinely reported to the clinicians responsible for patient care. It could be obtained if asked for, but at our center this did not occur in any case.

Histopathology

Biopsies were performed on clinical indication and processed with standard methods for light microscopy and C4d immunohistochemistry. The 2003 upgraded Banff 97 working classification of renal allograft pathology including AMR was followed because it was the classification used in the MCT [19].

Statistical analysis

Descriptive statistics include mean and standard deviation. A repeated measures ANOVA analysis was performed to evaluate differences between groups over time. Differences in proportions between groups were tested for significance with Fisher's two-sided exact test. A *P- value* of < 0.05 was considered significant. analysis were conducted using the SPSS (IBM Corporation, Somers, NY, USA) and SAS (SAS Institute Inc., Cary, NC, USA) software.

Results

Patient demographics

Demographic data of the 53 patients included in the MCT at our center is given in Table 1 of the recruited patients, all of which had a negative lymphocyte CDC XM, 60% (32/53) were tested in the flow cytometric LXM; 12% (4/32) tested positive and 88% (28/32) tested negative. In the EPCXM positive group, 3 patients also had a positive flow cytometric LXM test. Seven patients (13%) tested positive in the EPCXM test and all had Abs of IgG class. All patients were tested for the presence of HLA-Abs by solid-phase assays and 40/53 (76%) were non-sensitized (PRA < 10%), 8/53 (15%) were sensitized (PRA 10-80%) and 5/53 (9%) were highly sensitized (PRA > 80%). Of the patients with a positive EPCXM test, 3/7 (43%) patients were non-sensitized, 2/7 (29%) sensitized and 2/7 (29%) highly sensitized.

Rejections

The incidence of biopsy-proven acute rejection during the first three months was 71% among patients with a positive EPCXM compared to 11% in those with a negative EPCXM(5/7 vs. 5/46, *P = 0.002*). After the initial three months, three rejections occurred among patients with a negative EPCXM while patients with a positive EPCXM had no late rejections. Chronic Allograft Nephropathy (CAN) was seen in three patients with a negative EPCXM and in no patients with a positive EPCXM. All patients receiving kidneys from deceased donors (n = 25) in the MCT were included at our center. When these patients were analyzed separately the results were the same as for the whole group recruited at our center (data not shown). Types of rejections and mean time to rejection are presented in Table 2.

Kidney graft function

Renal function as assessed by mGFR was significantly higher in the EPCXM negative compared to the EPCXM positive group at 6 months (Figure 1A; 50 vs. 29 ml/min, *P = 0.01*). However, the difference decreased over time and became non-significant one to four years post-Tx. Similarly, SCr levels as a marker for renal function were significantly higher 3 and 6 months post-Tx in the patients with a positive EPCXM when compared to those with a negative EPCXM (Figure 1B; 183 vs. 118 μmol/l, *P = 0.01* and 172 vs. 124 μmol/l, *P = 0.02*). Over time, SCr values in patients with a positive EPCXM decreased slowly, while the SCr increased slightly in those with a negative EPCXM. As a result, the statistical difference between the two groups became non-significant from

one to four years post-transplantation (Figure 1B). In conclusion, the statistical difference in renal function between the EPCXM groups found at 3 and 6 months disappeared over time.

Discussion

Like in the whole patient population, EPCXM positive patients recruited at our center had significantly higher SCr levels three and six months after transplantation compared to EPCXM negative patients. The mGFR was lower in the EPCXM positive than in the EPCXM negative group at six months post-Tx. There was no statistically significant difference in SCr or mGFR between EPCXM positive and EPCXM negative patients at one-year post-Tx. In EPCXM negative patients, the SCr seemed to increase slowly over time from 1 to 4 years after transplantation. However, the SCr decreased over the same period in the EPCXM positive group making the difference between the groups statistically non-significant (Figure 1A). Similar observations were made by Jackson and coworkers, who in a study of 60 LD kidney recipients showed that EPCXM positive patients had higher SCr values and incidence of cellular rejection early (mean 50 days) post-Tx compared to EPCXM negative patients – a difference that disappeared late (mean 815 days) post-Tx [20]. A significant finding in that report was that Abs detected in the EPCXM, in contrast to anti-HLA Abs, were enriched for the IgG2 and IgG4 subclasses [20]. Despite the fact that IgG2 and IgG4 are poor complement activators, anti-endothelial cell Abs detected in the EPCXM can cause hyper acute rejection in the absence of complement activation and HLA DSA [21]. It is currently not clear why the presence of Abs against donor EPCs pre-Tx is associated with poor kidney graft function early (< one year), but not late (> one year), post-Tx. One possible explanation could be that the EPCXM positive patients with more early rejections initially received higher doses of CNI than the EPCXM negative patients with fewer early rejections. These higher, possibly nephrotoxic, doses of CNI might have been reduced over time leading to a decrease in SCr.

Of the 53 patients recruited into the MCT at our center and tested with an EPCXM test before kidney transplantation, 7 (13%) tested positive in the EPCXM as compared to 24% in the entire study population [16]. One reason for the lower number of EPCXM positive patients in our cohort may be the absence of patients of Afro-Caribbean origin. In the MCT, 58% of Afro-Caribbeans were EPCXM positive as compared to only 21% of patients of other origins [16]. Immunological and non-immunological factors contribute to the racial disparities observed for renal graft recipients both in terms of time on the waiting list as well as the outcome of the transplantation, with blacks being at a disadvantage compared to whites [22]. Black recipients appear to be stronger immune responders [23] and experience a higher frequency of pre-Tx positive lymphocyte crossmatch tests [24].

As in the MCT, patients recruited at our center with a positive EPCXM test had a higher incidence of acute cellular rejection (ACR) in the first three months. In fact, the incidence of rejection among ECPXM positive patients at our center was 71% compared to 46% in the MCT (P > 0.05) [16]. This may be explained by the

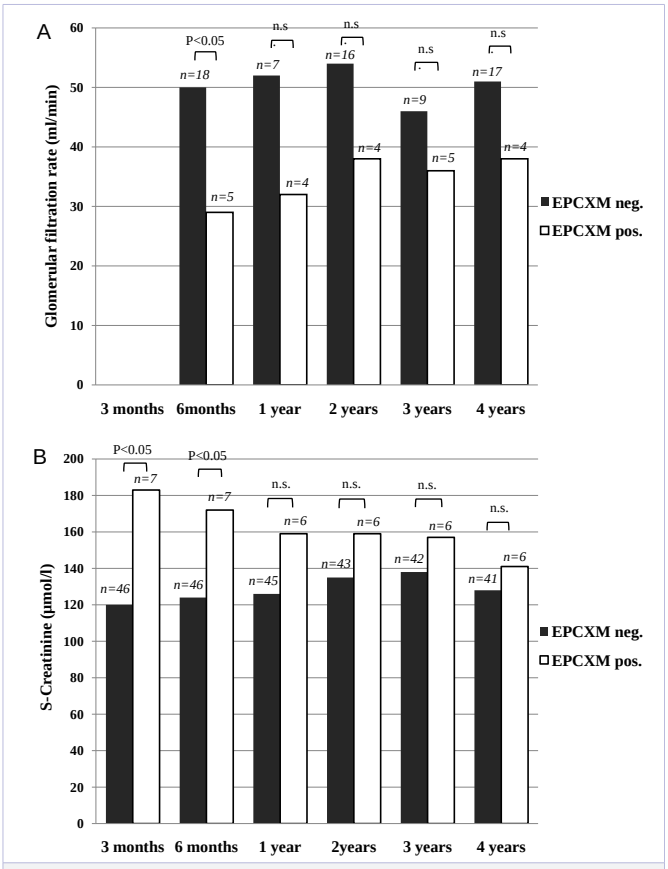

Figure 1: Measured glomerular filtration rate (A) and serum creatinine (B) in patients with positive (empty bars) and negative (filled bars) endothelial precursor cell crossmatch (EPCXM) tests. n.s, not significant

fact that only patients with EPC-reactive antibodies of IgG class were detected in our cohort, while EPC Abs of both IgG and IgM class were found in the entire study population. More sensitized patients were found in our EPCXM positive group compared to the ECPXM positive group of the MCT (57% vs. 29%; *P* > 0.05). No acute rejections were diagnosed more than three months after transplantation in the EPCXM positive patients, while three acute rejections were diagnosed in the EPCXM negative patients. We believe the early rejections, even though not AMR, to be associated with the presence of EPC Abs. Besides a direct effect of EPC antibodies on the ECs of the graft, they may potentiate antigen uptake and presentation, and thereby initiate a T cell-mediated ACR. Chronic Allograft Nephropathy (CAN) was also only seen in the EPCXM negative patient group. This is surprising considering the proposed role of Abs for the development of CAN [4], but a similar observation was made by Jackson and coworkers who showed no statistically significant difference between the IgG EPCXM positive and negative groups with regard to late (> 100 days post-Tx) rejections [20]. Because biopsies were performed on clinical indication and rejections occurred early in the EPCXM positive and late in the EPCXM group, this could explain why we only observed CAN in patients with a negative EPCXM.

The most important limitation of this follow-up study is the relatively low number of patients. However, advantages of

the study are that the results of the EPCXM were blinded to the transplant clinicians and that no patients were lost during the follow-up. A weakness of the EPCXM as it is performed today is that also HLA Abs will result in a positive EPCXM test [25]. Thus, unequivocal detection of Abs against non-HLA in the EPCXM is not feasible in sensitized patients with HLA Abs binding to donor EPC [25]. Therefore, it will be important for the future to identify the antigens responsible for positive EPCXM tests such that a solid phase assay with purified antigens can be developed. A number of candidate non-HLA has been described using various approaches including proteomics techniques [26-28]; antigens that should be tested against serum samples positive in the EPCXM test.

In conclusion, a positive pre-transplant EPCXM in kidney transplantation is associated with increased risk of early rejection and decreased renal function three and six months after transplantation. However, the negative effect of a positive EPCXM on renal function seems to disappear after one year and there does not seem to be an increased risk of late acute rejection or CAN based on a positive EPCXM. However, the risk of early rejections in patients with a positive EPCXM should not be neglected, because severe rejections [11] and even graft loss [21] have been reported as a consequence of donor-reactive anti-EPC Abs.

Authorship

MG, JH, MEB: research design, data collection, data analysis, and writing of the manuscript. AAM: data collection and analysis. LR: research design, data collection and analysis. J.H and M.E.B shares joint senior co-authorship.

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Conflict of Interest

J.H. is founder of AbSorber AB, the company manufacturing the XM-ONE® test. He is also a shareholder in Allenex AB, the majority owner of AbSorber AB, and his wife receives a royalty from XM-ONE® sales. No other author has any conflict of interest to declare.

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