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Changes in adenosine generating enzymes CD39 and CD73 upon reperfusion in clinical kidney transplantation

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Abstract

Ischemia reperfusion (I/R) injury is an inevitable consequence of organ transplantation. Its pathophysiology is complex and treatment is primarily supportive. Recent animal experiments suggest that extracellular adenosine may be a critical mediator in protection from renal I/R injury. However, information on adenosine production in human kidney transplantation is lacking. In this study changes in protein and mRNA expression of adenosine generating enzymes CD39 and CD73 in transplanted human kidneys are assessed. Results show that more CD39 protein is present in living compared to deceased donor kidneys before transplantation. CD39 mRNA expression was not different between groups and did not change upon reperfusion. However, CD73 mRNA expression was significantly downregulated after reperfusion, while its tissue protein expression did not change. Altogether, results show that living donor kidneys may be protected by higher pre-transplantation CD39 expression. Yet, this effect may be counteracted by the decrease in CD73 expression after reperfusion. Further studies will need to focus on the consequences of these enzyme changes for renal adenosine generation and kidney graft injury.

Introduction

Ischemia reperfusion (I/R) injury is an inevitable consequence of organ transplantation, and a major determinant of patient and graft survival.[1-3] The pathophysiology of I/R injury is complex and incompletely understood, and effective treatment is currently lacking. Recent animal experiments suggest that adenosine may be a critical mediator in protection from renal I/R injury.[4;5]

Adenosine is a nucleoside, acting as a signaling molecule when present in the extracellular space. Extracellular adenosine can be generated by metabolization of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine through an enzymatic reaction initiated by ectonucleoside-triphosphate-diphosphohydrolase-1 (CD39) and completed by the enzyme ecto-5'-nucleotidase (CD73) (Figure 1). Hypoxia and ischemia are known to induce CD39 and CD73.[6;7] Animals genetically modified by CD39 or CD73 deletion experience lower adenosine levels during ischemia and a vast increase in I/R induced inflammation and subsequent tissue damage in many organs.[6-9] Consequently, CD39 overexpression has beneficial effects in mouse renal I/R injury.[10]

Altogether, animal models provide clear evidence for the protective role of CD39 and CD73 activity in renal I/R injury. In this study, we report on changes in adenosine generation by CD39 and CD73 in human clinical renal I/R injury.

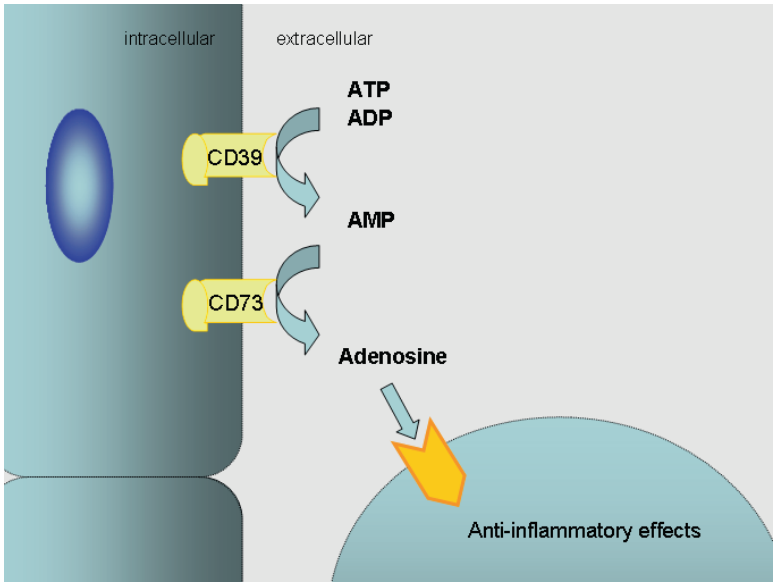

Figure 1: Schematic representation of extracellular adenosine generation from ADP and ATP by the enzymes ectonucleoside-triphosphate-diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidase (CD73).

Materials and methods

Patient population

Twenty-three patients receiving a kidney from a living donor (n=10), a brain dead donor (n=7) or a cardiac dead donor (n=6) were included for mRNA isolation from renal biopsies and subsequent expression analysis. From a different cohort of 10 living, 10 brain dead donor and 10 cardiac dead donor kidney graft recipients, renal biopsies were collected and processed for immunohistochemical analysis. Patient characteristics are shown in Supplemental Table 1. All kidneys were still functioning at 1 year after transplantation, but one patient in the mRNA cohort, that received a BDD kidney transplant died 6 months after transplantation (because of veno-occlusive disease ultimately causing hepatic failure). The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Table 1: Transplantation and outcome characteristics of the three different patient cohorts that were included. In each cohort material was collected from living donor (LD), brain dead donor (BDD) and cardiac dead donor (CDD) grafts during kidney transplantation.

	IHC			PCR		
	LD	BDD	CDD	LD	BDD	CDD
N	10	10	10	10	7	6
Donor age: mean (SD)	57(10)	48(16)	41(10)	52(8)	45(21)	41(13)
Donor gender (M:F)	5:5	5:5	6:4	2:8	3:4	4:2
WIT1 in min. (SD)	N/A	N/A	18(5)	N/A	N/A	18(6)
CIT in h. (SD)	2.8(0.5)	18.6(9.6)	17.8(3.4)	3.0(0.4)	17.4(7.6)	15.6(3.9)
WIT2 in min. (SD)	29(8)	28(4)	28(8)	29(7)	34(3)	28(5)
Recipient age: mean (SD)	54(16)	51(13)	45(15)	49(16)	53(14)	54(9)
Recipient gender (M:F)	4:6	7:3	7:3	6:4	4:3	3:3
CrCl day 30 (ml/min)	45(11)	63(30)	48(21)	56(17)	47(14)	50(8)
DGF	0/10	3/10	7/10	0/10	3/7	4/6
DGF duration in days (SD)	0	5(2)	13(6)	0	3(4)	7(6)

IHC: cohort of patients in which renal biopsies were used for immunohistochemical analysis.
PCR: cohort of patients from which renal biopsies were used for PCR analysis.
WIT1: first warm ischemia time.
CIT: cold ischemia time.
WIT2: second warm ischemia time.
DGF: delayed graft function.
DGF duration: dialysis after transplantation in days (SD).
CrCl: Creatinine clearance.

Operation and materials

Kidney transplantations were performed according to local standardized protocol. In living donors minimally invasive nephrectomy was performed. For cold perfusion and storage of the

kidney, Custodiol® Histidine-tryptophan-ketoglutarate solution (HTK) solution (Tramedico, Weesp, The Netherlands) was used. Deceased donor kidneys were perfused and stored with University of Wisconsin solution (UW) or HTK. The immunosuppressive regimen was based on induction therapy with an interleukin-2 receptor blocker (basiliximab, day 0 and day 4) and maintenance treatment with tacrolimus or cyclosporine A, in addition to mycophenolate mofetil and steroids in all groups.

Immunohistochemical analysis

To assess presence of CD39 and CD73, a renal cortical biopsy was obtained after cold storage from living, brain dead and cardiac dead donor kidney grafts. Only in the LD group, a second cortical biopsy was collected as well at 45 minutes after reperfusion. Immunohistochemical analyses were performed on 4 µm cryostat kidney tissue sections. Frozen sections were fixed in acetone for 10 min. Slides were washed and incubated with peroxidase blocking solution for 30 minutes and blocked with PBS containing 5% normal human serum (NHS) and 1% BSA for 45 minutes at room temperature. Then slides were incubated overnight at room temperature with the following primary antibodies: CD39 mouse monoclonal IgG1, 1:50 (eBioscience, Vienna, Austria); CD73 mouse monoclonal IgG1, 1:3200 (Hycult Biotechnology, Uden, Netherlands). Next, a peroxidase-conjugated goat anti-mouse IgG (1:200) secondary antibody was applied (DAKO, Glostrum, Germany), followed by sequential fluorescein isothiocyanate (FITC, amplification reagent, NENTM Life Science Products, Boston, MA, USA) and anti-fluorescein-HRP (DAKO, Glostrum, Germany). Staining was completed by incubation with 3,3' diaminobenzidine tetrahydrochloride (DAB)/ hydrogen peroxide (Sigma, St Louis, MO, USA) and counterstained with Mayer's Hematoxylin Solution (Merck, Darmstadt, Germany). At 100× magnification, 10 microscopic fields of each kidney section were quantified using computerized image analysis (ImageJ).

mRNA expression in human kidney biopsies

A renal cortical biopsy was obtained after cold storage, before transplantation, from living, brain dead and cardiac dead donor kidneys. A second, post-reperfusion biopsy was collected 45 minutes after reperfusion from living donor kidneys only. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70ºC. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads.[11] The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for further processing if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0).[12] cDNA was synthesized from 1 µg total RNA, using an oligo dT primer, RNase-OUT, M-MLV reverse transcriptase, 0.1 M-DTT

and buffers in a volume of 20 µL (all purchased from Invitrogen, Breda, The Netherlands). Quantitative real-time PCR was performed in duplicate by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (BioRad). The amplification reaction volume was 20 µL, consisting of 10 µL iQ SYBR Green PCR mastermix, 1 µL primers, 1 µL cDNA, and 8 µL water. Data were analyzed using Gene Expression Analysis for iCycler Real-Time PCR Detection System (BioRad). Expression of each gene was normalized against mRNA expression of the housekeeping gene GAPDH. The primer sequences are shown in Supplemental Table 2.

Table 2: Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Forward primer 5'->3'	Reverse primer 5'->3'	Supplier
CD39	AGCAGCTGAAATATGCTGGC	GAGACAGTATCTGCCGAAGTCC	Biolegio
CD73	GCAGACATTAACAAATGGAGG	CATCCGTGTGTCTCAGGTTG	Biolegio

Analysis

Clinical donor data were retrieved from Eurotransplant Foundation (Leiden, The Netherlands). Delayed graft function (DGF) was defined as the need for dialysis within one week after transplantation. Statistical analysis was performed using SPSS 17.0 statistical analysis software (SPSS Inc, Chicago, Ill). Graph error bars represent the standard error of the mean (SEM), unless otherwise stated. A *P*-value of less than 0.05 was considered significant.

Results

CD39

CD39 staining was positive in peritubular capillaries and in glomeruli. Already before transplantation, living donor kidneys had a significantly higher CD39 tissue expression as compared to both brain dead ($p=0.004$), and cardiac dead ($p<0.0001$) donor kidneys (Figure 2). After reperfusion, CD39 tissue expression did not change in living donor kidneys ($p=0.16$). CD39 mRNA expression was similar in grafts of all three donor types before transplantation ($p=0.27$). Upon reperfusion CD39 mRNA expression did not change ($p=0.49$).

CD73

CD73 signal was positive in endothelial cells and to a lesser extent in tubular epithelial cells. Before transplantation, CD73 protein and mRNA expression was equal in renal biopsies of the three different donor types ($p=0.52$, $p=0.13$, respectively, Figure 3). After reperfusion, CD73 tissue expression did not change ($p=0.20$). However, CD73 mRNA expression was significantly reduced after reperfusion in living donor ($P=0.002$) kidneys.

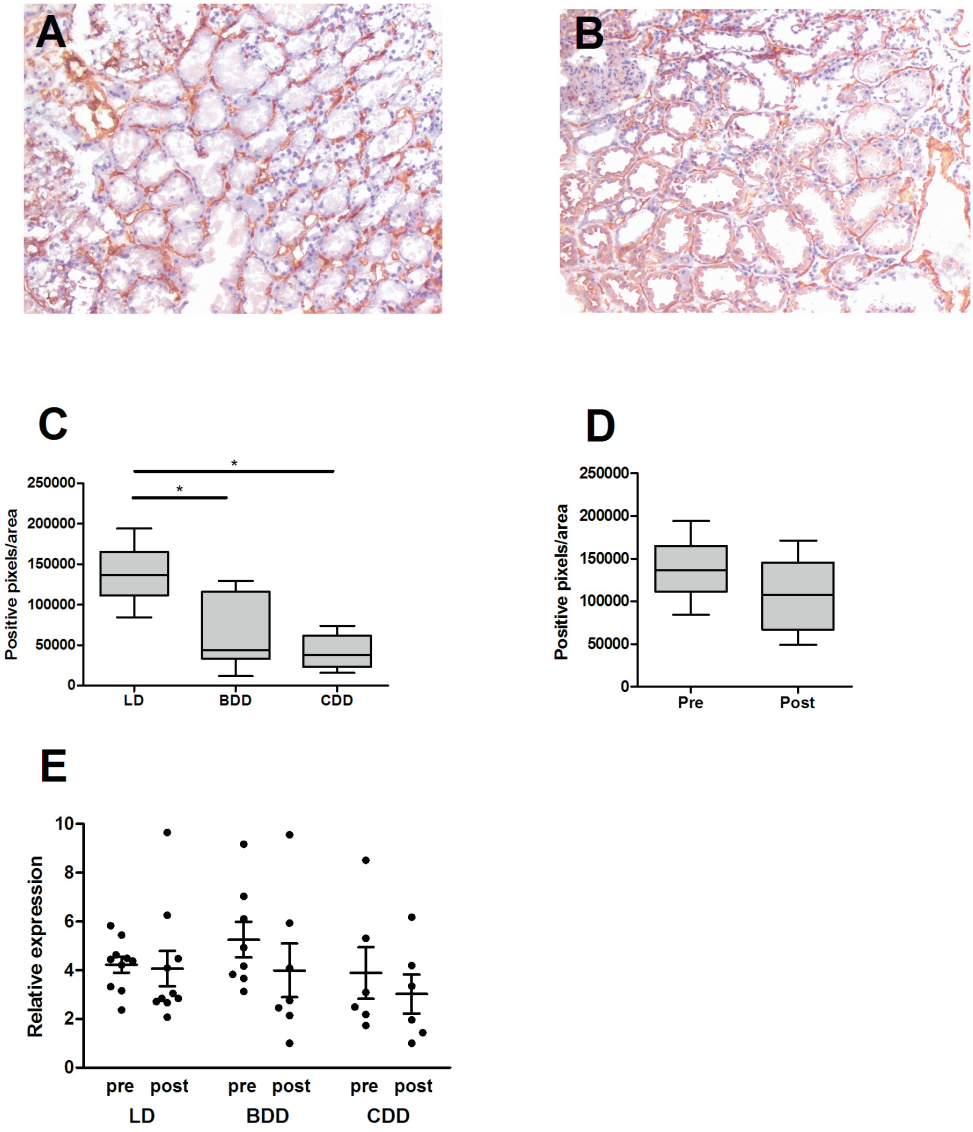

Figure 2: CD39 protein levels are higher in living than deceased donor kidneys.
CD39 staining was positive in peritubular capillaries and in glomeruli. Typical example of CD39 staining in **A** a pre-reperfusion biopsy and **B** a post-reperfusion biopsy of a LD kidney. **C** Before transplantation, CD39 tissue expression was significantly higher in LD kidneys as compared to BDD ($p=0.004$) or CDD kidneys ($p<0.0001$). **D** Upon reperfusion, there was no change in CD39 protein expression in living donor kidneys ($p=0.16$). The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. mRNA expression of CD39 was assessed in kidney biopsies. **E** CD39 mRNA expression was similar before reperfusion in all groups ($p=0.27$). Similar to protein expression, CD39 mRNA expression did not change upon reperfusion in LD, BDD and CDD kidneys ($p=0.87$, $p=0.57$, $p= 0.20$, respectively).

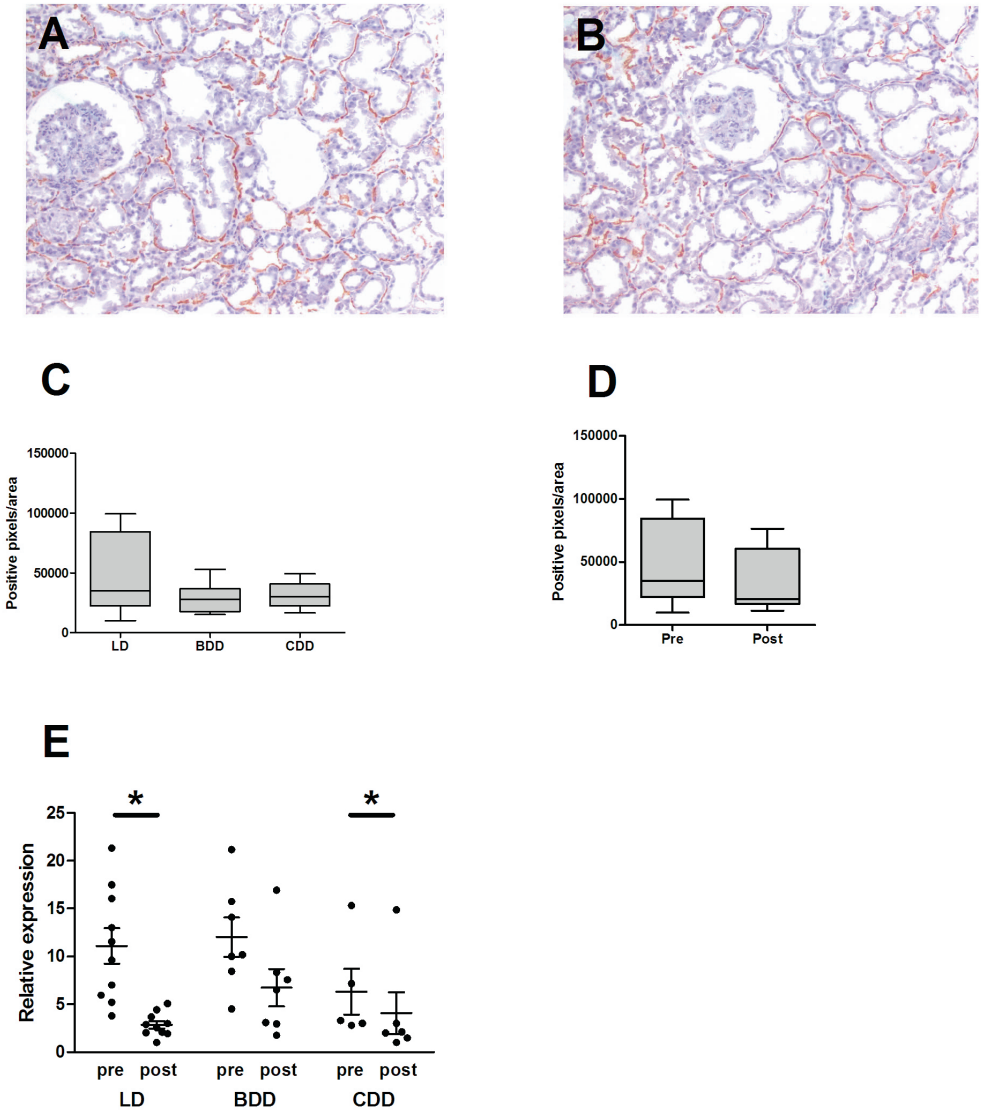

Figure 3: CD73 mRNA levels decrease after reperfusion.
CD73 staining was positive in endothelial and tubular epithelial cells. Typical example of CD73 staining in **A** a pre-reperfusion biopsy and **B** a post-reperfusion biopsy of a LD kidney. **C** Before transplantation, CD73 tissue expression was similar in kidneys of all three donor types (p=0.52). **D** 45 minutes after reperfusion, CD73 protein expression had not changed in living donor kidneys (p=0.20). The boxes run from the lower to upper quartile, the whiskers indicate the 5 to 95 percentile, while the horizontal line is set at the median. mRNA expression of CD39 was assessed in kidney biopsies. **E** CD73 mRNA expression was similar before reperfusion in kidneys of all three donor groups (p=0.13). While CD73 mRNA expression was massively down regulated after reperfusion (p=0.002) in LD kidneys, BDD kidneys showed no significant changes in expression although mean CD73 showed a trend towards decrease after reperfusion. In CDD kidneys the CD73 decrease did reach statistical significance. (P=0.043)

Discussion

I/R injury is a compulsory component of kidney transplantation, and its pathophysiology is still unclear. Although animal experiments have suggested protection from renal I/R injury by extracellular adenosine, data on adenosine generation in human renal I/R have been missing. In this study we show changes in adenosine generation by the enzymes CD39 and CD73 in transplanted human kidneys.

CD39 mediates the first step in the extracellular hydrolysis of ATP and ADP towards adenosine. Animal experiments show critical involvement of CD39 in renal I/R injury.[10;13] In human renal I/R injury, we found no changes in CD39 expression after reperfusion in kidney transplantation. However, before transplantation CD39 tissue expression was significantly higher in living compared to deceased donor kidneys. This may suggest a protective effect of CD39 in living donor kidneys, and a potential beneficial effect of CD39 over expression in humans as well.

CD73 is the enzyme that generates adenosine from AMP in the extracellular space. In contrast to CD39, results on the exact role of CD73 in renal I/R injury are conflicting. Although its essential role in ischemic preconditioning has been shown,[14] others report on a protective effect of CD73 deletion mediated by AMP accumulation or by nonenzymatic functions of CD73.[15-17] Here, we show a vast decrease in CD73 mRNA expression after reperfusion in both living and deceased donor kidneys. Since these are the first human data describing expression of these enzymes in renal I/R injury, we can only relate our results to previous experimental studies. These quite unambiguously show the in vitro upregulation of CD73 after hypoxia [6] and an increase in CD73 activity after ischemic preconditioning in mice.[14]

Although in this study CD39 and CD73 are central, when extrapolating results, it may be suggested that extracellular adenosine are involved in human renal I/R injury. In the transplantation setting, it is of interest that adenosine has been added to UW preservation solution. Although this adenosine appears critical in the beneficial effects of UW solution,[18] the effect of enhanced adenosine generation is probably many times larger, since the in vivo effect of adenosine lasts only minutes.[19]

In conclusion, we show that expression of adenosine generating enzymes CD39 and CD73 is different between living and deceased donor kidneys and changes after reperfusion. Further studies are needed to assess the consequences of these enzyme changes for renal adenosine generation and kidney graft injury.

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