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ORIGINAL ARTICLE

Fibrosis and matrix metalloproteinases in rat renal allografts

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Summary

The temporal activity and gene expression of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinase (TIMP) were investigated in a rat model of chronic allograft nephropathy. Gelatinolytic activity of MMP-2 and -9 were demonstrated by zymography, and MMP-2,-9 and TIMP-3 mRNA by *in situ* hybridization. The generation of fibrosis was determined as total collagen content/DNA. Significantly more latent and active MMP-2, as well as latent MMP-9, were seen in allografts than in autografts. Intense MMP-2 mRNA expression was demonstrated in the allografts during the first 20 days after transplantation, located mainly in the interstitium of the kidney. In addition, some tubular cells expressed MMP-2 mRNA. After day 20, MMP-2 gene expression was faint. MMP-9 mRNA expression in allografts was located mainly in the glomerulus. TIMP-3 mRNA expression was downregulated in allografts. MMP-2, MMP-9 and TIMP-3 seem to play a critical role in the development of fibrosis in the renal allograft.

Introduction

In clinical renal transplantation, chronic rejection is still a major problem. Despite short-term success, many renal allografts are lost in the long term due to chronic allograft nephropathy (CAN) [1]. The histological hallmark of CAN is progressive fibrosis in which extracellular matrix (ECM) turnover plays an important role [1]. Previously we developed an experimental model in which rat renal allografts, after an early inflammatory episode at 5–10 days post-operation, developed chronic rejection under triple-drug immunosuppression within 40–60 days [2]. In this experimental model, we demonstrate that inflammation induces collagen synthesis and the development of interstitial fibrosis [3].

Renal fibrosis manifests as an accumulation of collagens in the kidney [4,5]. The collagens are of great physiological importance as a support for the renal parenchyma and as a component of the basement membrane [6]. ECM is a dynamic network of proteins and proteoglycans. Under physiological conditions, the synthesis of matrix is balanced by its degradation. The degradative side of the balance is under the control of the proteolytic matrix metalloproteinases (MMPs), which in turn are regulated by tissue inhibitors of matrix metalloproteinases (TIMPs).

Several experimental models of interstitial fibrosis have demonstrated changes in the production of MMPs [7–10]. Results from recent clinical studies of renal allografts suggest that MMPs and TIMPs could play a role in the development of glomerular sclerosis and interstitial fibrosis [11–13]. To date, over 20 different MMPs have been identified, which are classified into four groups, according to their protein sequence and substrate specificity: interstitial collagenases; gelatinases; stromelysins, matrilysin, and metalloelastase; and membrane-type MMPs. The gelatinases (MMP-2 and MMP-9) are thought to play a role in both basement membrane

turnover (via collagen IV and V breakdown) and in interstitial matrix turnover (via gelatinolytic activity) [14].

The expression of MMP is regulated by a wide variety of compounds including inflammatory cytokines such as IL-1 [15] and growth factors such as TGF-β [16]. A second level of control of MMP activity is achieved through the secretion of TIMPs, of which four have been described [17]. TIMP-1 and TIMP-2 are capable of inhibiting the activities of all known MMPs. TIMP-3, but not TIMP-1 or TIMP-2, is abundantly expressed in normal adult mouse and rat kidneys [18,19]. In the transplant kidney the alteration in the ratio between TIMPs and MMPs may be an important molecular mechanism leading to the development of fibrosis.

We investigated the changes in MMP-2, MMP-9 and TIMP-3, in relation to the accumulation of collagens and the progression of renal fibrosis in chronically rejecting rat renal allografts. The renal transplantations were performed on a strain combination which developed chronic rejection under immunosuppressive therapy of triple drug treatment. Graft histology was performed on the explants to compare the time-related appearance of various changes in the allografts.

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 received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' (NIH). 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 [2]. 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. Immunosuppression consisted of triple drug therapy of methylprednisolone (2 mg/kg), azathioprine (2 mg/kg) and cyclosporine (5 mg/kg) daily, subcutaneously. Rats were killed and the grafts were harvested at different times after transplantation: 4, 7, 10, 20, 30, 40, 50, and 60 days postoperation ($n = 3$–6 at each time point), without nephrectomy of the other kidney.

Autotransplantations, with the same cold and warm ischemia times, were performed on 24 animals, three animals per each time point, under the same triple drug immunosuppression. These autografts were used as control material for the time-related follow-up harvested at the same time points. Nontransplanted normal rat kidneys served as the day 0 control samples.

Histology

Histological examination of the graft was performed on the explants in parallel. 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 [20]. The numerical chronic allograft damage index (CADI) was used to quantify the chronic alterations in the graft [21]. The CADI was formed of the six histopathological changes characteristic of chronic rejection, as described previously [21]: interstitial inflammation, fibrosis, glomerular sclerosis, mesangial matrix increase, vascular intimal thickening, and tubular atrophy.

Determination of total tissue collagen and DNA

The total collagen and DNA concentrations of the grafts were analyzed to calculate the net gain of matrix in the grafts. The grafts were homogenized in distilled water. The amount of total collagen and DNA in the tissue was determined from the homogenate as described earlier [22]. The collagen/DNA ratio was calculated, assuming that DNA directly reflects the number of nucleated cells and the amount of tissue in the graft.

In situ hybridization

Matrix metalloproteinase and TIMP mRNA expression were studied by *in situ* hybridization on paraffin-embedded grafts. The probes were from Bluescript plasmids (Stratagene, La Jolla, CA, USA): an 890-bp fragment of the rat MMP-2, a 518-bp fragment of the rat MMP-9 [23] and human TIMP-3 containing 600 bp [24]. *In situ* hybridization was performed mainly as described previously [25]. 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.

Extraction of metalloproteinases and gelatinolytic activity assay

Matrix metalloproteinase activity in kidney allografts and control kidneys was monitored by gelatin zymography as described [23]. Briefly, zymography was performed in SDS-polyacrylamide slab gel, containing 10% acrylamide and 0.1% gelatin as substrate. Proteins were stained with Coomassie blue G250. High Molecular Weight Standards and Low Molecular Weight Standards (Pharmacia Biotech, USA) were used for electrophoresis. Supernatant from the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated HT 1080 human fibrosarcoma cell line was used as positive control for gelatinolytic activity (kindly provided by Dr Karl Tryggvason, Karolinska Institutet, Stockholm). Enzymatic activity was determined by scanning bands using a densitometer (HP ScanJet IIc Scanner; Hewlett-Packard, Creeley, CO, USA) connected to a computer to measure the area produced by each peak (software, BioImage, Watford, UK).

Statistics

Data are expressed as mean ± SEM and Mann–Whitney *U*-test was used to compare the results between the groups. $P < 0.05$ was considered significant.

Results

Histological findings

The kidney allografts developed increasing fibrosis, tubular atrophy, and glomerular sclerosis 40–60 days after transplantation. These are generally accepted as the criteria for chronic rejection in the kidney allograft [1]. The inflammation associated with the process increased during the first week in the graft and began to decrease slowly after 30 days post-transplantation. The histological findings have been described in detail previously [2]. The time-related CADI values are shown in Fig. 1(a).

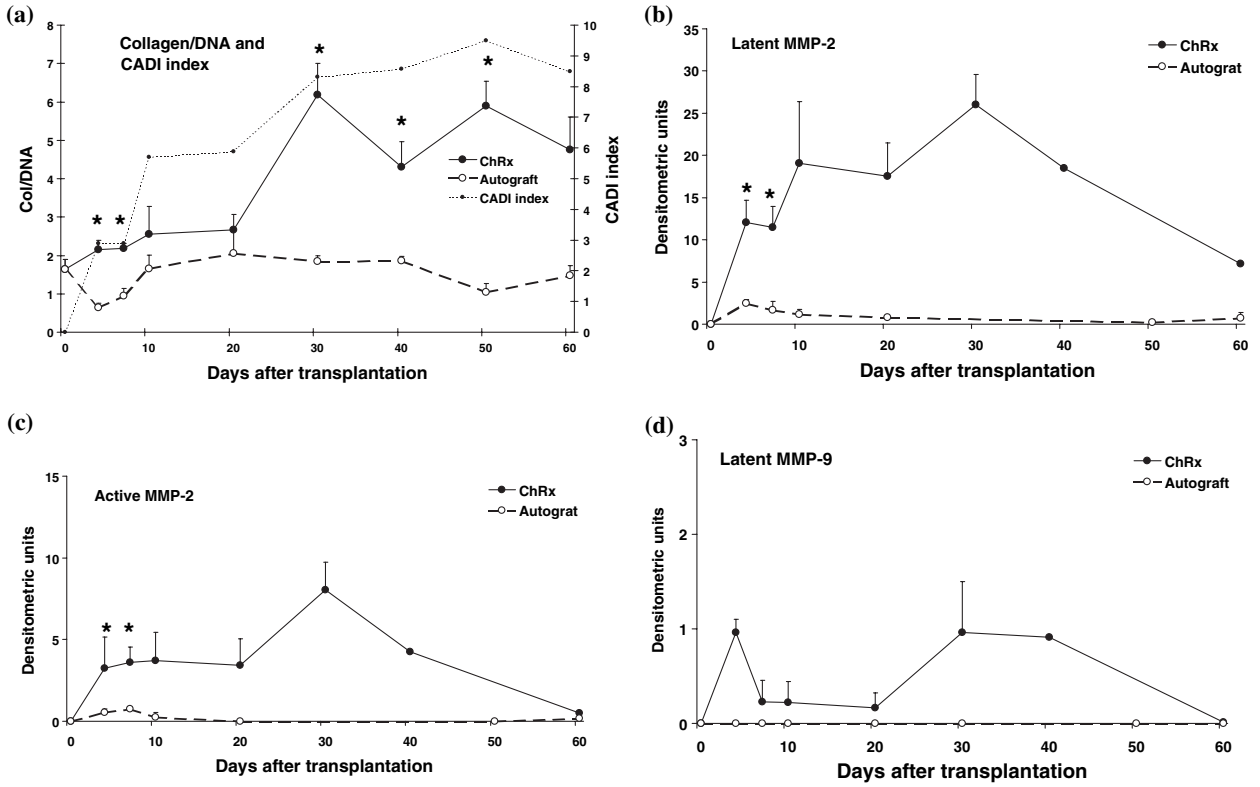

Figure 1 (a) The collagen/DNA ratio (in the allografts and autografts) and chronic allograft damage index (CADI) (in the allografts) over a period of 60 days after transplantation. (b–d) Gelatinolytic activity of rat renal allografts and autografts measured as densitometric units from zymography. In (b) and (c), the results of matrix metalloproteinase (MMP)-2 latent and active forms are given, and in (d) the results of MMP-9 latent form. Each point represents mean + SEM. *$P < 0.05$.

Generation of collagen

The total collagen and DNA concentrations of the grafts were analyzed. The collagen/DNA ratio was counted, assuming that DNA directly reflects the number of nucleated cells and the amount of tissue in the graft. Figure 1(a) demonstrates the time-related collagen/DNA ratio of the grafts over a period of 60 days after transplantation. A higher collagen/DNA ratio in the allografts compared with the control autografts was clearly evident, indicating the net gain of matrix in the kidneys. The difference between the two groups was statistically significant ($P < 0.05$) on days 30, 40, and 50.

Gelatinolytic activity of MMP-2 and MMP-9

Figure 2 shows typical gelatinolytic activities detected by zymography in allografts and autografts. The most prominent band of proteolytic activity in the allografts corresponded to MMP-2. The amount of this latent MMP-2 increased up to day 30, after which time point its level decreased (Fig. 1b). Proteolytic activity of active MMP-2 increased in the same way as latent MMP-2 but it was not as high (Fig. 1c). The levels of the latent and active MMP-2 in the autografts were much lower compared with the enzyme activities in the allografts. The statistical significance ($P < 0.05$) between the allografts and autografts was demonstrated on days 4 and 7 for the latent and active MMP-2. MMP-9 gelatinolytic activity was induced in the first days after transplantation and another increase was shown on day 30 (Fig. 1d). No MMP-9 gelatinolytic activity was noted in the autografts.

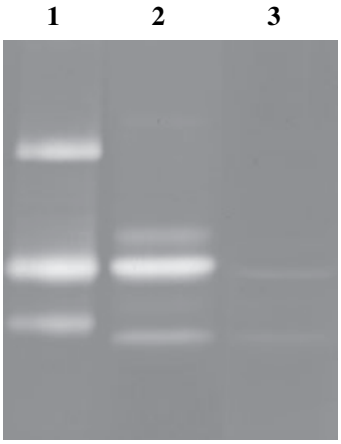

Figure 2 Gelatin substrate zymograph. Enzyme activity of media from 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated HT-1080 fibrosarcoma cell line served as a control (lane 1). Enzyme activity of rat renal allograft, day 6 (lane 2) and rat renal autograft, day 6 (lane 3).

MMP-2 and MMP-9 mRNA expression demonstrated by *in situ* hybridization

The expression of MMP-2, MMP-9, and TIMP-3 mRNA in renal allografts and autografts was demonstrated by *in situ* hybridization. MMP-2 mRNA expression was demonstrated in the allografts during the first 20 days after transplantation. MMP-2 mRNA gene expression located mainly in fibroblast-like cells in the interstitium in the juxtamedullary area of the kidney (Fig. 3a). In addition, some tubular cells expressed MMP-2 mRNA. After day 20, MMP-2 gene expression was almost nonexistent. In the autografts, a slight MMP-2 mRNA expression was seen during the first week after transplantation and only in fibroblast-like cells in the interstitium. MMP-9 mRNA expression in the allografts was located mainly in the scattered single cells of the glomerulus (Fig. 3b). Only occasionally, a few MMP-9 expressing glomerular cells were seen in the autografts.

The most prominent expression of TIMP-3 mRNA was seen in cells of the collecting ducts in the papilla from day 4 to day 50 and this was seen almost exclusively in autografts (Fig. 3c). In addition, tubular epithelial cells in the medullary area expressed TIMP-3 mRNA in the autografts (Fig. 3d) during the whole period of the experiment and in the allografts this was seen between days 7 and 20. Only on day 7, a few capillary endothelial cells in the allografts expressed TIMP-3 mRNA.

Discussion

Diffuse interstitial fibrosis as is seen in the histology of chronically rejecting human renal allografts correlates strongly with impaired graft function [21]. To better understand the molecular mechanisms of graft fibrosis, we studied MMP-2 and MMP-9 enzyme activity and gene expression of MMP-2, MMP-9, and TIMP-3 in relation to the accumulation of collagen and to the histological findings observed in a rat model of CAN [2].

The collagen/DNA ratio indicating the net collagen accumulation of the allograft was significantly increased compared with the autografts from 10 days after transplantation and continued up to day 40, after which time it decreased slightly. This coincided with the histological findings, where chronic rejection as defined by the CADI was seen on day 40.

In this rat model of CAN, both active and latent MMP-2, as well as latent MMP-9 enzyme activities were induced in the allografts compared with the controls. Peak values coincided with an increase in net collagen accumulation from day 30 onwards. There was no active MMP-9 either in the allografts or in the autografts, and the amount of active MMP-2 compared with the amount

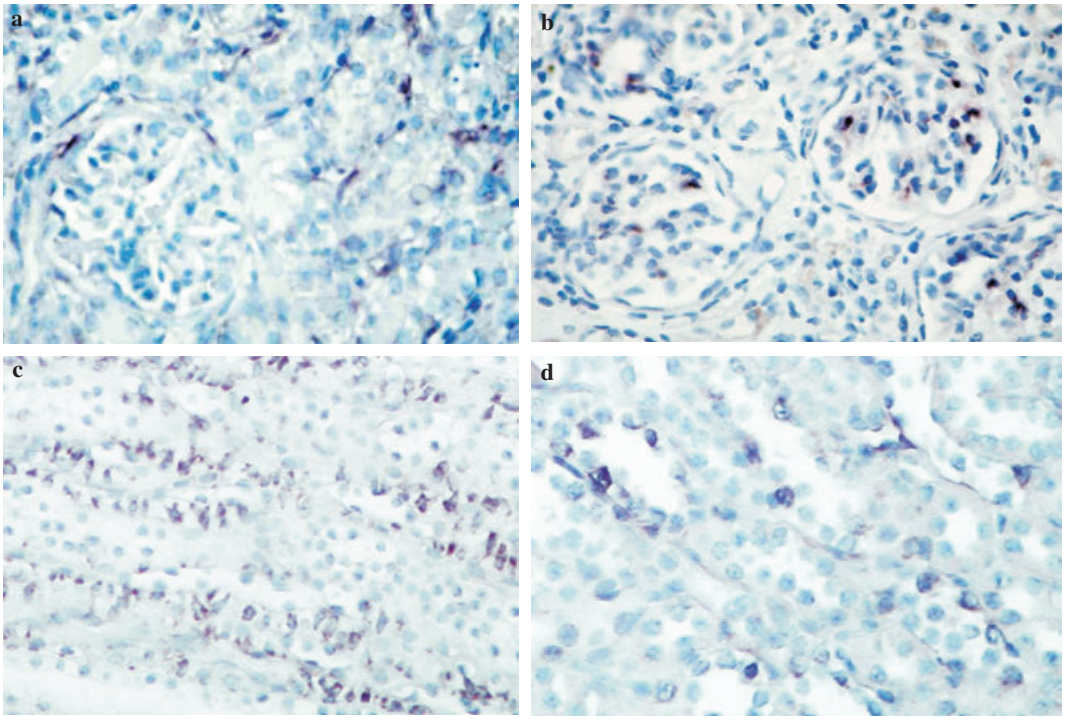

Figure 3 The expression of matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitors of matrix metalloproteinase (TIMP)-3 mRNAs in the renal allografts, demonstrated by *in situ* hybridization. An intense, positive expression of MMP-2 in the interstitial fibroblasts of the renal allografts (a). A positive signal for MMP-9 mRNA in the glomerulus of the allograft (b) and a positive signal for TIMP-3 mRNA in the cells of the collecting ducts (c) and in the tubular cells in the medulla of the autograft (d) (original magnification 400×).

of latent MMP-2 was much lower. These results indicate that the latent MMP-2 and MMP-9 forms generally remained unprocessed during chronic allograft rejection. As the equilibrium between the synthesis and the degradation of the collagens is displaced toward accumulation of collagen, our results indicate that this accumulation could be partly a consequence of the nonprocessing of both MMP-2 and MMP-9. Only a slight MMP-2 mRNA expression was seen during the first week after autografting, caused probably by the surgery itself. Otherwise, autotransplantation did not have any effect on the induction of MMPs. Thus, alloresponse seems to be important in the regulation of MMPs. However, intervention studies would be needed to confirm this suggestion.

Inflammatory cytokines such as TNF-α and IL-1 are responsible for the induction of MMP-2 and MMP-9. In our rat transplant model, the triple drug therapy prevented acute rejection, but after an inflammatory episode, the allograft developed chronic rejection. Thus, the regulation of MMPs is a consequence of alloresponse, and not of the drugs used.

Matrix metalloproteinase-9 is involved in inflammatory processes [15,26]. T-lymphocyte migration into tissues requires focal degradation of the basement membrane [27]. Transient adherence to fibronectin induces the production of activated forms of MMP-2 and MMP-9, as well as downregulation of TIMP by T-cell lines [27]. The increase in latent MMP-9 activity measured during the first week after transplantation could thus be the result of immune cell infiltration.

Altered interactions between tubular epithelial cells and tubular basement membrane play an important role in the progression of tubular atrophy, a characteristic feature of renal fibrosis. The initial influences for migration of activated tubular epithelial cells must include degradation or manipulation of the tubular basement membrane architecture. This is associated with increased production of MMP-2 and MMP-9 [28]. A role of active MMP-2 in CAN, as demonstrated in this study, could be the dysregulation of basement membrane integrity. The localization of MMP-2 mRNA in the tubular cells and in the interstitium corroborates this hypothesis.

A significant downregulation of TIMP-3 mRNA in allografts raises questions about its participation in response to injury during the process of the development of fibrosis in kidney allografts. Tubulointerstitial injury in CAN could result in an imbalance between apoptosis and

proliferation and thus the development of fibrosis. Dysregulation of TIMP-3 levels has a direct effect on apoptotic cell death [29].

In conclusion, MMP-2 and MMP-9 were upregulated and the inhibitory effect of TIMP-3 downregulated in kidney allografts. The development of fibrosis associated with chronic rejection is thus dependent on the regulatory mechanism of these molecules.

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