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Sequential analysis of adhesion molecules and their ligands in rat renal allografts during the development of chronic rejection

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Abstract Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are important in endothelial cell-leukocyte interactions. In this sequential study, the expression of ICAM-1 and VCAM-1 and their ligands LFA-1 and VLA-4 as well as major histocompatibility complex class II antigens (MHC class II), and interleukin-2-receptor (IL-2R) were investigated during the development of chronic renal allograft rejection in a rat model. The time-related expression of adhesion molecules and their ligands in the graft was correlated to the chronic allograft damage index (CADI). In association with an initial short immune activation, there was a significant ICAM-1 and VCAM-1 induction in the vascular endothelium and the tubular epithelium. In the interstitium, there was infiltration of lymphocytes expressing ligand molecules VLA-4 and LFA-1, as well as activation markers MHC class II and IL-2R. Thereafter, the expression declined together with the increase of CADI-values. In end-stage chronic rejection, there was practically no expression of ICAM-1 and VCAM-1. In the interstitium, there were only few ligand-expressing leukocytes. In conclusion, adhesion molecules and their ligands are involved in the induction phase of the process but no longer in the later stages of chronic rejection.

Key words Adhesion molecules · Chronic rejection

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 rejection. The findings of chronic renal allograft rejection are well defined [9, 10, 11, 16, 18, 22]. It includes perivascular and interstitial inflammation of various degrees, vascular intimal proliferation and thickening. In addition, glomerulosclerosis, tubular atrophy, and increasing fibrosis are seen. All these changes together cause deterioration of graft function. The changes associated with chronic rejection can be histologically evaluated and expressed by the Chronic Allograft Damage Index (CADI) [10, 11].

In the early immunological phase of the alloresponse, cell-cell interactions and T-cell activation are crucial. In this cellular cascade, rolling along the endothelium, adhesion to the vascular wall and migration from the circulation to the inflammatory site requires adhesion molecules, T-cell receptors and major histocompatibility (MHC)-antigens. The cytokine inducible expression adhesion molecules of the Ig-superfamily, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) is important for the firm adhesion of leukocytes expressing their ligands,

the integrin molecules, leukocyte function antigen-1 (LFA-1) and very late antigen-4 (VLA-4) [5, 14, 15, 21]. These adhesion molecules are upregulated by cytokines, such as IL-1, TNF-α and γ-interferon, which are produced during the immune activation of rejection.

The induction of these vascular adhesion molecules in the graft has been described as being associated with classical activation markers, the increase of MHC class II antigens and the appearance of interleukin-2 receptor (IL-2R) on the surfaces of lymphocytes during acute rejection [3, 7, 19, 25, 26]. Less is known about the involvement of adhesion molecules in chronic rejection. Based on a few reports of clinical transplantation, it has been suggested that the upregulation of peritubular capillary VCAM-1 may be one of the factors which are important in the development of chronic rejection [8, 12, 19, 27]. On the other hand, in rat models of chronic rejection, the expression of ICAM-1 has been found in the glomeruli and in vascular structures of renal allografts [1, 4, 6].

We have previously developed a model of chronic rejection in which the transplantations are performed in a high-responder rat strain combination which, under immuno-suppression of triple drug treatment with steroids, azathioprine and cyclosporine after an early inflammatory episode, develops chronic rejection within 40–60 days [20]. In this study, we investigate the sequential expression of ICAM-1 and VCAM-1 on various vascular structures and the presence of their ligand molecules, LFA-1 and VLA-4 expressing leukocytes in rat renal allografts during the development of chronic rejection. To relate the findings to the degree of immunological activation, the expression of the classical activation markers, IL-2R and MHC class II, was analyzed as well. We followed the time related expression of the aforementioned molecules in chronic rejection and correlated this to the CADI values, which showed the development of histological changes in the allograft.

Materials and methods

Animals

Male inbred rats (weighing 250–300 g) were used for transplantation. DA (RT1^a) were used as donors and BN (RT1^n) as recipients. 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 Helsinki University Central Hospital and the regional authorities.

Transplantations

Transplantations were performed using the modified technique described by Fisher and Lee [13]. Briefly, the animals were anesthetized using midazolam (Dormicum) and fentanyl-fluanisone (Hyp-

Table 1 Different expressions of adhesion molecules and MHC class II in the vascular structures and tubuli, and activation markers IL-2R and Class II and ligands LFA-1 and VLA-4 in interstitial leukocytes during acute (*AcRX*), end stage chronic rejection (*ChrRX*) and in normal rat kidney

	Arterial endothelium			Capillary endothelium			Glomerular endothelium			Tubular epithelium			Interstitial leukocytes			
	ICAM-1	VCAM-1	Class II	ICAM-1	VCAM-1	Class II	ICAM-1	VCAM-1	Class II	ICAM-1	VCAM-1	Class II	Class II	IL-2R	LFA-1	VLA-4
AcRX	$2.0 \pm 0.8^{a,b}$	$3.0 \pm 0.4^{a,b}$	$2.0 \pm 0.5^{a,b}$	$2.5 \pm 0.5^{a,b}$	$2.5 \pm 0.6^{a,b}$	2.0 ± 1.0^{a}	$2.0 \pm 0^{a,b}$	$1.5 \pm 0.6^{a,b}$	0.0 ± 0.0	$2.7 \pm 0.5^{a,b}$	$2.5 \pm 0.6^{a,b}$	$2.7 \pm 0.5^{a,b}$	$123 \pm 40^{a,b}$	$35 \pm 3^{a,b}$	$26 \pm 4^{a,b}$	$25 \pm 3^{a,b}$
ChrRX	0.3 ± 0.2	0.5 ± 0.4	0.2 ± 0.5	1.0 ± 1.0	0.6 ± 0.5	1.3 ± 1.0	0.7 ± 0.5	0.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.5	0.7 ± 0.4	0.8 ± 0.9	49 ± 22	2 ± 2	3 ± 2	2 ± 2
Normal	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.8 ± 0.4	26 ± 8	0 ± 0	0 ± 0	0 ± 0

[a] $P < 0.05$ compared to normal rat kidney; [b] $P < 0.05$ compared to chronic rejection

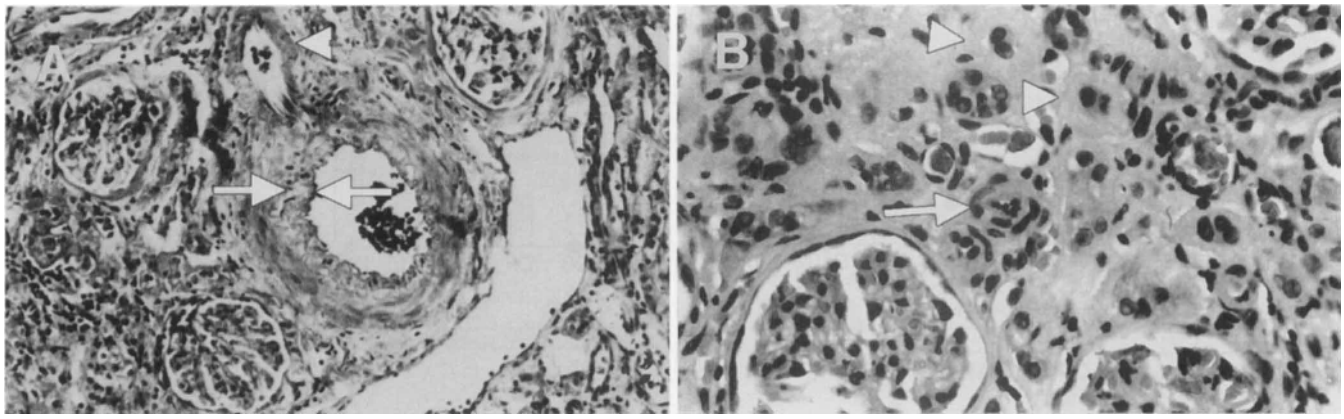

Fig. 1 A, B Histological findings of the end stage chronic kidney allograft rejection (H&E staining). **a** An artery with significant intimal thickening (*between the arrows*) and medial fibrosis. The *arrowhead* is pointing at an arteriolar branch (original magnification × 100). **b** An arteriole with narrowed lumen and thickened wall (*arrow*), surrounded by prominent interstitial fibrosis and atrophic tubuli (*arrowheads*). A glomeruli with somewhat increased matrix is also seen (original magnification × 200)

norm). The donor's right kidney, renal artery, vein, and ureter were dissected free from the surrounding tissues. The kidney was removed and flushed with ice cold Euro-Collins solution containing 500 IU of heparin/ml, until the kidney was macroscopically bloodless. The donor kidney was transplanted to the recipient's abdominal aorta and inferior vena cava below the renal vessel level by end-to-side anastomoses. Ureteral anastomosis was performed by end-to-end technique. The recipient's own right kidney was removed after the transplantation. The other own kidney of the recipient was left intact and used as internal control. Our experimental model of chronic rejection has recently been described in detail [20].

To prevent acute rejection, triple drug medication was used as basic immunosuppression in one group of rats. Treatment consisted of cyclosporine (5 mg/kg), azathioprine (2 mg/kg) and methylprednisolone (2 mg/kg) subcutaneuosly daily. For the time-related follow-up of chronic rejection for up to 60 days, 32 animals underwent triple drug immunosuppression and were killed at different time points after transplantation, days 5, 10, 15, 20, 30, 40, 50 and 60, three to five animals at each time point. Three normal kidneys from healthy rats without transplantation were used for the analysis of the time point 0. Autotransplantations with the same times for cold- and warm ischemia and reperfusion were performed on 32 animals; the left kidney was removed and replaced into the same animal by end-to-end anastomosis to the renal artery and vein, and ureter. Triple drug immunosuppression was given, and the autografts were used as controls of the surgical and reperfusion effects. For the time-related follow-up, the autografts were harvested at the same time points as the transplants, three to five animals at each time point.

Histology

Histological examination of the graft was performed on the explants removed in parallel on days 5, 10, 15, 20, 30, 40, 50 and 60 after transplantation, three to five grafts in each time point. The specimens were fixed in 10% buffered formalin and stained with hematoxylin-eosin and Masson's trichrome. The graft histology was evaluated according to the Banff criteria [17, 18]. The numerical chronic allograft damage index (CADI) was used to quantify the chronic alterations in the graft: the CADI was formed from the 6 histopathological changes characteristic of chronic rejection described previously [10, 11]: interstitial inflammation, fibrosis, glomerular sclerosis, mesangial matrix increase, vascular intimal thickening and tubular atrophy.

Immunostaining of tissue sections

Kidneys were snap frozen using liquid nitrogen, stored at –70 °C and sectioned using a cryostat. The sections (3–5 um) were fixed in cold acetone. An indirect immunoperoxidase technique and monoclonal mouse antibodies against rat MHC class II (Sera-Lab, Sussex, United Kingdom), IL-2R (Sera-Lab), LFA-1 (R&D Systems Europe LTD, Abingdon, UK), ICAM-1 (R&D Systems Europe), VCAM-1 (Dr. R. Lobb, Biogen, Cambridge, MA, USA) and VLA-4 (Pharmingen, San Diego, CA, USA) were used. The specificity of the antibodies used has been described by the producers. Normal mouse IgG antibody was used as a negative control for the mouse monoclonal antibodies. The own kidney of the recipient was used as internal negative control material for each rat. The frozen sections were first incubated with the monoclonal antibodies. A peroxidase-conjugated rabbit anti-mouse (DAKO A/S, Glostrup, Denmark) and a peroxidase-conjugated goat anti-rabbit (ZYMED Laboratories, San Francisco, CA, USA) were used as second and third antibodies, respectively. The reaction was revealed by 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Mayer's hemalum was used for counterstaining. The expression of adhesion molecules in the various structures of the kidney were all analyzed and the intensity of expression scored from 1 to 3. The numbers of infiltrating leukocytes in the interstitium positive for ligand molecules, IL-2-R and MHC class II were counted per high power visual field (magnification × 400, which makes 1.75 mm^2).

Statistics

The data was expressed as mean ± SD and, for comparison of results between the experimental groups and normal rat kidney, the *t*-test was used. P values < 0.05 were considered significant.

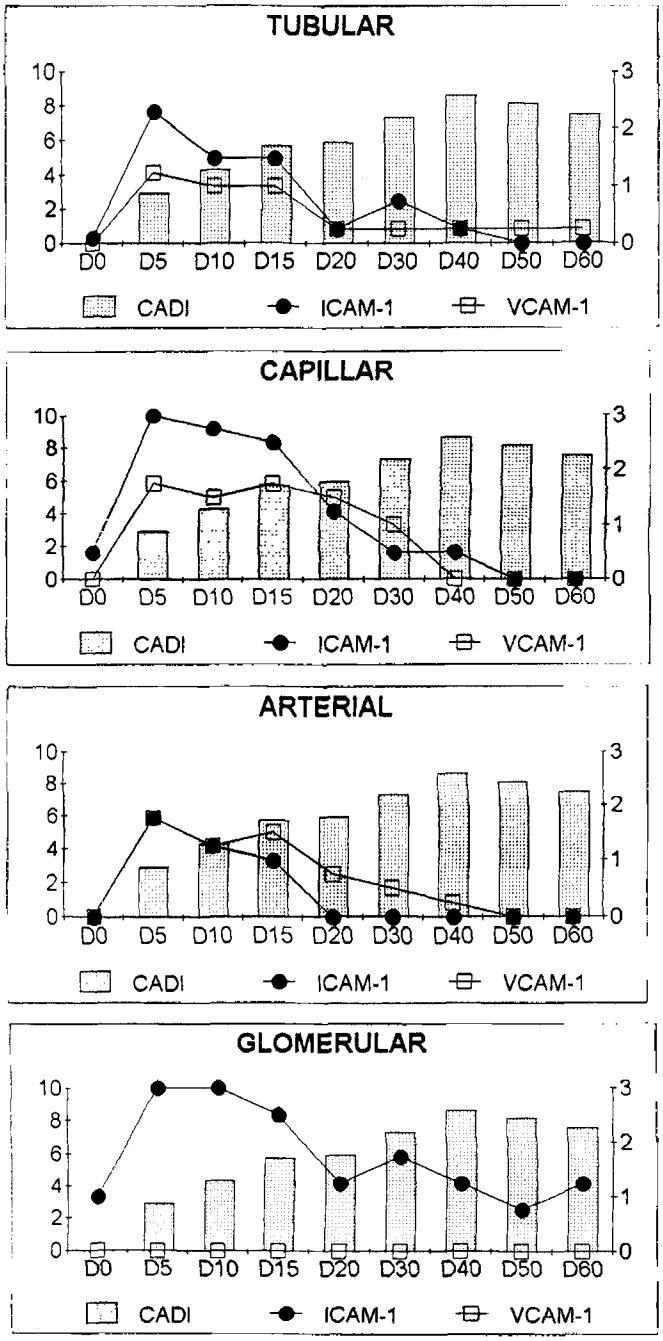

Fig. 2 The time related follow up and correlation between the intensity (scored 1–3) of expression of adhesion molecules ICAM-1 and VCAM-1 (mean ± SD) in various structures of the kidney allograft and the CADI index, during the course of the development of chronic rejection

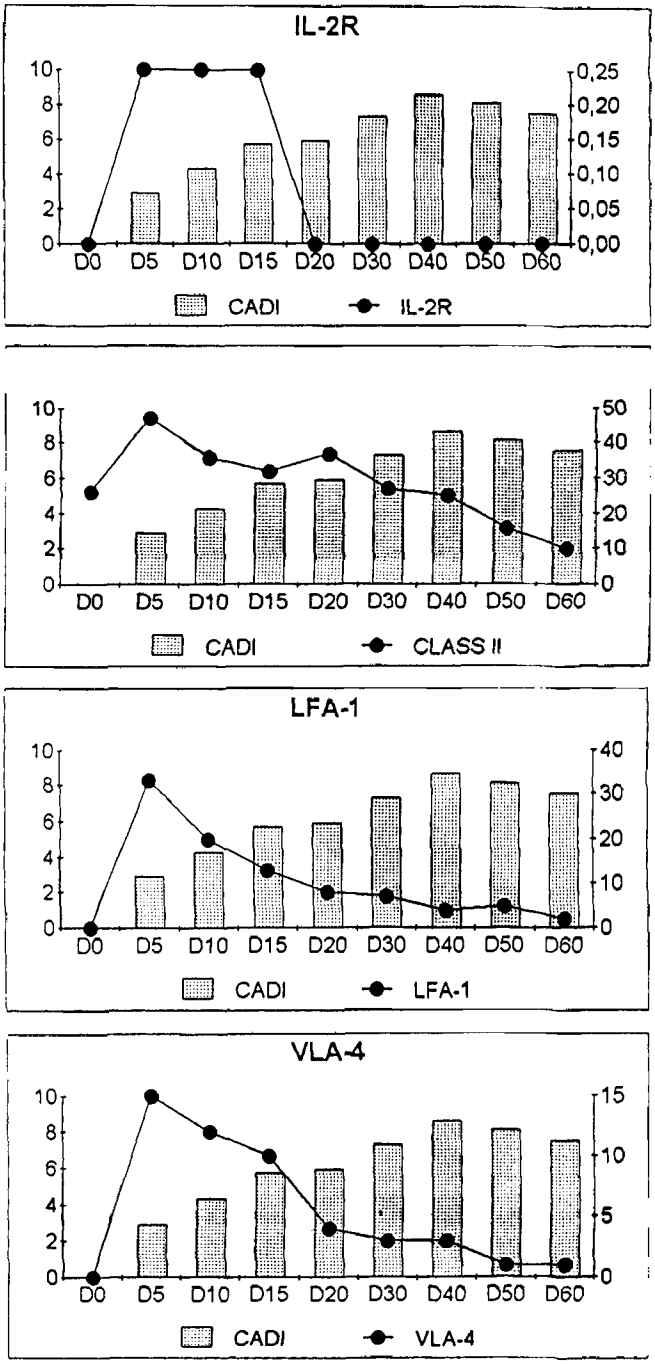

Fig. 3 The time related follow up and correlation between the number of graft infiltrating inflammatory cells in the interstitium (counted per high power visual field) expressing activation markers IL-2R and class II molecules, ligand molecules LFA-1 and VLA-4, and the CADI index (mean ± SD) during the course of the development of chronic rejection

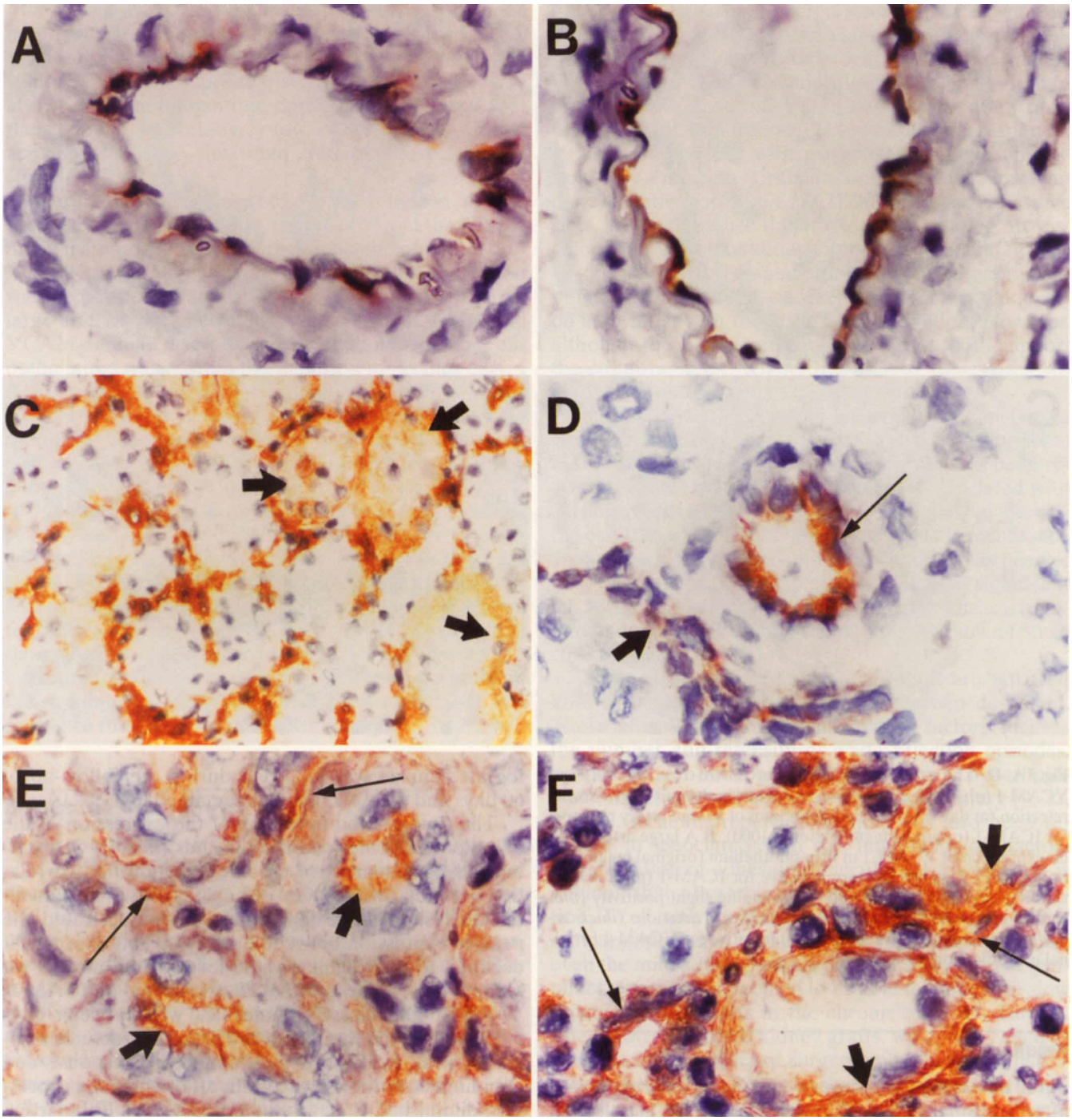

Fig. 4 A–F The expression patterns of ICAM-1(*on the left*) and VCAM-1 (*on the right*) in kidney allografts during the early inflammatory phase (at day 5) of the development of chronic rejection. **A** A large artery with a strong endothelial ICAM-1 expression (original magnification × 1000). **B** A close up view of a large artery with maximal positivity of VCAM-1 in the endothelium (original magnification × 1000). **C** An intense ICAM-1 expression of peritubular capillaries. Also note the positive staining in the tubuli (*arrows*) (original magnification × 400). **D** Arteriolar (*thin arrow*) and peritubular capillary (*thick arrow*) endothelium showing VCAM-1 expression (original magnification × 1000). **E** A strong ICAM-1 expression in peritubular capillaries (*thin arrows*) and tubular epithelial luminal surfaces (*thick arrows*) (original magnification × 1000). **F** Peritubular capillaries (*thin arrows*) with an intense VCAM-1 positivity. Also note the expression of VCAM-1 in the basal portions of tubular epithelium (*thick arrows*) (original magnification × 1000)

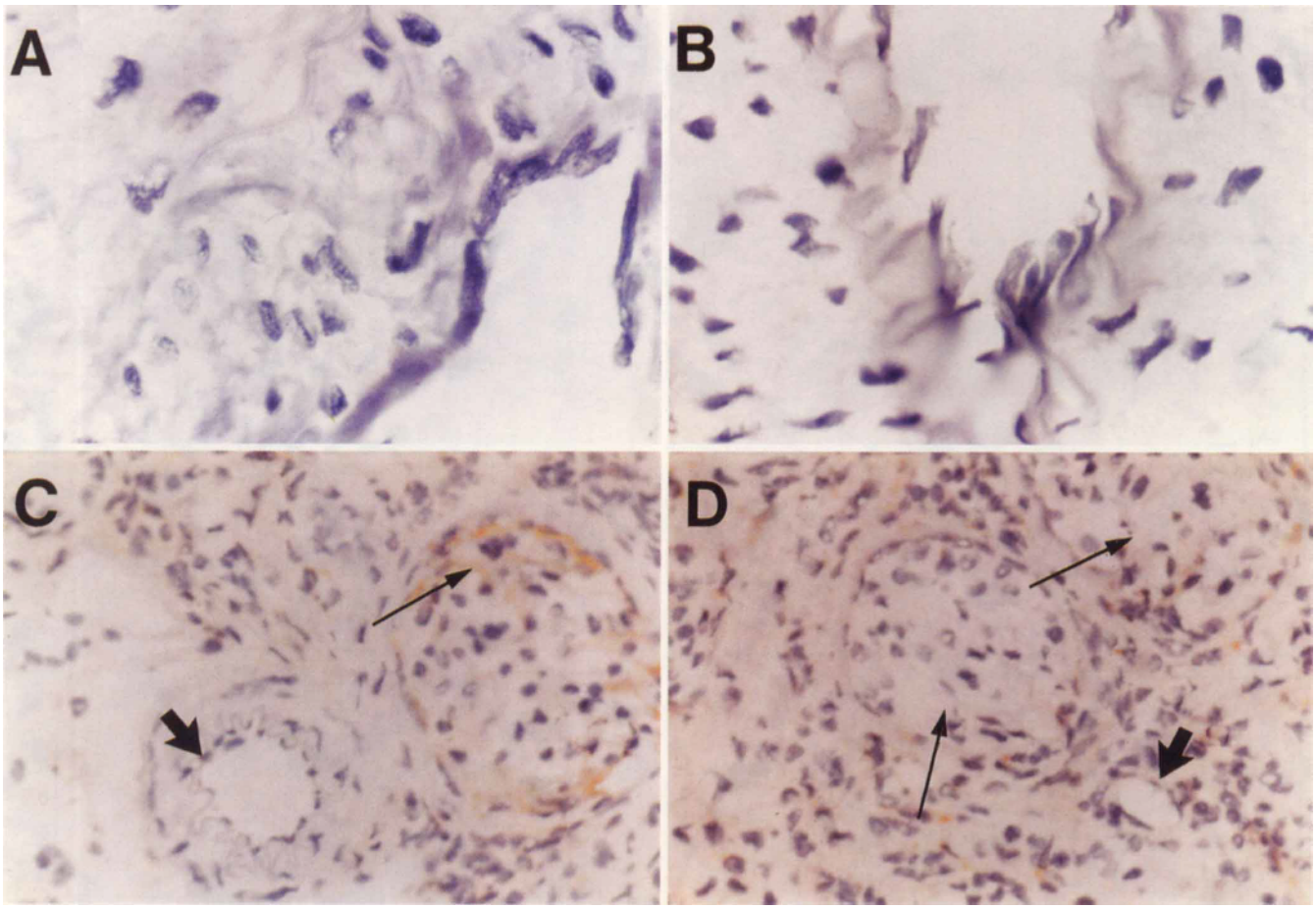

Fig. 5 A–D The expression patterns of ICAM-1(*on the left*) and VCAM-1 (*on the right*) in kidney allografts in the end stage chronic rejection (at day 60). **A** The endothelium of a large artery negative for ICAM-1 (original magnification × 1000). **B** A large artery with no expression of VCAM-1 in the endothelium (original magnification × 1000). **C** A small artery negative for ICAM-1 (*thick arrow*) with a glomerulus demonstrating a retaining slight positivity (*thin arrow*) (original magnification × 400). **D** An arteriole (*thick arrow*) and two glomeruli (*thin arrows*) negative for VCAM-1 (original magnification × 400)

Results

After an early inflammatory episode, appearing 5 days after transplantation, the characteristical histological findings of chronic rejection, such as vascular wall thickening, glomerular changes, tubular atrophy and interstitial fibrosis were developed in the kidney allografts during the follow-up of 60 days (Fig. 1). The CADI-index reached its highest value (7.9 ± 2.1) on day 40, and end-stage chronic rejection with remarkable fibrosis was seen in histology 40–60 days after transplantation. The histology of the own native kidneys in these animals was normal. In autografts, no evidence of chronic allograft damage was recorded during the follow-up of 60 days and the CADI values remained low (≤ 2.0).

The time related follow-up of the expression of the adhesion molecules and their ligands (mean ± SD) during the development of chronic rejection and correlation between CADI-index is shown in Figs. 2–3. Sequential analysis of adhesion molecules demonstrated the peak of expression between days 5–15, and after that it declined to approximately the level of the normal rat kidney. The highest value of expression of ICAM-1, VCAM-1 and class II molecules was between days 5–15, both in the capillaries and arterial structures associated with a temporary inflammatory response and lymphoid activation in the graft, after which the expression diminished to nearly its normal level. Tubular expression of ICAM-1, VCAM-1 and class II molecules was strongest on day 5. In the glomeruli, VCAM-1 expression was absent for the whole 60 day follow-up time. However, the ICAM-1 expression of glomerular capillary endothelium was increased between days 5 and 10. Figure 4. demonstrates the expression patterns of ICAM-1 and VCAM-1 in kidney allografts during the early inflammatory phase of the development of chronic rejection. In the interstitium, the number of

MHC class II, LFA-1 and VLA-4 positively stained mononuclear cells was highest on day 5, subsided thereafter, and by day 60 there were practically no positively stained cells. IL-2R positive stained lymphocytes were also seen in the interstitium during the early phase, but their number was relatively low, indicating a low T-cell activation during the mild inflammatory episode (Fig. 3).

During the development of chronic rejection, the adhesion molecule expression disappeared and the CADI-index increased (Fig. 2). At the end-stage of chronic rejection, practically no adhesion molecule expression was demonstrated in the grafts. The endothelium of large arteries became totally negative for ICAM-1 and VCAM-1. Small arterioles and peritubular capillary endothelia did not express the adhesion molecules either. Also, tubular epithelial expression of ICAM-1 and VCAM-1 disappeared. Only some slight retaining glomerular ICAM-1 staining was seen. Figure 5 shows the lack of expression of ICAM-1 and VCAM-1 in kidney allografts at the end stage of chronic rejection.

In autografts, only a slight temporal staining of ICAM-1 and VCAM-1 was recorded in the vascular and capillary endothelium at day 5 (not shown). A faint class II expression was recorded in the endothelium but also in the tubular epithelium at the same time. Thereafter the autografts showed the same pattern of staining as the own control kidneys of the rats with allografts. No inflammatory infiltrate was seen in the autografts and the staining pattern of LFA-1, VLA-4 and IL2-R was similar to that of the normal own kidneys.

Discussion

In our model, after a mild early inflammatory episode, characteristical histological findings of chronic rejection were developed within 60 days after transplantation [20]. In the follow-up, the induction of adhesion molecules occurred between days 5 and 15 and the expression peaked on day 5 after transplantation in all of the renal structures investigated. This was associated with a mild inflammatory response in the graft, infiltration of mononuclear cells expressing the corresponding ligand molecules but demonstrating a relatively low level of lymphocyte activation. After that, the expression of the adhesion molecules subsided. The correlation of these findings with the CADI values showed that the chronic allograft damage increased as the expression of adhesion molecules decreased. The inflammation phase with adhesion molecule involvement preceded the development of chronic changes in the graft.

In another model of chronic rejection in rat kidney, allografts were performed between the weak responder combination of F344 and LEW strains under cyclosporine monotherapy [6]. In this experiment, ICAM-1 induction and LFA-1 expression were associated with the accumulation of IL2-R expressing mononuclear cells within 10 days, but were recorded at all time points up to the development of glomerulosclerosis, interstitial fibrosis and intimal proliferation 12–16 weeks after transplantation [6]. In our model between the high responder strains DA and BN with triple drug immunosuppression, only a mild early inflammatory episode, a low lymphocyte activation with a few IL2-R and class II positive cells, is able to induce the development of allograft vasculopathy and interstitial fibrosis, recorded already 40–60 days after transplantation. Increased expression of ICAM-1 and LFA-1 were seen only for a certain period associated with the mild T-cell activation. However, although the models are quite different, both support the importance of ICAM-1/LFA-1 in the process of chronic rejection.

The VCAM-1/VLA-4 pair has important function in lymphocyte and monocyte extravasation during acute kidney allograft rejection [5]. Previously it has been reported that VCAM-1 expression is also associated with chronic rejection [19, 27], recorded especially in interstitial capillaries. Experimental models usually include the analysis of ICAM-1 induction but VCAM-1 has been of less interest. In our experimental study, significant VCAM-1 induction was associated with the early inflammatory episode, but was no more seen in end-stage chronic rejection.

The mechanisms of chronic rejection are not well known, but several risk factors have been described. There are alloantigen-dependent risk factors such as the number and intensity of acute rejection episodes, and alloantigen-independent factors such as hyperlipidemia, infections and ischemia/reperfusion injury [9, 22, 23, 24]. Ischemia/reperfusion injury has also been reported to associated with the immunological factors and induction of adhesion molecules [2]. However, it is obvious that alloresponse with inflammatory infiltration, T-cell activation and production, cytokines are of critical importance in the process [9, 22]. In our study, even the mild alloresponse, even with a low level of lymphocyte activation, seemed to be able to induce the slow process which results in the chronic allograft damage. The autotransplanted kidney grafts, which were used as controls for the effect of surgery and ischemia/reperfusion damage, did not show a significant induction of adhesion molecules, and no inflammatory reaction was seen either. The CADI values of the autografts were low and no chronic allograft damage was developed.

In the early phase of the alloresponse, adhesion molecules seem to play an important role; their presence on the endothelium of vessels causes the leukocytes to roll along the vessel wall, adhere to the endothelium, and finally to extravasate and migrate to the site of inflammation [5, 6, 14, 15, 21, 25, 26]. Although the inflammation subsided in our kidney transplant model of chronic re-

jection, the processes associated with the chronic allograft damage index proceeded. Once initiated by the inflammation, and with its induced secretion of various cytokines, the chronic process proceeds and is driven further by locally produced pericrine and autocrine factors. Thus, in clinical transplantation, even a mild subclinical alloresponse may also induce this process which in humans may take months or years to develop characteristic chronic allograft damage.

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