Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Expression of adhesion molecules and RANTES in kidney transplant from nonheart-beating donors

Manuel Gomez del Moral,[1]* Beatriz Aviles,[3]* Ingrid K. Colberger,[2] Yolanda Campos-Martin,[2] Javier Suela,[2] Joaquin Alvarez,[4] M. Jesús Perez-Contin,[5] Ana Sánchez-Fructuoso,[3] Alberto Barrientos[3] and Eduardo Martinez-Naves[2]

1 Department of Cell Biology, Faculty of Medicine, Complutense University, Madrid, Spain
2 Department of Immunology, Faculty of Medicine, Complutense University, Madrid, Spain
3 Department of Nephrology, Hospital Clinico San Carlos, Madrid, Spain
4 Department of Transplant Coordination, Hospital Clinico San Carlos, Madrid, Spain
5 Department of Surgery, Hospital Clinico San Carlos, Madrid, Spain

Keywords
adhesion molecules, intracellular adhesion molecule 1, kidney transplant, nonheart-beating donor, RANTES, vascular cell adhesion molecule 1.

Correspondence
Prof. Eduardo Martinez Naves, Departamento de Inmunología, Facultad de Medicina, Universidad Complutense de Madrid, Avda. Complutense S/N 28040 Madrid, Spain.
Tel.: 34-913941644 (42); fax: 34-913941641; e-mail: emnaves@med.ucm.es

*The authors are joint first authors and the order of authorship is arbitrary.

Received: 29 November 2003
Revised: 29 July 2004
Accepted: 26 October 2004

doi:10.1111/j.1432-2277.2004.00060.x

Summary

The main difference between cadaveric kidneys from donors with a heartbeat (HBD) and kidneys from nonheart-beating donors (NHBD) is related to warm ischemia/reperfusion time which constitutes an acute inflammatory process. On the contrary, brain death induces in HBD expression of pro-inflammatory adhesion molecules, making it important to evaluate this kind of molecules in both types of donors. Human renal biopsies from NHBD, HBD and normal kidneys (ischemia time = 0) were taken and frozen just before transplant. A semi-quantitative RT-PCR method was used to determine intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), lymphocyte function associated antigen (LFA-1), LFA-3, CD40, CD40 ligand (CD40L) and RANTES (regulated upon activation, normal T-cell expressed and secreted) gene expression. We have detected an elevated relative gene expression of ICAM-1, VCAM-1 and RANTES in NHBD biopsies compared with normal kidneys. In the case of RANTES, the gene expression from NHBD biopsies was higher than observed in HBD biopsies. The rest of genes were not augmented in any group. Preliminary data about early outcome of transplants indicates a correlation between pretransplant RANTES high gene expression levels and early post-transplant acute rejection. The gene expression of pro-inflammatory molecules like adhesion molecules and RANTES is augmented in kidneys from cadaveric NBD just before transplant. The expression is higher probably because of the prolonged warm ischemia period. A larger clinical study is necessary to clarify the effects of these variable expressions on the transplant outcome.

Introduction

The ever-increasing number of patients undergoing dialysis and the adoption of less restrictive transplant criteria have led to a shortage of kidneys for transplant. Although there is a need to extend the use of brain-dead donors with heartbeats, there is still room for additional sources of organs, and this has encouraged the use of nonheart-beating donors (NHBD).

The use of NHBD has been the subject of several reports [1–4], and it is clear that this type of donation is on the rise [5,6]. The widespread reluctance to accept NHBD as a source of kidneys for transplantation is in part the result of the warm ischemic insult sustained by

the kidneys, which has been associated with increased rates of delayed graft function (DGF) in NHBD compared with heart-beating donor (HBD) renal transplants. Higher rates of acute rejection in the presence of DGF have been previously been described [7]. Both DGF and acute rejection are detrimental to long-term graft survival. For these reasons NHBD kidneys are considered marginal organs. On the contrary, several publications do not show differences in the long-term survival between NHBD and HBD despite the higher incidence of DGF in the NHBD recipients [2,5,6], and the outcome in NHBD may be even more successful [8] (C. G. Koffman, personal communication).

Some of the differences between organs harvested from HBD and NHBD are related to ischemia/reperfusion (I/R). Reperfusion of blood through ischemic tissue results in a set of reactions that can cause injury to vascular and parenchymal cells. Pathologically, these are characterized by deposition of complement, upregulation of adhesion molecules, inflammatory cell infiltration and cytokine release. Activation of complement, platelets, neutrophils and macrophages following ischemia extends the initial damage [9]. Grafts with prolonged cold or warm ischemia times are more susceptible to short-term or long-term deterioration [10].

One of the hallmarks of cellular rejection is the expression of adhesion molecules on vascular endothelial and tubular epithelial cells (TEC). Adhesion molecule activation is initiated by cytokine release in renal ischemia and each of these occurs in donor kidney biopsies [11]. The effect of hypoxia on the expression of adhesion molecules have been studied *in vitro* on cultured human endothelial and tubular cells. Hypoxia at 37 °C is a stimulus that induces the synthesis and expression of the adhesion molecule, intracellular adhesion molecule 1 (ICAM-1) [12]. Takada *et al.* [13] demonstrated an increased expression of ICAM-1 in rat kidneys exposed to reperfusion after warm ischemia but not after cold ischemia. Renal proximal tubular cells are especially sensitive to exposure to hypoxia and these cells may secrete various cytokines [12,14].

Leukocyte adherence to endothelia, extravasation and migration to sites of tissue injury are mediated by adhesion molecules. Lymphocyte function associated antigen (LFA)-1 and very late antigen-4 have been found to constitute independent leukocyte adhesion molecular pathways for attachment to endothelia by interacting specifically with two cytokine-inducible endothelial cell surface molecules: ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1), respectively [15]. Increased expression of both ICAM-1 and VCAM-1 has been reported in acute rejection of experimental and human renal transplantation [16]. Using RNA *in situ* hybridization, it has been found that both ICAM-1 mRNA and VCAM-1 mRNA are induced *de novo* on vascular cells in acute rejection, which suggests that VCAM-1 acts in tandem with ICAM-1 in the rejection process [17].

Chemokines have been identified as specific attractants of leukocytes into inflamed tissue. In addition to their chemotactic activity, chemokines may induce the expression of cell adhesion molecules. Chemokines are produced and secreted by various cell types including leukocytes. In the renal parenchyma endothelial cells, TEC, mesangium cells and fibroblasts may produce chemokines either constitutively or in response to renal damage [18]. It has been suggested that RANTES has an role in allograft rejection [13,19].

CD40 ligand (CD40L), a product of activated T cells, is locally expressed in kidneys undergoing rejection. Furthermore, during rejection, CD40 expression not only is present on most graft infiltrating cells but also is increased on resident TEC. The TEC express CD40, and *in vitro* activation increases chemokine production. It has been demonstrated that during renal allograft rejection, local expression of both CD40 and CD40L is strongly increased [20]. Platelets express CD40L within seconds of activation *in vitro* and in the process of trombus formation *in vivo*. CD40L on platelets induces endothelial cells to secrete chemokines and to express adhesion molecules. CD40 is constitutively expressed at low levels in kidney suggesting that it is a natural partner for CD40L on platelets [21].

Both donor groups have special characteristics because of processes proceeding the engraftment, such as warm I/R in NHBD and renal damage associated with brain death in HBD, both of them developing an inflammatory process. In this context the contribution of organ donor risk factors, such as the expression of adhesion molecules in kidneys before transplantation, has been given only very limited consideration. To address this question, we investigated the levels of gene expression of these molecules in HBD and NHBD kidneys before transplantation using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). We also examined if these were associated with subsequent post-transplant rejection or impaired graft function in the NHBD group.

Subjects and methods

Study population

NHBD procedure

In 1989, a program to procure kidneys from NHBD was started at our hospital. The grafts were maintained from the moment cardiac arrest had been diagnosed until the time of procurement by cardiopulmonary bypass involving extracorporal circulation, external oxygenation and intense hypothermia [22]. In addition to the classical

prerequisites for the brain-dead donor, our current acceptance criteria for NHBD kidneys are:

1 Maximum time of oligoanuria before cardiac arrest: 60 min

2 Maximum time of warm ischemia (from the start of cardiac arrest to the start of perfusion): 120 min

3 Maximum pump perfusion time: 240 min

4 Donor age <55 years

A recent addition to the program is the procurement of organs from subjects who die suddenly on the street and are transported to the center for donation [23]. To be accepted as possible donors, these subjects were also required to fulfill the following criteria: (i) known cause of death, ruling out violence, (ii) nonbleeding injuries to the thorax or abdomen, (iii) external cardiac massage and mechanical ventilation performed within 15 min of the start of cardiac arrest, (iv) transfer of subjects to the hospital with external cardiac massage, mechanical ventilation and intravenous liquid perfusion, (v) no external signs of possible intravenous drug addiction to control the risk of HIV or hepatitis C or B positivity.

Family and legal consent was obtained for all kidneys procured in this manner. The organs obtained were classified according to Maastricht donor categories [24]. To include all of our donor types, a fifth category was added to this classification scheme: type V: unexpected cardiac arrest in intensive care.

Samples and donors

Seventeen biopsies were taken from kidneys obtained from NHBD and seven from HBD. The samples were taken just before the moment of engraftment and the graft recipients were informed and accepted the use of the biopsies in the study. Seven tissue samples were taken during extirpation from unaffected parts of different kidneys removed because of a renal tumor, considered as normal kidney tissue and were to be used as controls. The biopsies were immediately snap frozen in liquid nitrogen and stored at −70 °C. All recipients suffered DGF >5–7 days after transplantation and were biopsied to clarify the cause of graft dysfunction. A second biopsy was performed in three cases where the DGF persisted. Only the pretransplant biopsies were used in the study. The characteristics of different donor types and short outcome are summarized in Table 1. The transplant recipients were subjected to the same immunosuppressive treatment, i.e. triple therapy (cyclosporine or tacrolimus plus prednisone and mycophenolate).

Table 1. Donor and recipient characteristics, and short-term outcomes after transplantation.

	NHBD ($n = 17$)	HBD ($n = 7$)
Donor age (years)	30.8 ± 4.1	32.8 ± 5.2
Recipient age (years)	47.8 ± 16.1	37.1 ± 19
Warm ischemic time (min)	107 ± 20	0
Cold ischemic time (h)	22.3 ± 2.2	22.5 ± 3.1
Short-term outcome		
Acute rejection	7*	4
Acute tubular necrosis	13	3

All data are given as mean ± SD.
*Three after acute tubular necrosis.

Isolation and quantification of total RNA

Thirty milligrams of frozen biopsy tissue was immediately disrupted using a heat treated, RNase free potter and homogenized by passing the lysate through a 20-G (Ø0.9 mm) needle fitted to a syringe. The isolation of total RNA was performed using the commercial kit RNeasy Mini Kit (Qiagen, Hilden, Germany).

The concentration and purity of RNA in the final solution were determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a spectrophotometer (Ultrospec Plus Pharmacia, Uppsala, Sweden). The concentration of the original sample was calculated as: ($A_{260} \times$ dilution factor)/1000 = c (μg/μl). A ratio (A_{260}/A_{280}) between 1.7 and 2.0. was considered pure and used in the experiments.

Analysis of gene expression by semi-quantitative RT-PCR

Reverse transcriptase-polymerase chain reaction was used to identify expression of specific gene transcripts within renal biopsy tissue of the following genes: RANTES, ICAM-1, VCAM-1, LFA-1, LFA-3, CD40, C40L and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a highly expressed housekeeping protein used in this study to estimate the quality and quantity of RNAs.

Oligonucleotide primers were designed to amplify mRNA and to flank an intron site in the target sequence in order to easily detect genomic contamination (Table 2).

Standard curves for the different molecules were generated by serial dilutions of total RNA from normal kidney tissue and the dilutions were processed under identical RT-PCR conditions for each gene to assure that under these conditions the intensity of amplified bands was proportional to the amount of cDNA templates.

Reverse transcription for cDNA synthesis

One microgram of total RNA was reverse-transcribed into cDNA using 20 units of AMV Reverse Transcriptase (Genaxis, Nimes France). AMV reaction buffer 1x (25 mM Tris–HCl pH 8.30, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT) (Genaxis), 1 mM of dNTPs in Deoxynucleotide Mix

Table 2. Characteristics of PCR primers of target genes.

Gene	Sense	Sequence	Size of PCR product (bp)
GAPDH	+	AAGGTCGGAGTCAACGGATTT	983
	–	GGGCCATGAGGTCCACCAC	
ICAM-1	+	GTCCCCCTCAAAAGTCATCC	942
	–	AACCCCATTCAGCGTCACCT	
VCAM-1	+	GGTGGGACACAAATAAGGGTTTTGG	715
	–	CTTGCAATTCTTTTACAGCCTGCC	
LFA-1	+	AGATGGAGCCTCCCGTGCCC	568
	–	GGCACTCTGCACCTCACAGG	
LFA-3	+	TTACAACAGCCATCGAGGAC	306
	–	GGAGTTGGTTCTGTCTGGTT	
CD40	+	TCTGGGGCTGCTTGCTGA	721
	–	TGGAGCAGCAGTGTTGGAGC	
CD40L	+	CACAGCATGATCGAAACATACAAC	797
	–	CACTGTTCAGAGTTTGAGTAAGCCA	
RANTES	+	TTGCTCTTGTCCTAGCTTTGGGAG	628
	–	GCAGCGCCTCAGAAGCTCTTC	

(Roche, Basel, Switzerland), 1 μM Oligo-p(dT)15 Primer (Sigma-Genosys, St. Louis, Missouri, USA), and 40 units of ribonuclease inhibitor (TaKaRa Biomedicals, Kusatsu, Japan). The mixture was incubated at 25 °C for 10 min and then at 42 °C for 60 min. After cooling to 4 °C the reaction tube was stored at −20 °C.

PCR

Before the experiment, ideal PCR conditions such as the amount of RNA, the concentration of $MgCl_2$ as well as the number of amplification cycles were determined for each gene in order to work in exponential conditions.

For every reaction a 1 μl of cDNA was amplified with 1 unit of Biotools DNA polymerase (Biotools, Madrid, Spain) reaction buffer 1x (7.5 M Tris–HCl pH 8.0, 5 mM KCl, 2 mM $(NH_4)_2SO_4$) (Biotools), 2 mM $MgCl_2$, 0.2 mM of dNTPs (Gene Amp dNTP Mix with dTTP; Roche, Basel, Switzerland) and 1 μM of each oligonucleotide primer (Sigma-Genosys, UK). A negative control was prepared using the same mix (without cDNA).

cDNA amplifications were carried out in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research Inc., San Francisco, California, USA). After an initial heating to 95 °C during 5 min to denature, a temperature cycle was 1 min denaturation at 95 °C, 1 min of annealing at 60 °C (65 °C for LFA-1), 2 min of extension at 72 °C and finally 10 min of extension at 72 °C after 30 cycles (35 cycles for LFA-1). In each PCR experiment a parallel GAPDH amplification was performed.

The amplification of each gene was repeated at least three times for each sample from the initially isolated mRNA. The standard deviation of the mean of triplicates was always <5%.

Analysis of PCR products

Ten microlitre of each PCR product were loaded in an 1% agarose gel. DNA Molecular Weight Marker X was used to identify the sizes of products. The gel was trans-illuminated on a UV table and photographed using a video gel documentation system (Gel Doc 2000; Bio-Rad, Hercules, California, USA). The densitometry analysis was performed with the MultiAnalyst software (Bio-Rad) where the band intensity was measured.

The results were calculated as the ratio of the densitometry values in mean counts of each molecule and the values given for GAPDH under the same conditions.

Statistical analyses

The *t*-test or Mann-Whitney test were used to compare continuous variables (expressed as mean ± SD).

We have compared the expression of ICAM-1, VCAM-1, LFA-1, LFA-3, CD40, CD40L and RANTES between the two donor types (HBD or NHBD) as well as controls. In the case of NHBD, the group was divided and compared according to posterior short-term graft evolution.

Fluorimeter-based real-time PCR

Another semi-quantitative method was performed using real-time PCR technology for quantification of GAPDH, ICAM-1, VCAM-1 and RANTES mRNA expression in kidney biopsies. One microgram cDNA was obtained (cDNA Kit AMV; Roche) using random primer hexamers $[p(N)_6]$, upstream RNA poly A sequence. PCR amplification was performed using an ABI PRISM 7000 sequence detection system and Sybr green PCR Master Mix (Applied Biosystems, Foster City, California, USA). Primer pairs, with an amplicon between 70–100 bp, were designed on the basis of the sequences obtained from gene data bank, using Primer Express Software (Applied Biosystems). *GAPDH* (+): CTC TGC CCC CTC TGC TGA T, (−): GTG CAG GAG GCA TTG CTG A; *ICAM-1* (+): GGC AAC GAC TCC TTC TCG G, (−): CGC CGG AAA GCT GTA GAT G; *VCAM-1* (+): CAT GGA ATT CGA ACC CAA ACA, (−) ATG GAG GAA GGG CTG ACC A; *RANTES* (+): CAA CCC AGC AGT CGT CTT TGT, (−) CTC CCG AAC CCA TTT CTT CTC. The optimed runs were as follows: 95 °C 10 min and 40 times cycle of 95 °C 1 min, 60 °C 1 min and 72 °C 1 min. Finally, to determine gene expression, DNA amplification was detected reading Sybr green fluorescence.

Results

The mean ratios of each gene calculated from the densitometry values of three independent PCRs were grouped

according to donor type and clinical evolution and the mean results were compared between categories. Table 3 shows the relative expression of genes studied.

ICAM-1

The gene expression ratio of ICAM-1/GAPDH showed that the ICAM-1 expression was increased in kidney biopsies obtained from NHBD before transplantation compared with control biopsies ($P < 0.005$). No increase of ICAM-1 expression was found in the group of kidneys obtained from HBD compared with the control, neither any significant difference in expression between the HBD and NHBD groups. The clinical post-transplant evolution showed that the patients who developed acute rejection had received kidneys with an increased expression of ICAM-1 ($P < 0.05$) while the kidneys received by the group of patients who developed acute tubular necrosis (ATN) did not show any significant overexpression compared with the controls (Fig. 1; Table 3).

VCAM-1

In the case of VCAM-1 we found a significantly increased gene expression in biopsies from HBD ($P < 0.05$) as well as NHBD ($P < 0.005$) compared with normal kidney. No difference was found between the donor groups. The expression of VCAM-1 in the group which developed acute rejection after transplantation did not differ from the expression of the group who developed ATN (Fig. 1; Table 3).

RANTES

In NHBD kidney biopsies the RANTES gene expression was increased compared with normal kidney ($P < 0.005$) and HBD ($P < 0.005$), whereas no significant difference in expression between the HBD and control groups was seen. The clinical evolution showed that patients who suffered from acute rejection had received kidneys with an increased pretransplant level of RANTES ($P < 0.05$). In the ATN group no such pretransplantation overexpression was detected (Fig. 1; Table 3).

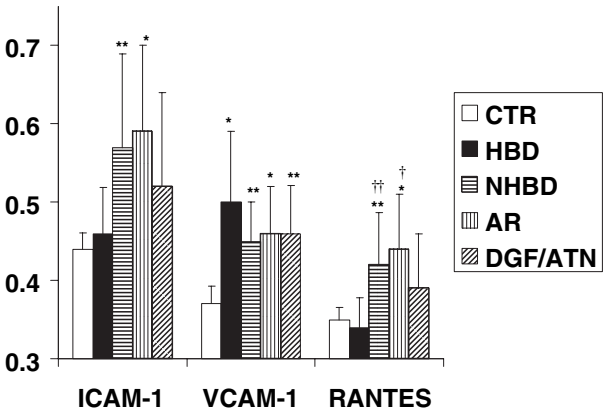

Figure 1 Different expression of ICAM-1, VCAM-1 and RANTES in human renal biopsies from heart-beating donors (HBD, $n = 7$), non-heart-beating donors (NHBD, $n = 17$) and normal kidney (CTR, $n = 7$). $*P < 0.05$, $**P < 0.005$ compared with normal kidney. $\dagger P < 0.05$, $\dagger\dagger P < 0.005$ compared with HBD.

We detected no difference in LFA-1, LFA-3, CD40 and CD40L gene expression levels between any of the groups investigated (Table 3).

Representative examples of bands obtained in an 1% agarose gel of the most outstanding molecules (ICAM-1, RANTES and VCAM-1) are shown in Fig. 2.

Real-time RT-PCR

In order to confirm these findings we repeated the mRNA quantification for GAPDH, VCAM-1, ICAM-1 and RANTES in our samples using real time RT-PCR analysis. The results using this technique were similar to those obtained previously. Figure 3 shows one representative experiment using this technique.

Table 3. Relative densitometry values of gene expression of ICAM-1, VCAM-1, RANTES, LFA-1, LFA-3, CD40 and CD40L in human renal biopsies from heart-beating donors ($n = 7$), nonheart-beating donors ($n = 17$) and normal kidney ($n = 7$) obtained before engraftment into the recipients.

	CTR	HBD	NHBD	AR	ATN
ICAM-1	0.44 ± 0.021	0.46 ± 0.059	0.57 ± 0.12**	0.59 ± 0.11*	0.52 ± 0.12
VCAM-1	0.37 ± 0.023	0.5 ± 0.09*	0.45 ± 0.05**	0.46 ± 0.06*	0.46 ± 0.061**
RANTES	0.35 ± 0.015	0.34 ± 0.038	0.42 ± 0.67**††	0.44 ± 0.08*†	0.39 ± 0.07
LFA-1	0.28 ± 0.019	0.30 ± 0.043	0.30 ± 0.022	0.29 ± 0.014	0.30 ± 0.029
LFA-3	0.38 ± 0.021	0.37 ± 0.029	0.36 ± 0.024	0.36 ± 0.019	0.36 ± 0.02
CD40	0.37 ± 0.025	0.35 ± 0.021	0.36 ± 0.030	0.35 ± 0.013	0.36 ± 0.033
CD40L	0.31 ± 0.018	0.33 ± 0.031	0.33 ± 0.024	0.33 ± 0.027	0.32 ± 0.019

The NHBD group is divided into two subgroups according to posterior graft evolution: AR and ATN. All data given as are mean ± SD.
$*P < 0.05$; $**P < 0.005$ compared with normal kidney.
$\dagger P < 0.05$; $\dagger\dagger P < 0.005$ compared with HBD.

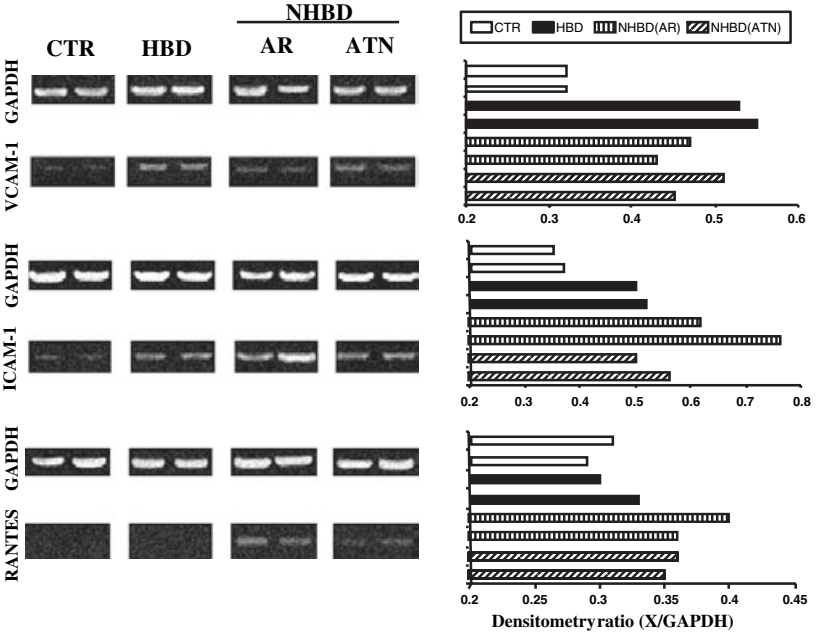

Figure 2 Bands obtained in an 1% agarose gel stained with EtBr after RT-PCR using specific primers. Representative examples of mRNA expression of ICAM-1, VCAM-1, RANTES and GAPDH in renal biopsy tissue taken before transplantation. The graphs represent the comparison of the densitometry ratio of ICAM-1, VCAM-1 or RANTES and the corresponding GAPDH in normal kidney (CTR), heart-beating donors (HBD) and nonheart-beating donors (NHBD). The NHBD group is divided into acute rejection (AR) and delayed graft function (DGF) according to the posterior graft evolution.

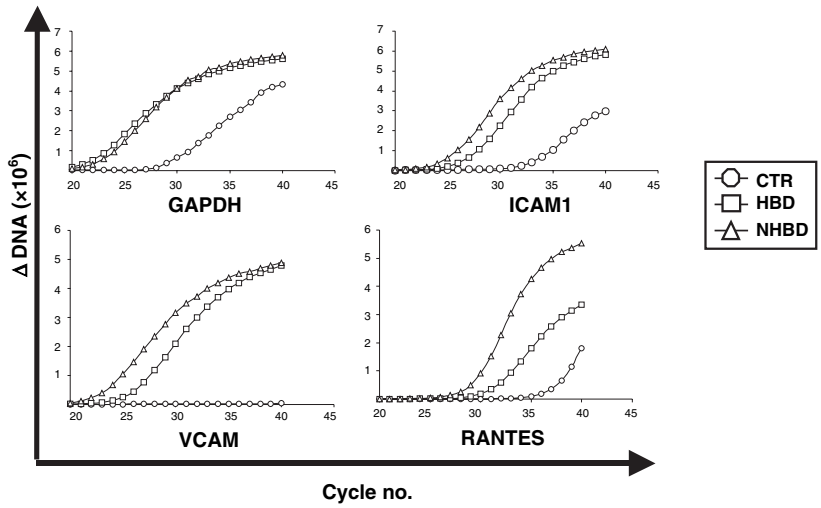

Figure 3 Representative real-time PCR cycle data of GAPDH, VCAM-1, ICAM-1 and RANTES mRNA expression in normal kidney (CTR), heart-beating donors (HBD) and nonheart-beating donors (NHBD).

Discussion

The increased ICAM-1 gene expression in kidney biopsies from NHBD observed in our study is consistent with previous observations: I/R injury is reported to increase mRNA [14] as well as ICAM-1 protein expression [11] and according to Takada *et al.* [13], cold I/R injury showed no *de novo* ICAM-1 expression compared with warm I/R injury, indicating the greater influence of the warm injury. Immunohistochemistry studies using monoclonal antibody have shown an increased staining of VCAM-1 in rat renal grafts with the length of ischemia times compared with native control kidneys [26]. In agreement with this finding, we observed a significantly augmented VCAM-1 mRNA expression in both HBD and NHBD donors.

The molecular basis for the selective homing of memory T cells to peripheral sites is not clear, although there is evidence for an important role of adhesion molecules ICAM-1 and VCAM-1 and chemokine receptors that involve interactions between endothelial cells and T cells [27]. Clinical studies have shown that I/R constitutes an acute inflammatory process involving cells surface adhesion molecules expression, and that these molecules are crucial for the recruitment and infiltration of effector cells into the postischemic tissue [10]. Hypoxia could directly activate the transcription of ICAM-1 through activation of Nuclear Factor Kappa B (NFkB) in TEC [12] or vascular endothelium.

In vitro studies have demonstrated that renal TEC with induced ICAM-1 and VCAM-1 expression are capable of

binding lymphocytes [26]. We believe that induction of tubular adhesion molecule expression may facilitate the infiltration of T lymphocytes after transplantation.

Leukocyte function associated molecule-1 (LFA-1) is considered a major neutrophil integrin. It is suggested that immediately after transplantation, neutrophils exploit rolling mechanisms, firmly adhere through the interaction of LFA-1 with ICAM-1 expressed on glomerular or endothelium of peritubular capillaries [10]. LFA-3, the ligand of LFA-2, is a co-stimulatory molecule of leukocytes. As expected and according to our results no pretransplant mRNA expression of LFA-1 or LFA-3 is augmented in any of our biopsy types as no infiltration is likely to be present.

The expression of CD40L represents one of the earliest and most specific T-lymphocyte activation markers during immune reaction [25]. CD40 is constitutively expressed at low levels in kidney [21]. Its expression is restricted to a limited number of TECs, and is increased on tubules in renal biopsies with inflammation [28]. CD40L, a product of activated T cells, is locally expressed in kidneys undergoing rejection [20]. Consistent with our hypothesis about the absence of infiltrating leukocytes we have not observed differences in CD40 and CD40L gene expression between experimental groups.

In the renal parenchyma endothelial cells, TEC, mesangium cells and fibroblasts may produce chemokines either constitutively or in response to renal damage [18].

Exposure of TECs *in vitro* to hypoxia not only increases expression of adhesion molecules but also increases the production of inflammatory mediators like RANTES [28]. Previous data have demonstrated that RANTES expression by T cells requires at least 2–3 days [19] implying TEC as the main source of the RANTES expression in pretransplantation kidney biopsies. The differential RANTES mRNA expression showed in our data between NHBD and HBD could be explained based on their different total ischemia times (NHBD: 22.3 ± 2.2 h plus 1.78 ± 0.33 h versus HBD: 22.5 ± 3.1 h) or because of more detrimental effects of warm ischemia (in this way, recent data [29] show that warm ischemia downregulate TGF-β 1 expression during the early phases of renal transplantation). In addition to their well-defined role as chemotactic mediators of target leukocytes, many chemokines have other distinct pro-inflammatory properties, including regulation of leukocyte extravasation, and activation of effector functions of leukocytes [25].

A positive correlation between adhesion molecule and chemokine expression in post-transplant renal biopsies and early acute rejection episodes has been previously observed [11]. Several studies have indicated that endothelial ICAM-1 and VCAM-1 were consistently induced in association with acute graft rejection [30] and have been shown to be upregulated on proximal tubules during renal allograft rejection [26], where ICAM-1 was induced even in areas without inflammatory infiltrates [25]. RANTES mRNA is expressed during cell-mediated transplant rejection. It has been detected in infiltrating mononuclear cells and renal tubular epithelium by *in situ* hybridization, and RANTES protein was localized to mononuclear cells, tubular epithelium and vascular endothelium [19]. Although the number of samples is small, preliminary data in this study suggest a significant correlation between previously elevated ICAM-1 and RANTES mRNA expression in NHBD kidneys and early post-transplant acute rejection.

Despite being associated with poor initial graft function, recent data show that the long-term allograft survival of NHBD kidneys does not differ significantly from the results of HBD [1–4,6] and LD transplants [1]. However, the duration of warm ischemia reported for NHBD in these studies was <60 min (107 ± 20 min in our study).

It seems like there is an increased gene expression of pro-inflammatory molecules in kidneys from NHBD just before transplant that may influence on the short time outcome. However a larger study is necessary to evaluate the effects of these and other donor-related parameters on the variable expression of adhesion molecules and to determine their possible influence on the early as well as long -term post-transplant events.

Acknowledgements

This work was supported by a grant from the Fondo de Investigaciones Sanitarias (FIS) (Ref. 00/0468) and Red temática de trasplantes (Ref. CØ3/Ø3) of the Spanish Ministry of Health.

References

1. Nicholson ML, Horsburgh T, Doughman TM, *et al.* Comparison of the results of renal transplants from conventional and nonheart-beating cadaveric donors. *Transplant Proc* 1997; **29**: 1386.
2. Weber M, Dindo D, Demartines N, *et al.* Kidney transplantation from donors without a heartbeat. *N Engl J Med* 2002; **347**: 248.
3. Koffman G, Gambaro G. Renal transplantation from non-heart-beating donors: a review of the European experience. *J Nephrol* 2003; **16**: 334.
4. Sanchez-Fructuoso AI, de Miguel Marques M, Barrientos A. Non-heart-beating donors: experience from the Hospital Clinico of Madrid. *J Nephrol* 2003; **16**: 387.
5. Sanchez-Fructuoso AI, Prats D, Torrente J, *et al.* Renal transplantation from non-heart beating donors: a promising

alternative to enlarge the donor pool. *J Am Soc Nephrol* 2000; **11**: 350.
6. Metcalfe MS, Butterworth PC, White SA, *et al.* A case-control comparison of the results of renal transplantation from heart-beating and non-heart-beating donors. *Transplantation* 2001; **71**: 1556.
7. Cho YW, Terasaki PI, Cecka JM, *et al.* Transplantation of kidneys from donors whose hearts have stopped beating. *N Engl J Med* 1998; **338**: 221.
8. Sánchez-Fructuoso AL, Prats D, Fernández-Pérez E, *et al.* Study of nonimmunological factors that could be contributed to development of acute vascular rejection. *Transplant Proc* 2002; **34**: 360.
9. Troppmann C, Gillingham KJ, Benedetti E, *et al.* Delayed graft function, acute rejection, and outcome after cadaver renal transplantation. The multivariate analysis. *Transplantation* 1995; **59**: 962.
10. Dragun D, Hoff U, Park JK, *et al.* Ischemia-reperfusion injury in renal transplantation is independent of the immunologic background. *Kidney Int* 2000; **58**: 2166.
11. Schwarz C, Regele H, Steininger R, *et al.* The contribution of adhesion molecule expression in donor kidney biopsies to early allograft dysfunction. *Transplantation* 2001; **71**: 1666.
12. Combe C, Burton CJ, Dufourco P, *et al.* Hypoxia induces intercellular adhesion molecule-1 on cultured human tubular cells. *Kidney Int* 1997; **51**: 1703.
13. Takada M, Nadeau KC, Shaw GD, *et al.* The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J.Clin Invest* 1997; **99**: 2682.
14. Kelly KJ, Williams Jr WW, Colvin RB, *et al.* Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 1996; **97**: 1056.
15. Fuggle SV, Sanderson JB, Gray DW, *et al.* Variation in expression of endothelial adhesion molecules in pretransplant and transplanted kidneys-correlation with intragraft events. *Transplantation* 1993; **55**: 117.
16. Jeong HJ, Lee HH, Kim YS, *et al.* Expression of ICAM-1 and VCAM-1 in renal allograft rejection. *Transplant Proc* 1998; **30**: 2953.
17. Park SY, Kim HW, Moon KC, *et al.* mRNA expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in acute renal allograft rejection. *Transplantation* 2000; **69**: 2554.
18. Grau V, Gemsa D, Steiniger B, *et al.* Chemokine expression during acute rejection of rat kidneys. *Scand J Immunol* 2000; **51**: 435.
19. Pattison J, Nelson PJ, Huie P, *et al.* RANTES chemokine expression in cell-mediated transplant rejection of the kidney. *Lancet* 1994; **343**: 209.
20. Woltman AM, de Haij S, Boonstra JG, *et al.* Interleukin-17 and CD40-ligand synergistically enhance cytokine and chemokine production by renal epithelial cells. *J Am Soc Nephrol* 2000; **11**: 2044.
21. Henn V, Slupsky JR, Grafe M, *et al.* CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998; **391**: 591.
22. Gomez M, Alvarez J, Arias J, *et al.* Cardiopulmonary bypass and profound hypothermia as a means for obtaining kidney grafts from irreversible cardiac arrest donors: cooling technique. *Transplant Proc* 1993; **25**: 1501.
23. Alvarez J, Iglesias J, Pulido O, *et al.* Type I non-heart-beating donors: policy and results. *Transplant Proc* 1997; **29**: 3552.
24. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. *Transplant Proc* 1995; **27**: 2893.
25. Kamoun M. Cellular and molecular parameters in human renal allograft rejection. *Clin Biochem* 2001; **34**: 29.
26. Koo DD, Welsh KI, McLaren AJ, *et al.* Cadaver versus living donor kidneys: impact of donor factors on antigen induction before transplantation. *Kidney Int* 1999; **56**: 1551.
27. Hill PA, Main IW, Atkins RC. ICAM-1 and VCAM-1 in human renal allograft rejection. *Kidney Int* 1995; **47**: 1383.
28. van Kooten C, Daha MR, van Es LA. Tubular epithelial cells: a critical cell type in the regulation of renal inflammatory processes. *Exp Nephrol* 1999; **7**: 429.
29. Lario S, Mendes D, Bescos M, *et al.* Expression of transforming growth factor-beta 1 and hypoxia-inducible factor-1 alpha in an experimental model of kidney transplantation. *Transplantation* 2003; **75**: 1647.
30. Alpers CE, Hudkins KL, Davis CL, *et al.* Expression of vascular cell adhesion molecule-1 in kidney allograft rejection. *Kidney Int* 1993; **44**: 805.