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CMV increases tubular apoptosis through the TNF-α–TNF-R1 pathway in a rat model of chronic renal allograft rejection

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Abstract

Introduction: Destruction of transplanted kidneys through chronic allograft nephropathy [CAN], also known as chronic rejection, is the greatest obstacle in successful kidney transplantation. Causes behind CAN are many, from pre-transplant causes to infections. Viral infections, especially CMV, are a risk factor for chronic rejection. We have previously developed a rat kidney transplant model, in which CMV enhances the development of chronic rejection under triple drug treatment. In this model we have now further studied the routes of apoptosis in virus induced early CAN vs. the routes of apoptosis in a later developing non-infectious CAN.

Materials and methods: Renal transplantations were performed in a strain combination of DA/BN under immunosuppression. One group of animals was infected with RCMV and the other was left uninfected. The grafts were harvested on days 3–40 after transplantation. Apoptotic cells were visualised by in situ terminal transferase mediated dUTP nick end labelling [TUNEL] from paraffin embedded, formalin fixed kidney grafts. Cytokines were labelled imunohistochemically from frozen sections, among them tumour necrosis factor alpha [TNF-α] and its receptor-protein 1 [TNF-R1] as well as CD 95 [FAS], caspase 3 and CD14. The results were semi-quantitatively scored from 0 to 3+ over various tissues structures separately.

Results: In the CMV infected grafts, we could demonstrate a more intense TUNEL reaction in tubular epithelium [2.0 ± 1.0 vs. 0.8 ± 0.5 at day 14, $P<0.05$] as well as an earlier increase in the expression TNF-α in the vascular endothelium [$2.0+1.0$ vs. $0.0+0.0$ at days 3–5, $P<0.05$] than in the non-infected group. There was also an earlier increase in the tubular TNF-R1 expression [$2.2+0.8$ vs. $1.0+0.0$ at days 5–7, $P<0.05$]. There was no difference in the expression of CD14, caspase 3 or FAS between the groups.

Conclusions: CMV enhanced development of CAN was associated with tubular apoptosis and concomitant increase of TNF-α-TNF-R1, rather than the FAS–FAS-ligand activation.
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Keywords: Rat kidney transplantation; Chronic rejection; CMV; TNF-α; TNF-R; Apoptosis

1. Introduction

The slow decline in transplanted kidney function due to the alloresponse was formerly called Chronic Allograft Rejection and is now referred to as Chronic Allograft Nephropathy [CAN] because different causes lead to this same end result, and the causes can not be defined on histology alone. Finding means for preventing chronic allograft failure [CAF], which is the effect of CAN is the greatest challenge for renal transplantation programs today. The factors leading to CAN are many and they are not all fully understood [1,2].

Allograft rejection is a T-cell mediated process with the major findings of interstitial lymphocyte predominant inflammation with tubulitis in histology [3]. Apoptosis of the tubular epithelium is playing an important part in the process of acute rejection episodes, that ultimately, when persistent or recurring, is one of the causes of CAN [4–6]. Vascular rejection is another process, that may or may not be present in CAN [3]. Endothelial cell dysfunction, leading to medial dysfunction, neointima formation and apoptosis of cells in the vessel wall goes through the activation of caspase 3 [7]. In chronic rejection of human cardiac allografts apoptotic cells could be detected within the

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arterial wall and in perivascular areas. Apoptotic cells included T cells, macrophages/monocytes and vascular endothelial cells [8].

In renal transplants, in addition to lymphocytic and endothelial apoptosis, also increased tubular epithelial apoptosis has been shown in the grafts undergoing acute and chronic rejection [3,7]. In acute rejection, cytotoxic CD8+ lymphocytes have been shown in close proximity to tubular epithelial cells in areas of damaged tubuli [8]. These lymphocytes contain perforins, that cause cell necrosis, but they can also induce apoptosis in the target cells, such as epithelial cells, at least through the FAS-pathway [2,9,10].

We have previously developed a rat model for CAN [11]. In this model we have showed that RCMV infection enhances the development of CAN. Non-infected grafts all developed, after an early inflammatory episode, chronic rejection within 40 to 60 days after transplantation whereas RCMV infected grafts ended-up with CAN in 20 days [12]. Especially, CMV infection increased vascular changes and interstitial fibrosis, and enhanced tubular atrophy.

The aim of the present study was to determine the routes of apoptosis in our model of CMV augmented CAN development. This is a further study performed on the same renal transplant material of the experiments on this model, described previously [11,13,14]. We have now visualised the intragraft apoptosis by the TUNEL reaction and correlated it with the expression of TNF-α, TNF-R1, FAS/FASL, CD 14 caspase 3, TP 1 and TRT in the graft in CMV infected and non-infected rat kidney allografts that developed CAN under triple drug treatment.

2. Materials and methods

2.1. Transplantation

This is a further study on the rat renal transplant material described previously [11,13,14]. Male inbreed DA [RT1^{a}] rats that weighed between 200–300 g were used as kidney donors, and BN [RT1^{n}] male rats as recipients. The grafted animals received triple drug treatment: Methylprednisolone [2 mg/kg/day], Azathioprine [2 mg/kg/day], and Cyclosporine [5 mg/kg/day] subcutaneously. 4–6 animals were sacrificed at each time point in each group. Autotransplantations were performed on 20 animals, with three to four animals per each time point, to which triple drug immunosuppression was also given and the autografts were used as control material for the time-related follow-up harvested at the same time points. Non-transplanted normal rat kidneys served as the day 0 control samples. The animals were treated according to the international principles of laboratory animal care. The study was approved by the committee for experimental research of Helsinki University Central Hospital and by the regional authorities of Uusimaa in Finland.

2.2. RCMV infection

Half of the animals were injected with 10^5 plaque forming units of rat cytomegalovirus [RCMV, Maastricht strain] intra abdominally on the first postoperative day. Established infection was confirmed by viral culture in fibroblasts. The virus was demonstrated in the culture after the appearance of cytopathic effects by indirect immunofluorecense, using a mixture of mouse monoclonal antibodies against RCMV early and late antigens [12]. Direct demonstration of RCMV was performed in parallel from frozen sections of the grafts, by immunofluorecense staining, using the same monoclonal antibodies against RCMV antigens. The other half of the animals was left uninfected [12].

2.3. Histology

Kidneys were harvested at regular intervals after transplantation [days 3, 5, 7, 14, 20, 30, 40]. The central sagittal sections of the kidneys were fixed in phosphate buffered formalin for normal histology and in situ hybridisation's, whereas the ends of the kidneys were snap frozen for cytokine immunohistochemistry. Normal histology was analysed for CAN from H&E and trichrome stained preparations, the chronic allograft damage index [CADI] was used [15] to confirm the development of CAN. The CADI is formed of six histological changes characteristic of CAN: interstitial inflammation and fibrosis, glomerular sclerosis and matrix increase in the mesangium, vascular intimal thickening ant tubular atrophy.

2.4. Apoptosis

Apoptotic cells were visualised with the in situ terminal transferase mediated dUTP nick end labelling method [TUNEL, Boehringer, Mannheim, Germany]. Paraffin sections from formalin fixed kidney transplant material were used. Procedures were done exactly as described by the TUNEL-kit provider. Shortly; 4 μm sections were deparafined in xylene, hydrated in graded alcohols, immersed in – proteinase K for digestion of membranes,−0,3% H2O2 for blocking of endogenous peroxidase, −0.1%Triton® for permeabilisation of membranes, – TUNEL reaction mixture and finally in Converter-POD and Diamino-benzidine. Counter staining was done with diluted Mayer's hemalaum. TUNEL-positive reaction in the kidneys was semi-quantitatively scored from 0–3 according to the amount of positively stained nuclei over different structures of the organ.

2.5. Immunohistochemistry

Kidneys were snap frozen in liquid nitrogen, stored at −70 °C and later sectioned in a cryostat. Frozen sections were fixed in cold acetone, immunostained by specific monoclonal or polyclonal antibodies. An indirect immunoperoxidase technique was used. A peroxidase conjugated rabbit anti-mouse [DACO, Glostrup, Denmark] and a peroxidase conjugated goat anti-rabbit [ZYMED, San Diego, CA, USA] were used as second and third antibody respectively. The reaction was revealed by 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Normal mouse IgG antibody was used as a negative control for the mouse monoclonal antibodies. The native kidney of the recipient served as an internal negative control in each animal. Diluted Mayer's hemalaum was used for counter staining. All positive reactions were semi-quantitatively scored from 1 to 3 in all structures separately.

The route for apoptotic signalling was sought for with immunostainings using the antibodies against FAS [A-20, sc-1023, an affinity purified rabbit polyclonal antibody raised against mouse FAS protein cross reacting with the rat protein, Santa Cruz Biotechnology, Santa Cruz, CA, USA], TNF-α [AB-210-NA, a goat anti-porcine polyclonal antibody that cross reacts with the rat protein, R&D Systems, Abingdon, UK], TNF-R1 [H-5, sc-8436, a mouse monoclonal IgG2b antibody raised against a human protein, still cross-reacting with rat epitopes, Santa Cruz Biotechnology], activated caspase-3 [D175, a rabbit polyclonal anti-human protein. Cell Signalling, Beverly, MA, USA], and CD14 [M-305, sc-9105; a rabbit polyclonal antibody raised against a mouse protein, still cross reacting with the rat protein. Santa Cruz Biotechnology]. Loss of telomerase activity was assessed with immunostainings on frozen sections as described above, with rabbit polyclonal antibodies against telomerase associated protein [TP1, E-20, sc-6373, an affinity purified polyclonal goat anti-mouse antibody. Santa Cruz Biotechnology] and telomerase reverse transcriptase [TRT, sc-7212, a polyclonal rabbit anti-human antibody, cross-reacting with the corresponding rat protein. Santa Cruz Biotechnology].

2.6. Statistical analysis

The data were expressed as mean ± SD, and for comparison of the results between infected and non-infected recipient groups Student's t test was used. *P*-values < 05 were considered significant.

3. Results

3.1. TUNEL-expression

Strong tubular TUNEL reaction was recorded over arterial medial cells and over tubular epithelium, especially in the inflamed areas in both CMV infected and non-infected groups. In the CMV infected

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