Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Cytomegalovirus enhance expression of growth factors during the development of chronic allograft nephropathy in rats

Kaija Inkinen,[1,2] Anu Soots,[2] Leena Krogerus,[3] Raisa Loginov,[1,2] Cathrien Bruggeman[4] and Irmeli Lautenschlager[1,2]

1 Department of Virology, Helsinki University Central Hospital and Helsinki University, Helsinki, Finland
2 Department of Surgery, Helsinki University Central Hospital and Helsinki University, Helsinki, Finland
3 Department of Pathology, Helsinki University Central Hospital and Helsinki University, Helsinki, Finland
4 Department of Medical Microbiology, University of Maastricht, The Netherlands

Keywords
chronic rejection, cytomegalovirus, growth factors, kidney.

Correspondence
K. Inkinen PhD, Transplant Unit Research Laboratory, Helsinki University Central Hospital, Meilahti, P-Floor, PO Box 340, FIN-00029 HUS, Helsinki, Finland.
Tel.: 358 9 471 71754; fax: 358 9 471 75866; e-mail: kaija.inkinen@hus.fi

Received: 15 July 2004
Revised: 7 January 2005
Accepted: 26 January 2005

doi:10.1111/j.1432-2277.2005.00113.x

Summary

Cytomegalovirus (CMV) accelerates chronic rejection (CRX) in a model of rat kidney allograft. In this model, the expressions of transforming growth factor beta 1 (TGF-β), platelet-derived growth factor (PDGF)-AA, PDGF-BB and connective tissue growth factor (CTGF) were investigated with and without CMV. Transplantations were performed under immunosuppression. One group of animals was infected with CMV and the other was left uninfected. The grafts were harvested on days 3–60 after transplantation. Growth factor proteins were demonstrated by immunohistochemistry, and mRNAs by *in situ* hybridization. A significantly more intense and earlier endothelial TGF-β (2.4 ± 0.8 vs. 1.0 ± 0.0; $P < 0.05$) and PDGF-AA (1.8 ± 0.4 vs. 1.0 ± 0.0; $P < 0.05$) expressions, confirmed by mRNA hybridization, occurred in the CMV group compared with the noninfected group. PDGF-BB appeared in a few inflammatory cells only. In addition CTGF appeared earlier and has more intense in the CMV group (2.5 ± 0.6 vs. 1.2 ± 0.5) and the number of CTGF mRNA-positive fibroblasts (57 ± 9 vs. 3 ± 4; $P < 0.05$) was significantly higher. Thus, CMV enhanced expression of TGF-β1, PDGF-AA and CTGF during the development of CRX.

Introduction

Cytomegalovirus (CMV) infection is thought to be one of the risk factors for chronic rejection (CRX). Clinical and experimental evidence indicates the accelerating role of CMV infection in the development of CRX in heart, lung and liver [1]. In renal transplantation, however, little is known about CMV and CRX or chronic allograft nephropathy (CAN), and clinical reports are controversial [2]. We have previously developed an experimental model in which rat renal allografts, after an early inflammatory episode at 5–10 days postoperatively, develop CRX under triple drug immunosuppression within 40–60 days [3]. In this experimental model, we have demonstrated that CMV prolongs and increases graft inflammation, accelerates and enhances the development of CRX and ends up with prominent interstitial fibrosis within 20 days [4].

The histologic findings of CRX (or CAN) are well defined and include atherosclerosis, interstitial fibrosis, glomerulosclerosis, multilayering of the peritubular capillaries and tubular atrophy [5]. Growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor beta 1 (TGF-β) are known to play an important role in smooth muscle cell (SMC) proliferation and in the induction of matrix protein synthesis. PDGF is a dimeric molecule composed of A or B-chains, assembled in different combinations,

creating PDGF-AA, PDGF-AB or PDGF-BB. PDGF-A is expressed by both intimal and medial SMC in arteries with chronic vascular rejection, whereas PDGF-B is mainly located in infiltrating monocytes [6]. Upregulated expression of PDGF-A by endothelial cells has been demonstrated in arteries during acute and CRX [6,7]. TGF-β is a multifunctional polypeptide growth factor which plays a major role in the regulation of extracellular matrix formation, fibrosis, arteriosclerosis, angiogenesis and inflammation [8]. Involvement of TGF-β with CRX has been observed [9]. Connective tissue growth factor (CTGF) is one of the downstream mediators of TGF-β, modulating fibroblast cell growth and extracellular matrix production. CTGF gene expression is induced strongly by TGF-β1 but not by other growth factors [10]. CTGF is important in renal fibrosis in various renal diseases and renal chronic transplant rejection [11].

In our previous study, we demonstrated that CMV infection enhanced chronic renal allograft rejection in a rat model [4]. In the present study, the effect of CMV infection on the development of vascular intimal thickening and fibrosis was further investigated at the molecular level from the same material. The expression of TGF-β1, PDGF-A and PDGF-B and CTGF at the protein and gene levels (except for PDGF-B) at various time points were examined in the presence of rat CMV (RCMV).

Materials and methods

Rats

Inbred DA ($RT1^a$) and BN ($RT1^n$) male rats of 200–300 g were used. The animals were fed with regular rat food and tap water *ad libitum*. The animals were treated according to the international principles of laboratory animal care. The study was approved by the Committee for Experimental Research of the Helsinki University Central Hospital and the regional authorities.

Renal transplantations

Transplantations were performed in a rat strain combination of DA → BN, as described previously [3]. The animals were anesthetized with midazolam (Dormicum®; Roche, Basel, Switzerland) and fentanyl-fluanisone (Hypnorm®; Janssen, Buckinghamshire, UK). The grafts were flushed and stored in heparinized Euro-Collins' solution on ice. The total ischemic time was 30 ± 10 min. The immunosuppression consisted of triple drug therapy of methylprednisolone (2 mg/kg), azathioprine (2 mg/kg) and cyclosporin A (5 mg/kg) daily, administered subcutaneously. One group of animals was infected with RCMV and the other was left uninfected (see below).

RCMV infection

One group of allograft recipients which also received triple drug treatment, were infected with the RCMV Maastricht strain by inoculation with 10^5 plaque-forming units of RCMV intraperitoneally, 1 day after renal transplantation. The presence of RCMV infection in the graft was confirmed 6–7 days after inoculation by viral culture and direct antigen detection as described for the same material in our previous publication [4]. The CMV-infected and noninfected rats were killed and the grafts were harvested at different times after transplantation: 3–5, 6–7, 10–14, 20, 30, 40, 50 and 60 days postoperation. Nontransplanted normal rat kidneys served as the day 0 control samples. The rat kidney transplant material was the same as in our previous publications [4,12,13].

The total number of grafts was 59; 30 rats in the CMV-infected group and 29 rats in the noninfected group. There were five rats in both groups harvested 3–4, 6–7 and 10–14 days after transplantation. On day 20, there were three rats in the CMV group and four rats in the noninfected group; on day 30, six and five rats; on day 40, five and six rats, respectively.

Histology

Histologic examination of the graft was performed on the explants. The specimens were fixed in normal buffered formalin and stained with hematoxylin–eosin and Masson's trichrome. Graft histology was evaluated according to the Banff criteria [14]. The numerical chronic allograft damage index (CADI) was used to quantify the chronic alterations in the graft [15]. The CADI was formed of the six histopathological changes, characteristic of CRX, as described previously [15]: interstitial inflammation, fibrosis, glomerular sclerosis, mesangial matrix increase, vascular intimal thickening and tubular atrophy. The examination of the histologic specimens was performed blindly by two observers.

Immunohistochemistry

The TGF-β, PDGF-AA and CTGF proteins were demonstrated in frozen sections (3–5 μm) of the explanted grafts by immunohistochemistry. Indirect immunoperoxidase staining, using a polyclonal pan-specific antibody against TGF-β (R&D Systems, Minneapolis, MN, USA), polyclonal anti-PDGF-AA and PDGF-BB (Genzyme Diagnostics, Cambridge, MA, USA) and a polyclonal antibody against CTGF (ABcam Limited, Cambridge, UK) was used. Before staining, the sections were treated with chloroform to eliminate nonspecific reactions as a result of endogenous

peroxidase. A peroxidase-conjugated goat anti rabbit antibody (Zymed, San Francisco, CA, USA) was used as a secondary antibody. The reaction was revealed using 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Mayer's hemalum was used as a counterstain. The intensity of the expression of each growth factor was scored from 0 to 3.

In situ hybridization

The TGF-β1, PDGF-A and CTGF mRNA expressions were studied by *in situ* hybridization on paraffin-embedded grafts. The probe for TGF-β1 was from a bluescript plasmid (Stratagene, La Jolla, CA, USA) containing a 513-bp fragment (1260–1773 bp) of the human TGF-β1, for PDGF-A from a bluescript plasmid containing a 305-bp fragment (774–1078 bp) of the rat PDGF-A and for CTGF a pRc/CMV plasmid containing approximately, 1.0 kb fragment of human CTGF (kindly provided by Dr G. Grotendorst, University of Miami School of Medicine, FL, USA). *In situ* hybridization was performed mainly as described previously [13]. Paraffin sections (3–5 μm) of the kidney transplants were deparaffinized and hydrated through descending ethanol concentrations. Sections were hybridized at 60 °C with RNA-probe. Digoxigenin-labeled probes were detected following the methods from the DIG-detection kit (Boehringer Mannheim, Mannheim, Germany). After color substrate incubation, the slides were counterstained with hematoxylin. Staining was considered positive when seen with the antisense probe only.

Statistics

The data were expressed as mean ± SEM and Mann–Whitney *U*-test was used to compare the results between the groups. *P*-values of <0.05 were considered significant.

Results

Histologic findings

The CMV-infected allografts ended up with CRX, with characteristic vascular changes and prominent interstitial fibrosis within 20 days after transplantation. In the non-infected grafts, CRX was seen only after 40 days. The Histologic findings have been described in detail in our previous publication [4]. The Histologic changes were examined blindly and the results were quantified and expressed as CADI values. The CADI value in CMV-infected allografts was significantly higher on day 6–7 (5.8 ± 0.3 vs. 2.9 ± 0.7; $P < 0.01$) when compared with the noninfected allografts, because of the significantly more intense inflammation. In CMV-infected grafts, the CADI value (9.0 ± 0.3 vs. 5.9 ± 0.9; $P < 0.05$) reached its maximum value, together with maximal histologic signs of CRX and prominent interstitial fibrosis, on day 20. In

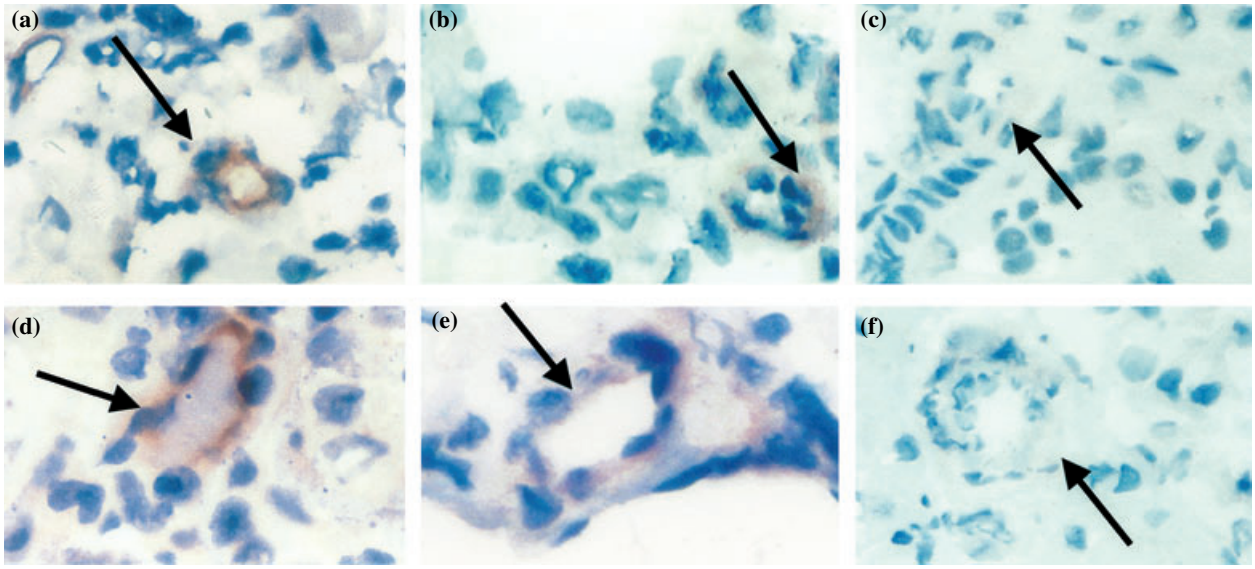

Figure 1 A clear transforming growth factor beta 1 (TGF-β) expression of the endothelial cells (arrows) in the cytomegalovirus (CMV)-infected (a) and a faint expression in the noninfected renal allograft (b) and corresponding expressions of platelet-derived growth factor (PDGF)-AA (d and e) on day 7 after transplantation demonstrated by immunohistochemistry. The normal control kidneys showed no TGF-β or PDGF AA (c and f) (original magnification 400×).

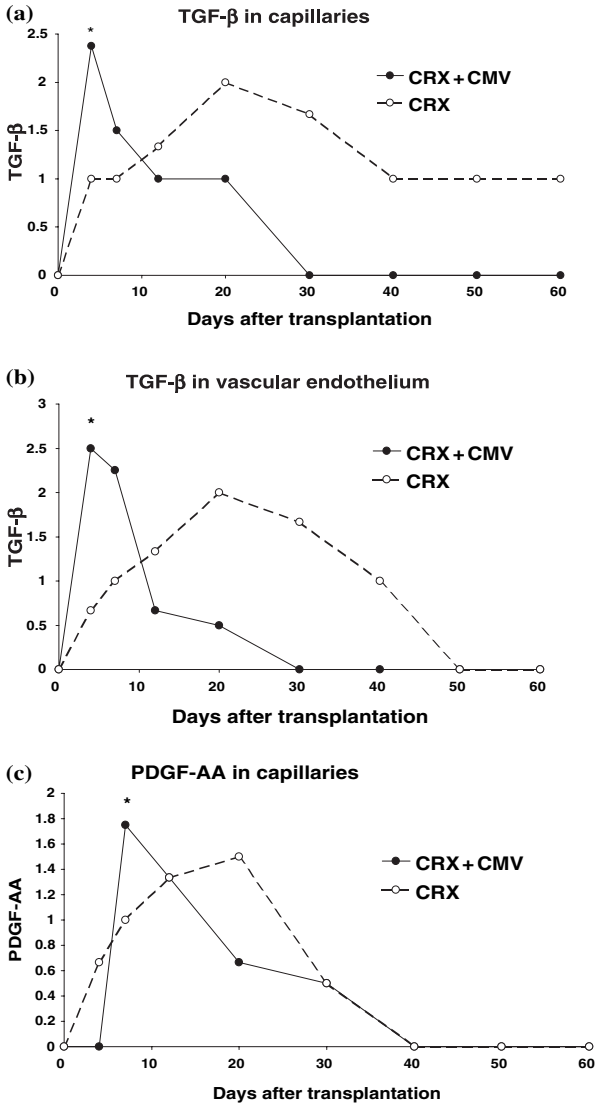

Figure 2 The expression of transforming growth factor beta 1 (TGF-β) in capillaries (a) and in vascular endothelium (b) and the expression of platelet-derived growth factor (PDGF)-AA in capillaries (c), demonstrated by immunohistochemistry. *$P < 0.05$.

the noninfected allografts, the maximal CADI value was reached on day 40, together with the histologic criteria of CRX.

Expression of TGF-β and PDGF

In immunohistochemistry, the expression of TGF-β was seen both in the capillary and vascular endothelium of the grafts. In CMV-infected rats, the maximum expression of TGF-β was reached at day 3–5 and it was significantly more intense compared with the noninfected group in the capillaries (2.4 ± 0.4 vs. 1.0 ± 0.0; $P < 0.05$) and in the vascular endothelium (2.5 ± 0.5 vs. 0.7 ± 0.3) (Figs 1 and 2). The noninfected grafts reached a lower peak expression of TGF-β at day 20 after transplantation (2.0 ± 0.0 for capillaries and 2.0 ± 0.0 for vascular endothelium). Moreover, the expression of PDGF-AA in the capillaries of CMV grafts (Figs 1 and 2) peaked earlier, at day 6 and 7, and was more intense than in the noninfected grafts (1.8 ± 0.3 vs. 1.0 ± 0.0). The peak capillary expression of PDGF-AA in the noninfected graft was seen on day 20 (1.5 ± 0.5) (Fig. 2). The expression of PDGF-AA in the vascular endothelium was very faint in both groups and there were no significant differences between the CMV-infected and the noninfected grafts (data not shown). The expression of PDGF-BB was seen only occasionally in interstitial inflammatory cells in both groups and there were no significant differences between the CMV-infected and noninfected grafts (not shown). For this reason the gene expression was not analyzed. The capillaries and vascular endothelium in the normal kidneys did not express TGF-β1 and PDGF-AA (Figs 1 and 2).

The expression of TGF-β1 and PDGF-A mRNA in the capillary and in the vascular endothelium was demonstrated by *in situ* hybridization. In CMV-infected grafts, TGF-β1 mRNA expression was located in the capillary and vascular endothelium, and was observed for a longer time period than in the noninfected grafts. In CMV-infected grafts, PDGF-A mRNA expression was located both in the capillaries and in the vascular endothelium during the first week after transplantation. The expression of TGF-β1 and PDGF-A mRNA in the normal kidneys was almost nonexistent. The expression of TGF-β1 and PDGF-A mRNA expressions are shown in Fig. 3.

Expression of CTGF

The CTGF protein was located in interstitial fibroblasts. The expression of CTGF peaked earlier (at days 10–14) and was significantly more intense in the RCMV-infected grafts (2.5 ± 0.6 vs. 1.2 ± 0.5; $P < 0.05$) than in the noninfected grafts (Figs 4a and 5). Normal kidneys demonstrated only very faint CTGF expression in the interstitial fibroblasts (Figs 4a and 5). The CTGF mRNA-positive fibroblasts were located in the area of the juxtamedullary cortex (Fig. 5). CTGF mRNA expression peaked earlier in the CMV-infected than in the noninfected grafts. The number of CTGF mRNA-positive interstitial cells was significantly higher (57 ± 9 vs. 3 ± 4; $P < 0.05$) in the infected animals than in the uninfected animals, on day 10–14 post-transplantation (Fig. 4b). In the normal kidney only few positive interstitial fibroblasts were recorded (Figs 4b and 5).

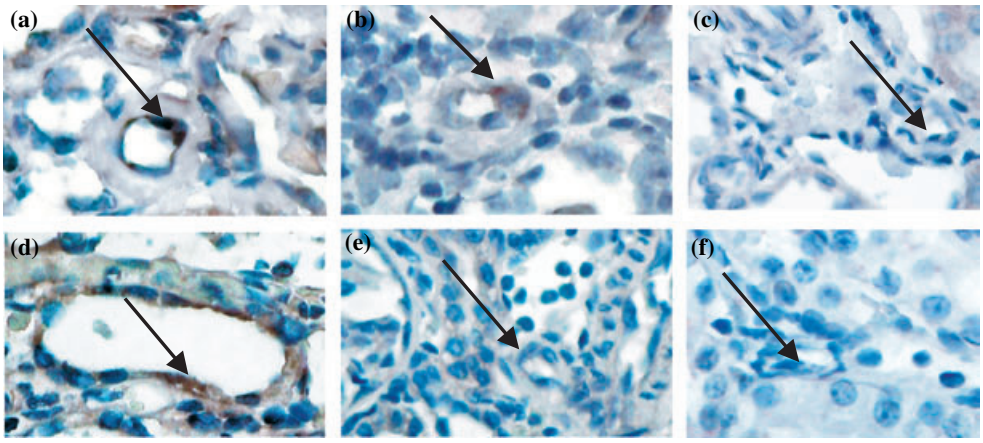

Figure 3 A strong positive signal for transforming growth factor beta 1 (TGF-β1) (a) and platelet-derived growth factor (PDGF)-A (d) mRNAs localized in the endothelial cells (arrows) of the cytomegalovirus (CMV)-infected renal allografs demonstrated by *in situ* hybridization. The minor signals seen in the non-CMV-infected grafts (b and e) and in the totally negative control kidneys (c and f) (original magnification 400×).

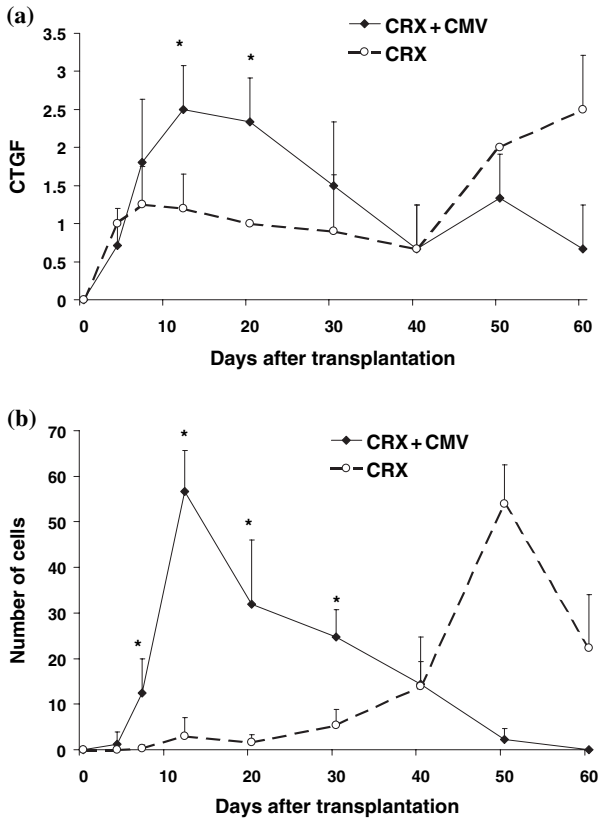

Figure 4 The intensity of connective tissue growth factor (CTGF) expression (a), demonstrated by immunohistochemistry and number of CTGF mRNA-positive cells (b) demonstrated by *in situ* hybridization. $*P < 0.05$.

Discussion

We previously used the RCMV Maastricht strain to study the effect of viral infection on the development of chronic renal allograft rejection in the rat [4]. In this experimental model of renal transplant nephropathy, we have now demonstrated that CMV infection enhanced and accelerated the expression of endothelial TGF-β and PDGF-AA and upregulated the corresponding gene expressions. In CMV-infected grafts, the expression of TGF-β peaked earlier and it was significantly more intense at the protein level compared with the noninfected group. In the CMV-infected group the expression of PDGF-A in the capillaries peaked earlier and was more intense than in the noninfected grafts.

TGF-β and PDGF-AA are known stimuli for SMC migration and proliferation in experimental vascular injury and may, in an autocrine and/or paracrine manner, promote further intimal expansion and lesion progression in vasculopathy [6,16]. In a previous study on a rat aorta allograft model of CRX, RCMV infection enhanced TGF-β expression and PDGF-BB in the vascular wall [16]. In our study RCMV infection seems to have no effect on the expression of PDGF-BB. Other studies suggest that PDGF-AA plays a role in the development of intimal lesions mediated by macrophage-derived cytokines in cardiac allograft arteriosclerosis [16]. The importance of arterial PDGF-AA in the development of chronic renal allograft rejection has also been demonstrated in another study using a rat transplant model [7].

Interstitial fibrosis is an important parameter in the chronic renal allograft. Our present data show a strong increase of CTGF expression at the interstitial sites in the CMV-infected grafts. CMV enhanced the appearance of CTGF mRNA-expressing fibroblasts in the graft. Our previous observations on the effect of CMV in the development of interstitial fibrosis [4] can now be linked with increased and enhanced induction of CTGF. CTGF induces fibroblast proliferation and extracellular matrix synthesis

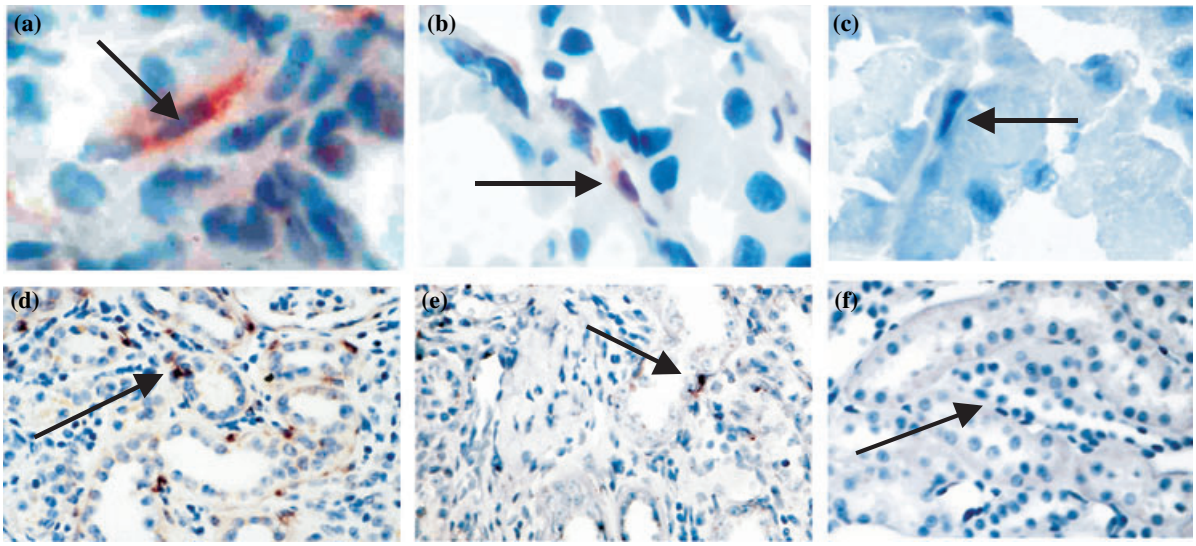

Figure 5 A strong positive expression of connective tissue growth factor (CTGF) in the interstitial fibroblasts (arrow) of the cytomegalovirus (CMV)-infected renal allografts (a), a weak expression in the non-CMV-infected renal allografts (b) and no expression in the control kidney (c) demonstrated by immunohistochemistry (original magnification 1000×). A strong positive signal for CTGF mRNA in numerous interstitial fibroblasts of CMV-infected allografts (d), a weaker positive signal for CTGF mRNA in a few interstitial fibroblasts of non-CMV graft (e) and no expression in the control kidney (f) demonstrated by *in situ* hybridization (original magnification 400×).

[10] and in our previous study, collagen synthesis was increased in CMV-infected grafts [13].

Various mechanisms could be suggested by which CMV infection enhances the expression of growth factors in chronic kidney allograft rejection. We have previously shown in the same transplant model that CMV prolongs and increases inflammation and increases the number of macrophages in the graft [4]. CMV enhances T-cell activation and the expression of adhesion molecules of the vascular endothelium [12]. Increased inflammatory response induced by CMV may stimulate the expression of growth factors indirectly, by proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α). CMV directly induces TNF-α [17] and upregulates IL-1β gene expression, which may lead to the production of IL-1 by mononuclear cells [18]. These cytokines stimulate the synthesis of PDGF-A and TGF-β [19,20]. However, the central role of growth factors, especially TGF-β, in the pathogenesis of CMV-related changes, can also be explained by the fact that CMV infection induces the transcription and secretion of TGF-β1 [21]. Further effects of TGF-β on fibroblasts are mediated by CTGF [10]. In normal TGF-β-induced repair response, CTGF expression is dependent of TGF-β [22]. However, both in CRX and in CMV-enhanced CRX, TGF-β expression was located in vascular structures and not in fibroblasts, although a few interstitial TGF-β-positive single cells were occasionally seen. In this model of CAN, the intense CTGF expression in fibroblasts during the development of fibrosis was possibly mediated by TGF-β, which was not produced by fibroblasts and was due to a paracrine effect [10].

These inflammatory phenomena, in response to injury induced by CMV infection, may lead to endothelial cell and SMC damage, and to the exposure of SMC to cytokines and peptide growth factors, which can lead to the enhanced intimal thickening observed in CMV-infected allografts. Growth factors secreted by endothelia and SMC induce the migration and proliferation of SMC during vasculopathy. The early expression of both TGF-β and PDGF-A in the vascular endothelium of CMV-infected grafts explains the early transplant vasculopathy. It is thought that growth factors may, via paracrine and autocrine activity involve the development of vessel neointima, besides having the potential to stimulate the neighboring SMC of the arterial vessel media to replicate or migrate and hence promote further neointimal expansion. This phenomenon is thus enhanced by CMV. In addition the early induction of interstitial fibrosis in our model can be explained by the CMV-induced increase of the inflammatory reaction in the graft. Inflammatory response increases many cytokines and prolonged production of these cytokines can lead to excessive matrix accumulation and chronic fibrosis.

In conclusion, CMV enhanced and accelerated the expression of endothelial TGF-β1 and PDGF-AA proteins in renal allografts ending up with CRX. Our results suggest that the CMV-associated enhancement of CAN is

mediated by TGF-β1 and the PDGF A-chain. CMV also enhances fibroblast CTGF expression in the graft during the development of interstitial fibrosis.

Acknowledgements

The authors thank Saija Kotola, Tarja Markov, Raisa Loginov and Stephen Venn for technical assistance and Kari Savelius for animal care.

This work was supported by grants from the Helsinki University Central Hospital Research Funds (EVO).

References

1. Lautenschlager I. Role of cytomegalovirus infection in the processes of organ allograft rejection. In: Scholz M, Rabenau H, Doerr H, Cinatl JJ, eds. *CMV-related Immunopathology*, Vol. 21. Basel: Karger, 1998: 142–157.
2. Dickenmann MJ, Cathomas G, Steiger J, Mihatsch MJ, Thiel G, Tamm M. Cytomegalovirus infection and graft rejection in renal transplantation. *Transplantation* 2001; **71**: 764.
3. Soots A, Lautenschlager I, Krogerus L, Saarinen O, Ahonen J. An experimental model of chronic renal allograft rejection in the rat using triple drug immunosuppression. *Transplantation* 1998; **65**: 42.
4. Lautenschlager I, Soots A, Krogerus L, *et al.* Effect of cytomegalovirus on an experimental model of chronic renal allograft rejection under triple-drug treatment in the rat. *Transplantation* 1997; **64**: 391.
5. Paul LC. Chronic allograft nephropathy: an update. *Kidney Int* 1999; **56**: 783.
6. Alpers CE, Davis CL, Barr D, Marsh CL, Hudkins KL. Identification of platelet-derived growth factor A and B chains in human renal vascular rejection. *Am J Pathol* 1996; **148**: 439.
7. Savikko J, Kallio EA, Taskinen E, von Willebrand E. The effect of acute rejection and cyclosporin A-treatment on induction of platelet-derived growth factor and its receptors during the development of chronic rat renal allograft rejection. *Transplantation* 2002; **73**: 506.
8. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994; **331**: 1286.
9. Paul LC, Saito K, Davidoff A, Benediktsson H. Growth factor transcripts in rat renal transplants. *Am J Kidney Dis* 1996; **28**: 441.
10. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev* 1997; **8**: 171.
11. Ito Y, Aten J, Bende RJ, *et al.* Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int* 1998; **53**: 853.
12. Kloover JS, Soots AP, Krogerus LA, *et al.* Rat cytomegalovirus infection in kidney allograft recipients is associated with increased expression of intracellular adhesion molecule-1 vascular adhesion molecule-1, and their ligands leukocyte function antigen-1 and very late antigen-4 in the graft. *Transplantation* 2000; **69**: 2641.
13. Inkinen K, Soots A, Krogerus L, Bruggeman C, Ahonen J, Lautenschlager I. Cytomegalovirus increases collagen synthesis in chronic rejection in the rat. *Nephrol Dial Transplant* 2002; **17**: 772.
14. Racusen LC, Solez K, Colvin RB, *et al.* The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; **55**: 713.
15. Isoniemi HM, Krogerus L, von Willebrand E, Taskinen E, Ahonen J, Häyry P. Histopathological findings in well-functioning, long-term renal allografts. *Kidney Int* 1992; **41**: 155.
16. Lemström KB, Aho PT, Bruggeman CA, Häyry PJ. Cytomegalovirus infection enhances mRNA expression of platelet-derived growth factor-BB and transforming growth factor-beta 1 in rat aortic allografts. Possible mechanism for cytomegalovirus-enhanced graft arteriosclerosis. *Arterioscler Thromb* 1994; **14**: 2043.
17. Haagmans BL, van den Eertwegh AJ, Claassen E, Horzinek MC, Schijns VE. Tumour necrosis factor-α production during cytomegalovirus infection in immunosuppressed rats. *J Gen Virol* 1994; **75**: 779.
18. Iwamoto GK, Monick MM, Clark BD, Auron PE, Stinski MF, Hunninghake GW. Modulation of interleukin 1 β gene expression by the immediate early genes of human cytomegalovirus. *J Clin Invest* 1990; **85**: 1853.
19. Phan SH, Gharaee-Kermani M, McGarry B, Kunkel SL, Wolber FW. Regulation of rat pulmonary artery endothelial cell transforming growth factor-β production by IL-1 β and tumor necrosis factor-α. *J Immunol* 1992; **149**: 103.
20. Funayama H, Ikeda U, Takahashi M, *et al.* Human monocyte-endothelial cell interaction induces platelet-derived growth factor expression. *Cardiovasc Res* 1998; **37**: 216.
21. Yoo YD, Chiou CJ, Choi KS, *et al.* The IE2 regulatory protein of human cytomegalovirus induces expression of the human transforming growth factor β1 gene through an Egr-1 binding site. *J Virol* 1996; **70**: 7062.
22. Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, Leask A. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J Biol Chem* 2001; **276**: 10594.