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Expression of vascular cell adhesion molecule-1 in kidney allograft rejection

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Expression of vascular cell adhesion molecule-1 (VCAM-1) in kidney allograft rejection. VCAM-1, a leukocyte adhesion molecule expressed by cytokine-activated endothelial cells in culture, may mediate mononuclear leukocyte infiltration in vessels and interstitium in solid organ allograft rejection. Using the avidin-biotin immunoperoxidase technique and an affinity-purified rabbit polyclonal antisera to recombinant human VCAM (rVCAM Ab) which works in methyl Carnoy's fixed tissues, we studied the expression of this molecule in biopsies of transplanted kidneys ($N = 34$) with and without features of rejection and allograft nephrectomies ($N = 17$) as well as nontransplanted control tissues ($N = 26$). The rVCAM Ab showed a population of reactive endothelial cells limited to sites of prominent subendothelial leukocytic cell infiltration in arteries and veins, and occasional peritubular capillaries (PTC) in rejecting allografts. Endothelial expression of VCAM was rarely identified in biopsies showing interstitial rejection only or cyclosporine toxicity, usually in PTC, and was only rarely encountered in nontransplanted control tissues. Apparent *de novo* expression of VCAM-1 by arterial smooth muscle cells and mesangial cells was present in cases of severe rejection. In addition, a population of cells (DC) with dendritic morphology was identified by rVCAM Ab within sites of lymphoid cell aggregation in rejecting allografts. Further evidence that these cells represent true DC was obtained by identification of VCAM-1 positive, morphologically similar cells in both germinal centers and interfollicular areas of all seven reactive lymph nodes tested; and by similar staining of these cells in the allografts and lymph nodes by antibodies to nerve growth factor receptor and the complement receptor CR1, previously shown to recognize DC. DCs were generally not seen in uninflamed normal control organs or portions of allografts uninvolved by lymphoid aggregates. Enhanced tubular epithelial cell expression of VCAM-1 was also present in rejecting allografts. All staining could be abolished by absorption of the antisera with VCAM-1 transfected, but not ICAM-1 or ELAM-1 transfected, CHO cells. *In situ* hybridization studies utilizing a cDNA probe to human VCAM-1 demonstrated mRNA production by glomerular, tubular and vascular cells corresponding to sites where the protein was immunohistochemically localized. This study provides evidence that: (1) endothelial cell expression of VCAM-1 may define sites of acute vascular inflammation in renal transplant rejection; (2) VCAM-1 is expressed by some arterial smooth muscle cells during vascular rejection; (3) VCAM-1 expression by mesangial cells and tubular cells may be upregulated in transplant rejection; and (4) there is probably a population of VCAM-1 expressing DC that migrates into host kidneys and participates in the cellular rejection process.

Acute vascular rejection in muscular arteries in renal allografts is characterized by subendothelial infiltration by effector immunocompetent and inflammatory cells, principally lymphocytes and monocyte/macrophages [1]. Vascular (as well as interstitial) rejection is often a focally distributed process, and so it is important to define the mechanisms which determine the sites of leukocyte attachment and migration through arterial endothelium that initiate the rejection process. It has been shown recently that a number of inflammatory or immune mediators, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) can induce the expression of proteins on the surface of cultured endothelial cells that act to increase the adhesiveness of these cells for specific leukocyte populations, including lymphocytes and monocytes [2, 3]. Modulation of the expression or configuration of such adhesion proteins on the endothelium or similar modulation of the corresponding ligands on circulating leukocytes might be especially important in establishing the endothelial and subendothelial injury characteristic of vascular rejection in solid organ allografts, and might also be vital to localization of the intraparenchymal inflammatory infiltrates mediating interstitial rejection in such allografts.

Currently, five endothelial proteins have been molecularly cloned [4–9] and have been shown to be involved in leukocyte adhesion in humans [10–14]: E-selectin, endothelial leukocyte adhesion molecule-1 (ELAM-1); P-selectin (platelet activation dependent granule-external membrane, PADGEM; granule membrane protein-140, GMP-140, CD62); intercellular adhesion molecule-1 (ICAM-1, CD54); ICAM-2; and vascular cell adhesion molecule-1 (VCAM-1, INCAM 110). Studies of ICAM-1 in human and experimental renal transplantation have provided evidence of up-regulated expression of this molecule on endothelial and tubular cells and indicated that leukocyte binding to ICAM-1 is an important component of both vascular and interstitial rejection processes [15–20]. A role for E-selectin or P-selectin in renal allograft rejection has not been reported. However, studies of VCAM-1 in cardiac transplant biopsies have localized up-regulated expression on capillary endothelium which is associated with areas of mononuclear cell infiltration, suggesting a role for this molecule in binding leukocytes

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in vivo and in determining the sites of active rejection [21, 22]. Recently, upregulated endothelial expression of VCAM-1 during episodes of human pancreatic and hepatic allograft rejection has also been demonstrated [23, 24]. Finally, a recent study by Briscoe et al has demonstrated up-regulated expression of VCAM-1 by tubular and vascular endothelial cells in a series of eleven renal allograft biopsies [25]. One other study, using an antibody to VCAM-1 without documentation of its specificity, has also reported on its expression in renal transplantation [26]. In this study, we have utilized a recently characterized polyclonal antiserum raised against recombinant human VCAM-1 (rVCAM) to evaluate the contribution of this molecule to renal vascular rejection [23]. In the present study we have established that this antiserum recognizes an epitope that is preserved in tissue fixed in methyl Carnoy's solution, evaluated the distribution of the VCAM-1 molecule in normal fixed and frozen human kidney tissue, and identified sites of up-regulated VCAM-1 expression in rejecting renal allografts. Our studies confirm and expand on the findings of Briscoe et al by demonstrating VCAM-1 expression on arterial endothelial cells and smooth muscle cells during the course of vascular rejection, and demonstrate a population of VCAM-1 expressing dendritic cells within aggregates of leukocytes in rejecting allografts. The application of *in situ* hybridization techniques allows us to conclude the VCAM expression identified by each of these cell types is the result of active local production of this peptide rather than a result of passive, local entrapment or phagocytosis of secreted or released VCAM-1 from other cells.

Methods

Tissue selection

A total of 34 renal allograft biopsies were utilized in this study. Allograft biopsies were obtained as core needle biopsies, and comprised the following categories: (1) 21 day protocol biopsies in patients undergoing combined kidney and pancreas transplantation ($N = 7$); (2) cases of acute cyclosporine nephrotoxicity as determined by subsequent clinical course and compatible biopsy findings ($N = 5$); (3) cases of mild interstitial inflammatory infiltration of uncertain significance, not clearly related to rejection ($N = 3$); (4) cases of acute cellular rejection, of at least moderate severity and demonstrating features of tubulitis ($N = 11$); (5) cases of acute vascular rejection, as previously illustrated [1] ($N = 6$); (6) and cases of chronic rejection ($N = 2$). Allograft kidneys ($N = 17$) excised for irreversible rejection were also utilized. These cases invariably had features of cellular (interstitial) and vascular rejection. The infiltrates of cellular rejection were usually of moderate to severe intensity, but focally distributed within the renal parenchyma, so that portions of tissue showed only minimal inflammatory infiltration. Many of these nephrectomy specimens also contained occasional, irregularly distributed lymphoid aggregates similar to those previously described in other solid organ allografts [19]. Arterial vessels showing intimal inflammation as well as uninvolved vessels were distributed both in areas of prominent interstitial inflammation and in areas in which this process was mild and even absent.

All biopsies were obtained from patients under conditions of routine immunosuppression protocols employing cyclosporine and prednisone. The biopsies were obtained prior to more specific or intensified therapies such as administration of OKT_3. The nephrectomy specimens in general were exposed to multiple courses of routine and intensified immunosuppression prior to excision, and represent a heterogeneous sample from a clinical standpoint.

Normal human kidney tissue ($N = 23$) was obtained from kidneys surgically excised because of the presence of a localized neoplasm. Tissues utilized for this study were obtained from macroscopically normal portions of kidney located at some distance from the neoplastic process. Additionally, normal tissue was obtained from fresh cadaver kidneys unable to be utilized for transplantation ($N = 3$).

Because VCAM-1 has been identified within dendritic cells in lymphoid tissues [23, 27], the reactivity of the antisera used in this investigation was also studied in seven reactive lymph nodes and tonsils, surgically removed for lymphoid hyperplasia.

All tissues were fixed in methyl Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid) for at least 12 hours and then processed, paraffin embedded, and sectioned using conventional techniques.

Normal tissues were also snap frozen and utilized unfixed for identical immunohistochemical studies to evaluate possible loss of antibody sensitivity when studying the fixed tissues employed for the remainder of this study.

Immunohistochemistry

Briefly, sections of methyl Carnoy's fixed tissue were deparaffinized with xylene and graded ethanol's, blocked with 3% hydrogen peroxide, and washed with PBS (138 mM NaCl, 2.7 mM KCl, 3.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3). The tissue was then incubated with one of the primary murine monoclonal antibodies (see below), or rabbit polyclonal antisera, and subsequently processed using a avidin-biotin immunoperoxidase method with 3,3′-diaminobenzidine (with nickel enhancement) as the chomogen as previously described [28, 29]. Sections were counterstained with methyl green or hematoxylin. For all samples, negative controls for the immunohistochemical procedures consisted of substitution of the primary antibody with both irrelevant murine monoclonal antibodies, or non-immune rabbit sera, and PBS. Positive controls included concurrent staining of fixed human tonsil, a tissue with detectable constitutive expression of VCAM-1 on dendritic cells [23, 27] as previously described, and fixed normal human kidney, a tissue with detectable constitutive expression of VCAM-1 on parietal epithelial cells [27].

Antibodies

Vascular cell adhesion molecule-1. Rabbit polyclonal antisera was raised against a recombinant form of human VCAM-1 (rsVCAM) that was purified to homogeneity by immunoaffinity chromatography as previously described [30]. NZW rabbits, 3 to 4 kg, were immunized with purified rsVCAM (1 mg), emulsified (1:1) in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA). The rabbits were boosted with rsVCAM (1 mg) in incomplete adjuvant, at monthly (3×) and then bimonthly intervals. Bleeds were taken 7 to 14 days after each boost. Rabbit antisera was affinity purified by passage over protein A, with the IgG fraction then passed over an affinity resin of human rsVCAM-1 immobilized on Affigel (8 mg

rsVCAM-1 ml of resin). The antisera was eluted with buffer at pH 3.0, dialyzed into PBS, aliquoted and stored at −80°C.

Absorption studies of VCAM-1. Reactivity and specificity of this antibody in fixed tissues was established using methyl Carnoy's fixed cell aggregates of Chinese hamster ovary cells either transfected or untransfected (negative control) with VCAM cDNA (provided by Dr. Margaret Rosa, Biogen, Cambridge, Massachusetts, USA), which were then shown to express VCAM-1 at the cell surface by appropriate binding of leukocyte cell lines as well as binding inhibition assays.

Further, Chinese hamster ovary (CHO) cells transfected with VCAM-1, ICAM-1, and ELAM-1 and demonstrating surface expression of each of these molecules as well as untransfected CHO cells, were maintained in culture as previously described [30]. Culture plates were washed with PBS, scraped, and pelleted after centrifugation at 1200 RPM. Cell pellets were fixed in methyl Carnoy's solution, and processed and embedded in paraffin for tissue immunohistochemistry using procedures detailed above. The rabbit polyclonal antisera to recombinant VCAM-1 was demonstrated to be reactive with the cell surface of CHO cells transfected with VCAM-1, but not with untransfected cells or those transfected with ICAM-1 or ELAM-1 (data not shown).

Cell pellets, each containing approximately 25×10^6 total cells, of VCAM-1, ICAM-1, and untransfected CHO cells were also collected in serial dilutions of PBS, and then incubated for 60 minutes at room temperature with aliquots of rabbit polyclonal anti-VCAM-1 antisera. After incubation, the suspensions were again centrifuged at 1200 RPM and the supernatants collected for incubation on tissue sections of both the cell pellets as noted above, and on methyl Carnoy's fixed tissue sections of human tonsil. Identification of tissue binding was determined with the avidin-biotin immunoperoxidase technique detailed above, and tissues were counterstained with methyl green.

Nerve growth factor receptor-5 antibody (NGFR5) and nerve growth factor receptor-2 antibody (NGFR-2)/dendritic cell markers. NGFR5 is a monoclonal antibody originally developed to study the expression of p75 nerve growth factor receptor in tumors and normal tissues [31]. Among normal tissues, in addition to expected neural immunostaining, NGFR5 has been demonstrated to react with several non-neural cell types, including lymphoid follicular dendritic cells [31, 32]. NGFR2 is a second murine monoclonal antibody with functional blocking characteristics which targets epitopes of p75 nerve growth factor receptor distinct from those recognized by NGFR-5 [33]. In this study we used the NGFR5 and NGFR2 antibodies as independent confirmatory immunolocalization markers of dendritic cells in lymphoid aggregates. A fourth, commercially available, antibody to follicular dendritic cells (DRC-1, Dako Corporation, Carpinteria, California, USA) reactive with the complement receptor CR1 (CD35) was also subsequently utilized on those nephrectomy specimens and biopsy specimens with sufficient tissue remaining after initial immunohistochemical studies.

Leukocyte markers. Immunophenotypic characterization of infiltrating leukocytes was performed as previously described [1]. Commercially available antibodies were used to identify populations of monocytes/macrophages (anti-CD68, monoclonal antibody KP-1, Dako Corporation [34], T lymphocytes (anti-CD3, Dako Corporation) [35], and B lymphocytes (anti-CD20, monoclonal antibody L26, Dako Corporation) [36, 37].

Endothelial markers. Endothelial cells were identified by lectin binding studies using Ulex europaeus I lectin (Vector Laboratories, Burlingame, California) as previously described [38, 39].

Smooth muscle cell markers. Murine monoclonal antibody α-SM-1 (Dako Corp.) has been characterized by tissue immunohistochemistry and Western blotting [40], and has been previously demonstrated to recognize smooth muscle α-actin in methyl Carnoy's fixed tissues [41, 42]. We have previously demonstrated the specificity of the increased glomerular expression of α-smooth muscle actin expression detected by tissue immunohistochemistry with this antibody by concurrent Northern analysis for α-actin mRNA synthesis in isolated glomeruli obtained in a rat model of mesangiolytic injury [41].

In situ *hybridization*

Riboprobe preparation. One microgram of 1.1 kilobase (kb) fragment of the human VCAM-1 gene, including 0.3 kb of the 3′-untranslated region, in the expression vector was transcribed into an antisense riboprobe using the T3 polymerase, as previously described [43], using 250 μCi^{35} S-UTP (New England Nuclear) as the radioactive label. After a 60 minute incubation at 37°C, the cDNA was digested by adding 1 U RQ1 DNase (Promega) and incubation at 37°C for an additional 15 minutes. Free nucleotides were separated using a sephadex G-50 column. A sense riboprobe was also transcribed for control hybridizations from a 1.25 kb fragment of the human VCAM-1 gene using T7 polymerase. Probes were stored at −70°C and used within seven days of synthesis.

In situ *hybridization.* Arterial tissue from nephrectomy specimens which had been fixed in 10% neutral buffered formalin and embedded in paraffin were deparaffinized according to standard protocal. *In situ* hybridization was then performed as previously described [43, 44]. After the tissue was air dried, it was dipped in NTB2 nuclear emulsion (Kodak) and exposed in the dark at 4°C for one to two weeks. After developing, sections were counterstained with hematoxylin and eosin. Controls included simultaneous procedures performed on replicate sections using the sense riboprobe described above, and the use of fixed cell pellets of the VCAM-1 transfected and untransfected CHO cells as additional positive and negative controls.

Double labeling immunocytochemistry

Methyl Carnoy's fixed, paraffin embedded tissues were sectioned and mounted on aminopropylmethoxysilane (APTS) coated slides. After deparaffinization and rehydration, the slides were incubated with rabbit-anti-VCAM-1 diluted in PBS plus 1% BSA overnight at 4°C. After washing, sections were incubated with goat-anti-rabbit IgG-gold (Amersham, Arlington Heights, Illinois, USA) diluted in PBS plus 1% BSA and 0.1% gelatin for one hour at room temperature. Sections were washed, and the gold was visualized with an intense M silver enhancement kit (Amersham). The sections were then incubated sequentially with: (1) anti-α-smooth muscle actin or anti-CD-68; (2) biotinylated horse-anti-mouse IgG (Vector Laboratories, Burlingame California, USA); and (3) avidin-biotin-alkaline phosphatase complex (Vector laboratories). The alkaline phosphatase was developed with a red substrate kit (Vector

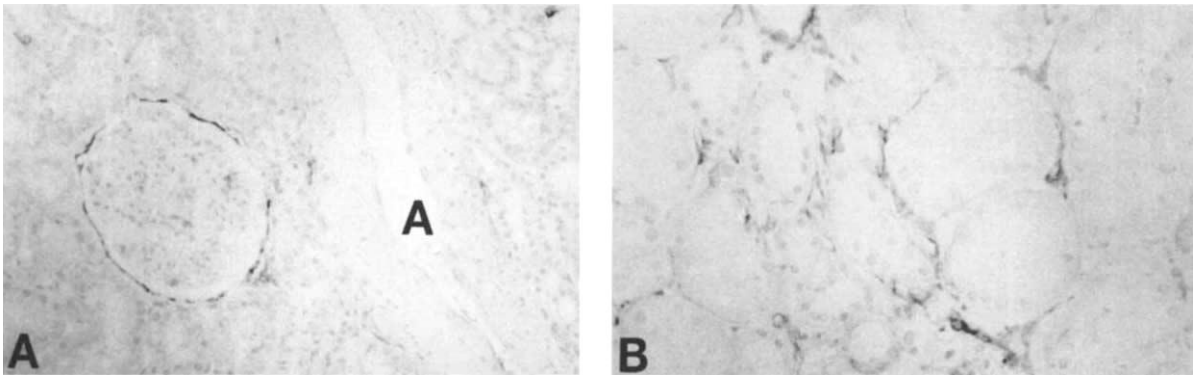


Fig. 1. *(A) Normal human kidney immunoreacted with anti-VCAM-1 antisera.* There is strong expression of VCAM-1 by glomerular parietal epithelium, but not by other glomerular structures, interstitium, or adjacent muscular artery. **(B)** Normal human kidney. Focally, VCAM-1 expression by peritubular capillary endothelium was present. Although not present in this section, focal basolateral expression of VCAM-1 by tubular epithelium was also present. A, 75×. B, 220×.

Fig. 2. *Arterial expression of VCAM-1 in vascular rejection.* **(A)** Muscular artery with acute rejection showing lifting of the endothelium and subendothelial accumulation of mononuclear inflammatory cells. No neointimal proliferation characteristic of chronic vascular rejection is present. **(B)** Large muscular renal artery with features of both acute (inflammatory infiltration) and chronic (neointimal proliferation) vascular rejection, double labeled for VCAM expression in black (immunogold technique) and α smooth muscle actin in red (alkaline phosphatase technique). The wall of another muscular artery segment incompletely profiled in this plane of section is present in the lower left portion of the photograph. **(C)** Higher power view of artery shown in 2B shows acute changes of endothelial swelling and subendothelial and intimal infiltration by leukocytes. Hematoxylin and eosin. **(D)** Higher power view of artery in B. There is prominent expression of VCAM-1 by swollen endothelium, and well as co-localization of VCAM-1 expression and actin expression by some neointimal smooth muscle cells (arrows). **(E)** Same artery and double label technique as D, except the primary anti-VCAM-1 antisera has been replaced by antisera absorbed with VCAM-1 expressing CHO cells. The VCAM-1 staining of endothelial and smooth muscle cells has been abolished, but α-actin staining is unchanged. **(F)** Same artery as in C through E. Double labeling shows VCAM-1 expression in black, and an infiltrating population of $CD68^+$ monocytes/macrophages in red. **(G, H)** Chronic vascular rejection in a smaller interlobular muscular artery from a different case than A or C, double labeled for VCAM-1 in black and α-actin in red. Colocalization of VCAM-1 and α-actin expression to smooth cells is again apparent in G, with VCAM-1 localization in these cells and the endothelial cells again abolished with the use of an absorbed sera in H. A, C-H, 220×. B, 60×. Reproduction of this figure in color was made possible through support from Biogen, Inc., Cambridge, Massachusetts, USA.

Laboratories) and the slides were counterstained with methyl green. Negative controls included substituting anti-VCAM-1 antibody which had been absorbed with VCAM-1 positive CHO cells for the primary anti-VCAM-1 antibody, and substituting normal mouse IgG for the anti-α-smooth muscle actin and anti-CD-68.

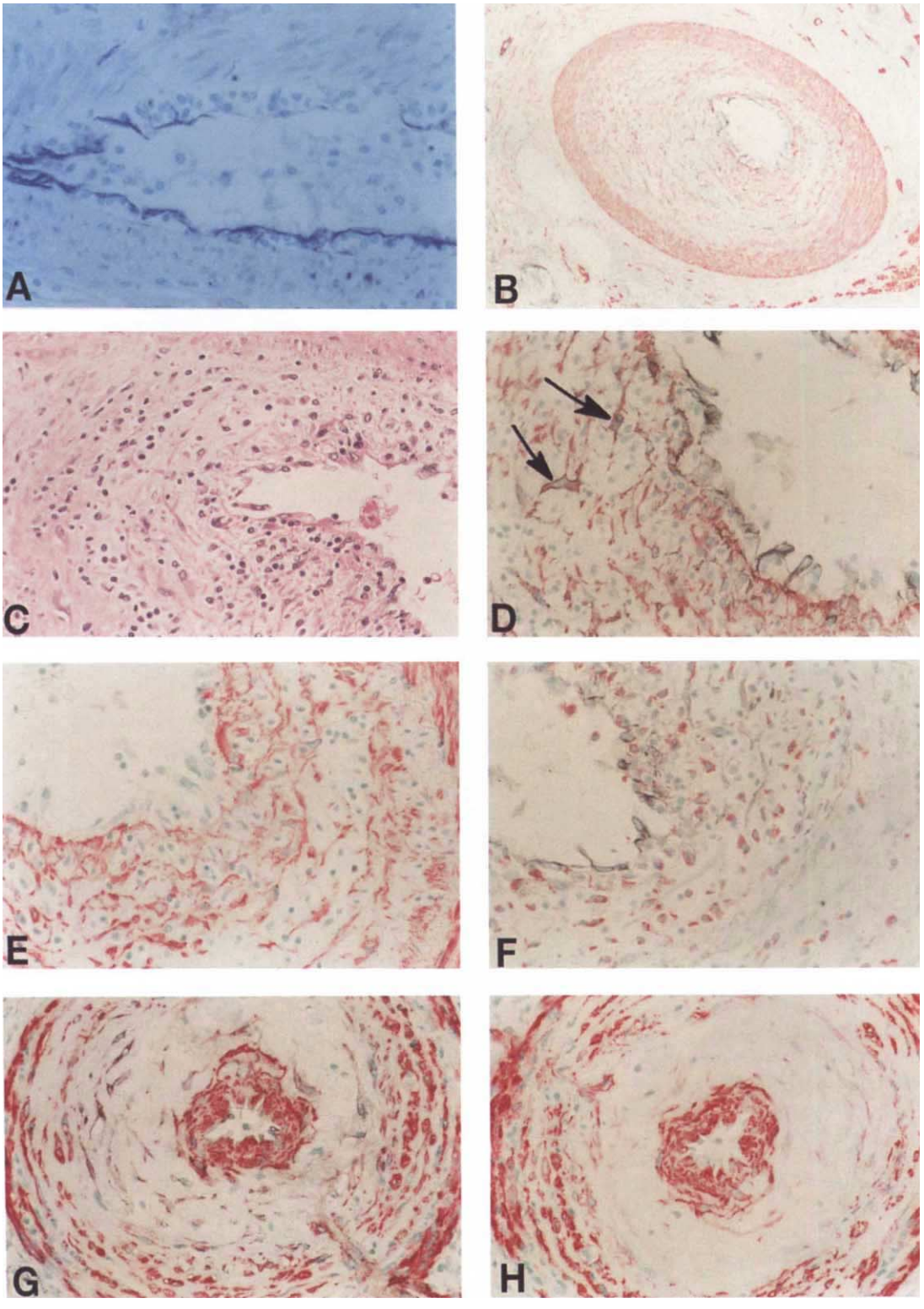
Results

Normal kidney

Our immunohistochemical studies revealed that a detectable level of VCAM-1 is only rarely expressed by endothelial cells lining arterial, venous, peritubular capillary, or glomerular capillary beds in normal kidney. Rare expression by arterial endothelium, usually involving only a single vessel within a large histology sample obtained from a nephrectomy, was present in 6 of 26 cases. The only kidney structure which reliably expresses significant amounts of VCAM-1 is glomerular parietal epithelium (Fig. 1A), a pattern identical to that described by Rice et al in a study on frozen kidney tissues using a different antibody reactive with the VCAM-1 molecule [27]. This finding, while of unknown significance, proved useful in the study of transplant specimens as an internal positive control for assessing the adequacy of immunostaining procedures and interpreting the validity of otherwise negative results. A more variable finding was VCAM-1 expression by tubular epithelial cells, a finding found in a minority (generally less than 10%) of such tubular segments in 60% of the normal kidneys studied. Occasionally, regional VCAM-1 expression by peritubular capillaries could be identified (Fig. 1B). Staining patterns were similar in frozen and fixed kidney sections, but with appreciably better morphologic preservation in the fixed tissues.

Transplant kidney biopsies without rejection

Patterns of VCAM-1 expression in the kidneys was generally similar to those encountered in normal kidneys. VCAM-1 expression by arterial endothelium, smooth muscle cells, or mesangial cells specifically was not a feature of the protocol transplant biopsies or those showing mild inflammatory infiltration of uncertain significance. The biopsies indicative of cyclosporine toxicity were noteworthy for one case with widespread VCAM-1 expression by tubular segments, and a second case showing VCAM-1 expression by arterial endothelium in a single muscular artery segment without evidence of inflammatory cell infiltration. Clinical follow-up of the first of these two cases confirmed the diagnosis of cyclosporine toxicity; no immediate clinical episodes of rejection subsequent to biopsy occurred. The second patient whose biopsy showed focal arterial expression of VCAM-1 developed a severe episode of biopsy-proven vascular rejection less than one month after this initial biopsy. The later biopsy was submitted fixed in its entirety in formalin, and VCAM-1 expression could not be ascertained.



Transplant kidneys with features of rejection

The variety of forms and chronologic stages of injury present in the tissues available for this study allowed the identification of five distinct sites of VCAM-1 expression in rejecting kidney. The first and most striking pattern of VCAM-1 expression involved the arterial vasculature. Focal, but frequently pronounced staining of the endothelial lining of muscular arteries

could be identified in those vessels showing features of acute vascular rejection, that is, with features of endothelial separation from the underlying intima and/or internal elastic membrane, adherence of mononuclear leukocytes to damaged endothelium, and the subendothelial infiltration of mononuclear leukocytes (Fig. 2). Such expression of VCAM-1 was typically more widespread and characterized by more intense staining than the isolated arterial expression noted in the normal kidneys and single cyclosporine nephrotoxicity case noted above. The endothelial expression of VCAM-1 was determined by obvious morphologic appearance and location of cells reactive with the VCAM-1 antisera, as well as similar staining patterns obtained in replicate sections in which endothelial binding by the lectin Ulex I was assessed. Immunophenotypic characterization of the infiltrating leukocytes within the arterial intima revealed that virtually all could be identified as belonging to T-lymphocyte ($CD3^+$) and/or monocyte/macrophage ($CD68^+$), but not B-cell ($CD20^+$) lineages. Only occasional instances of peritubular capillary expression of VCAM-1 could be identified, often in or near areas of prominent cellular rejection with extensive interstitial aggregation of mononuclear leukocytes.

Some arterial vessels with features of both acute rejection and more chronic neointimal sclerosing changes (chronic vascular rejection) showed expression of VCAM-1 by non-leukocytic spindled cells present in the neointima as well as variable expression by the smooth muscle cells comprising the media of these vessels (Fig. 2B-H). Double immunolabeling of histologic sections with a monoclonal antibody to the smooth muscle cell marker α-smooth muscle actin, and the anti-VCAM-1 antisera, demonstrated that these spindled cells were smooth muscle cells (Fig. 2). VCAM-1 expression by smooth muscle cells, often, but not invariably, was accompanied by adjacent infiltration of the tissue of arterial neointima by T cells and monocyte/macrophages. VCAM-1 expression by medial smooth muscle cells was generally not accompanied by inflammatory cell infiltration. More specific histologic patterns which corresponded to VCAM-1 expression by arterial smooth muscle cells were not discerned.

The second pattern of VCAM-1 expression was identified within the large tissue samples provided by allograft nephrectomies, and was related to the presence of occasional, distinct interstitial lymphoid aggregates, which by immunophenotypic characterization usually could be shown to be predominantly composed of T cells ($CD3^+$) with a smaller number of B cells ($CD20^+$) (Fig. 3). Less commonly, these aggregates contained a more prominent B cell component, comprising up to half of the cells present (Fig. 3D). Within these aggregates was a small but distinct population of cells with dendritic morphology which showed focally prominent expression of VCAM-1 (Fig. 3B), NGFR (Fig. 3E) and CD35 (not shown). By both morphologic appearance and this immunohistochemical characterization, these cells could be identified as dendritic cells similar to those found in lymph nodes and tonsils.

The third pattern of VCAM-1 expression was focal expression by glomerular mesangial cells (Fig. 4). Double immunolabeling techniques generally revealed that most VCAM-1 expressing mesangial cells also expressed α-smooth muscle actin (Fig. 4B), a finding that has been shown previously to correlate with mesangial cell injury or activation in both experimental animals and humans [41, 42]. This pattern of mesangial cell expression of VCAM-1 was most prominent in cases also showing VCAM-1 expression by vascular smooth muscle cells. It was not associated with specific glomerular abnormalities such as glomerulonephritis or acute allograft glomerulopathy; no cases exhibiting these features were available for this study.

The fourth pattern of VCAM-1 expression included the persistent widespread expression without discernible changes from normal kidneys of VCAM-1 by glomerular parietal epithelial cells. The fifth pattern was the persistent and frequently upregulated expression of VCAM-1 by a subpopulation of tubular cells (Figs. 4A, 5). This tubular expression was more widespread in the allograft nephrectomies as compared with normal kidneys, which were the pathologic specimens where the amount of tissue available for examination allowed for such comparisons to be made. Tubular expression was typically basolateral in distribution (Fig. 4A), although at times VCAM-1 expression was detected over the entire cell surface. In some cases tubules expressing VCAM-1 could be identified as proximal tubules; most often a distinction between proximal and distal tubular cell expression could not be made because of the extent of tissue injury. In most allograft kidneys, VCAM-1 expression involved only a minority of the tubular segments and could not be clearly correlated with concomitant features of infiltration of tubular segments by mononuclear leukocytes. In most rejection biopsies, however, tubular expression of VCAM-1 was clearly more extensive than control biopsies and was in some cases widespread. Such cases often, but not always, had adjacent accumulations of inflammatory cells in the interstitium.

Studies in which the primary antisera to VCAM-1 was replaced by PBS or non-immune rabbit serum, or when the primary antisera was absorbed with VCAM-1 expressing CHO cells, resulted in abolishing the specific staining patterns described above (Fig. 2E, H).

In situ *hybridization*

The *in situ* hybridization procedures revealed that cell types previously identified as expressing VCAM-1 by immunocytochemical techniques also expressed mRNA for VCAM-1. Specifically, in rejecting kidneys, the VCAM-1 probe hybridized to mesangial and parietal epithelial cells within the glomerulus, arterial endothelium and neointimal smooth muscle cells in arteries demonstrating acute and chronic rejection, and epithelial cells of the distal tubules (Fig. 6). The VCAM-1 mRNA probe also hybridized to lymphoid aggregates in allografts in a distribution consistent with production by dendritic cells. However, because of the compressed cellularity of these aggregates, the fact that dendritic cells extend thin cell processes throughout the aggregates, and the lack of distinct morphologic features of dendritic cells, the radioisotopic labeling could not be unequivocally localized solely to dendritic cells in these areas.

Discussion

This study adds to a growing body of evidence that VCAM-1 expression is up-regulated in the course of solid organ transplant rejection [21–26]. In kidney allografts, VCAM-1 may be expressed by multiple cell types, including parietal epithelial cells and mesangial cells in the glomerulus, endothelial cells and smooth muscle cells of the arterial wall, endothelial cells of the interstitial capillaries, dendritic cells within the interstitium,

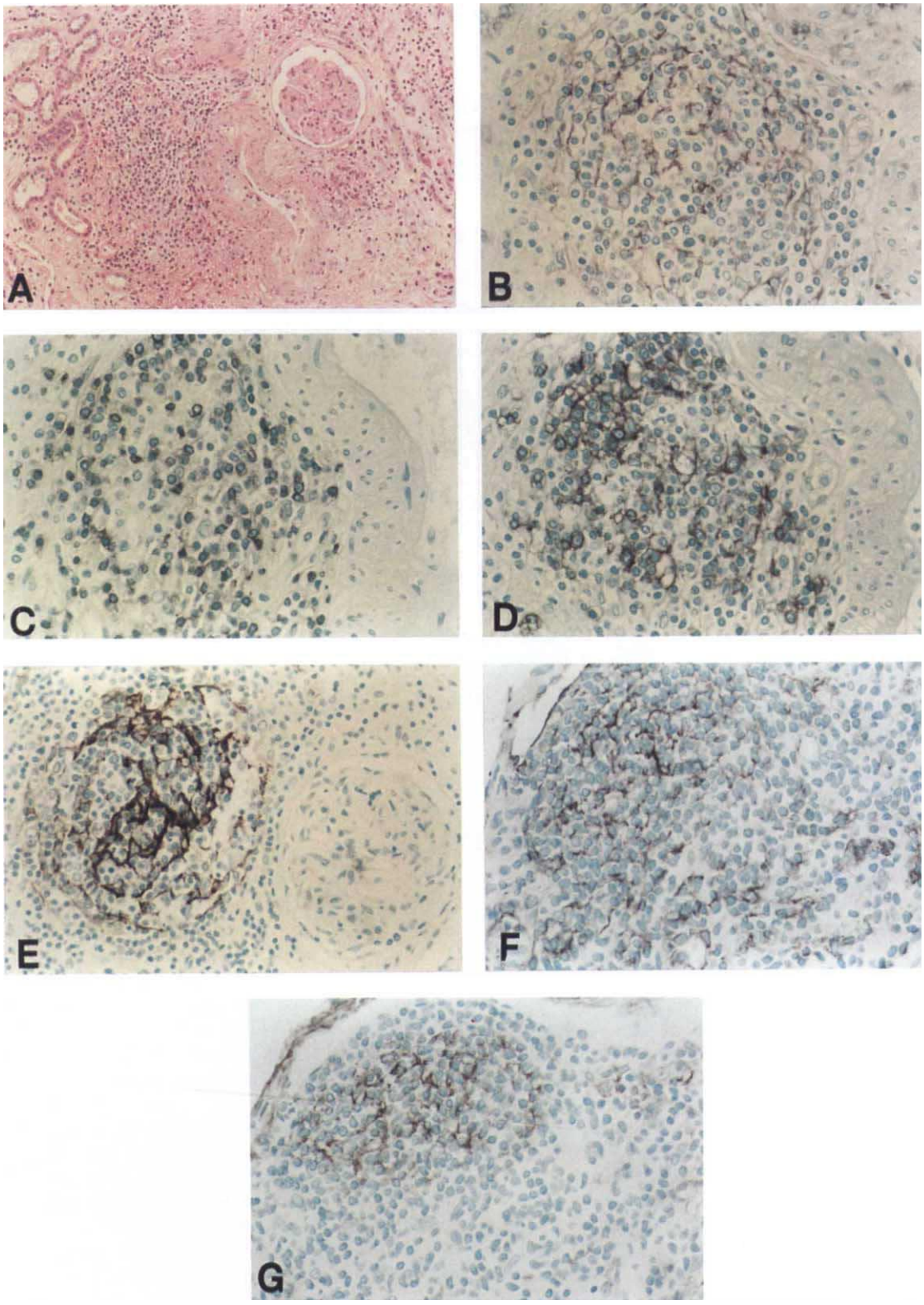


Fig. 3. *(A) Advanced allograft rejection; with an interstitial lymphoid aggregate.* **(B)** A population of dendritic cells expressing VCAM-1 within this aggregate. **(C)** Many $CD3^+$ T-lymphocytes are present in this aggregate. **(D)** Many $L26^+$ B-cells are also present in this aggregate, although the number of B cells encountered in such aggregates is usually less. **(E)** A similar lymphoid aggregate showing expression of p75 nerve growth factor receptor (NGFR) by the same dendritic cells that express VCAM-1. **(F)** Another lymphoid aggregate in a renal allograft biopsy different from that illustrated in D and E showing expression of VCAM-1. **(G)** Replicate (but not immediately adjacent) section of the same lymphoid aggregate illustrated in F showing expression of NGFR by same dendritic cells seen to express VCAM-1. A, 110×. B-D, 330×. E, 220×. F, G, 330×. Reproduction of this figure in color was made possible through support from Biogen, Inc., Cambridge, Massachusetts, USA.

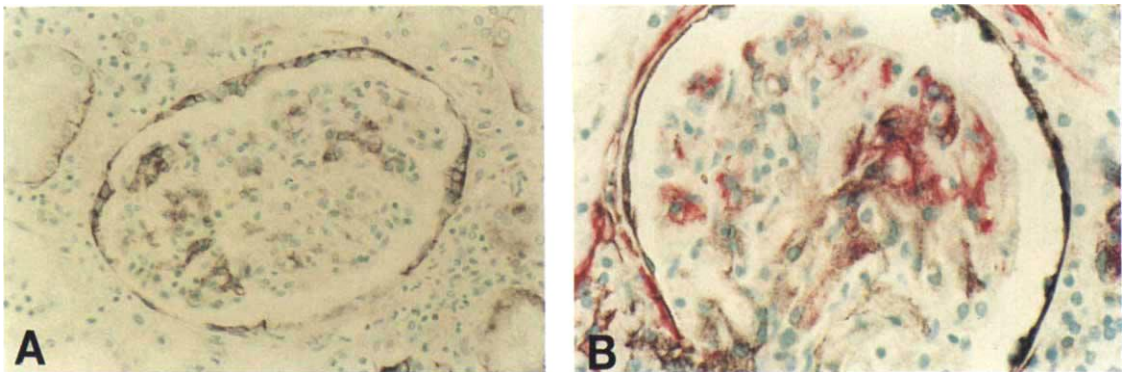


Fig. 4. (A) *Glomerular expression of VCAM-1 in a case of severe rejection.* Prominent parietal epithelial cell expression of VCAM-1 persists, while mesangial expression is now prominent. Adjacent tubules show enhanced, basolateral expression of VCAM-1 as well. (B) Double labeling of glomerulus from same case shows VCAM-1 expression in black, α-actin expression in red. The up-regulated expression of VCAM-1 by mesangial cells is accompanied by up-regulated expression of α-actin. A, 220×. B, 330×. Reproduction of this figure in color was made possible through support from Biogen, Inc., Cambridge, Massachusetts, USA.

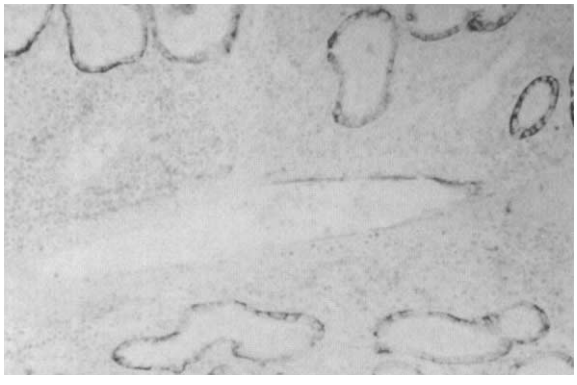


Fig. 5. *Same case but different artery as in Figure 2A.* There is an adjacent population of tubular cells showing up-regulated expression VCAM-1, with staining intensity most pronounced at the basolateral surface. 100×.

and some tubular epithelial cells. The expression of VCAM-1 by two of these cell types in human kidneys—the vascular smooth muscle cell and a population of interstitial dendritic cells indistinguishable from those identified in lymphoid organs and lymphoid aggregates in other sites—has not been recognized previously. Because VCAM-1 appears to have known biologic functions that serve both to recruit and activate certain classes of leukocytes, the up-regulated expression of VCAM-1 at specific tissue sites of injury suggest mechanisms that may account for the focality of the leukocytic accumulation and tissue injury that is characteristic of transplant rejection.

Perhaps the most striking finding of this study is the VCAM-1 expression in muscular arteries engaged in acute and chronic vascular rejection. This study demonstrates that VCAM-1 expression can be identified on muscular artery endothelium at the time of acute vascular rejection, and can be localized to areas where T cells and monocyte/macrophages infiltrate and accumulate in the subendothelial space. This finding underscores the significance of the endothelial VCAM-1 expression, because it is T lymphocytes and monocytes/macrophages which are among the leukocyte subpopulations which constitutively express the VLA-4 integrin (CD49d/CD29) on their cell surface, and hence would be the cell populations expected to adhere to cells with surface VCAM-1 expression [2, 3]. The endothelial expression of VCAM-1 in muscular arteries is only rarely seen in the cohort of normal tissues, and it is only rarely present in the cohort of transplant biopsies without detectable rejection. These findings provide evidence for a role for up-regulated expression VCAM-1 in attracting specific leukocyte populations to parenchymal sites of rejection.

Equally striking in this study is the prominent expression of VCAM-1 by smooth muscle cells of the entire vascular tree. VCAM-1 was focally present in both large and small arteries, and expression focally extended even to individual glomerular arterioles in nephrectomy specimens removed for severe, irreversible rejection. The neointimal proliferation characteristic of chronic vascular rejection was especially notable for expression of VCAM-1. Double immunolabeling procedures allowed us to ascertain α-actin expressing smooth muscle cells were the principle cell type expressing VCAM-1 in this locale. Our *in situ* hybridization studies indicate this expression of VCAM-1 is due to active synthesis of this peptide rather than non-specific adherence or endocytosis of VCAM-1 secreted by other cells such as circulating leukocytes.

The significance of VCAM-1 expression by vascular smooth muscle cells is uncertain. However, smooth muscle cell expression of VCAM-1 has been recently recognized in other settings of vascular injury, notably atherosclerosis in humans [44] and in a primate model of dermal injury mediated by the cytokines tumor necrosis factor and IL-4 [45]. Based on the observation that elevated VCAM-1 expression by smooth muscle cells in human atherosclerosis may be associated with focal inflammation of the plaque, it has been proposed that this up-regulated expression as well as up-regulated smooth muscle cell expression of other molecules important in the immune response such as class II MHC peptides may be reflective of an "activated" state that allows the smooth muscle cell to participate in the immune/inflammatory response.

A third principal finding is the identification of VCAM-1 expressing dendritic cells within lymphoid aggregates that were present in some nephrectomy specimens. We and others have previously demonstrated VCAM-1 expression on both follicular

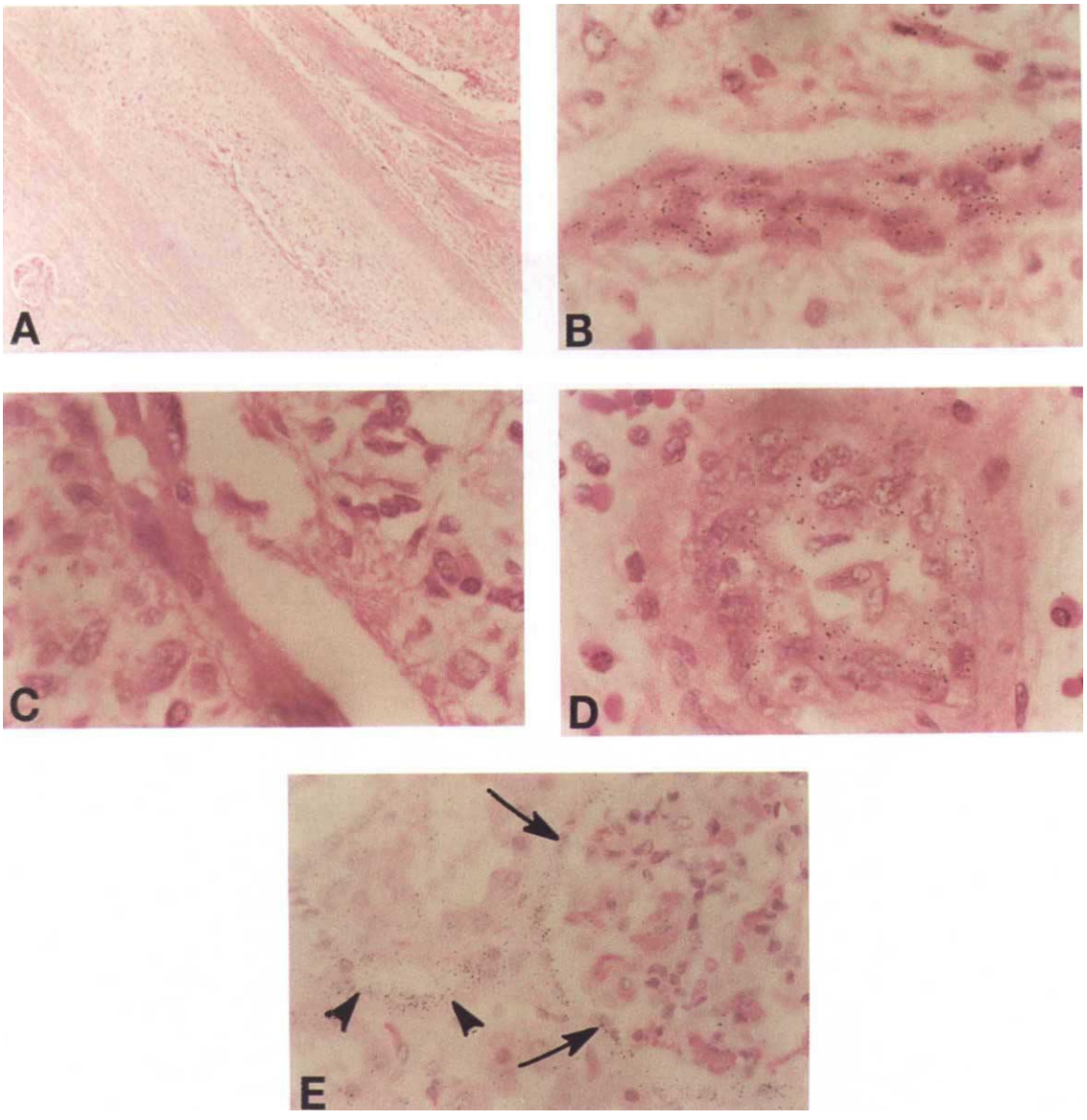


Fig. 6. (**A**) *Low power view of muscular artery from allograft nephrectomy studied by* in situ *hybridization with antisence probes for VCAM-1 mRNA*. There are features of neointimal proliferation (chronic rejection) and superimposed acute rejection (endothelial swelling, inflammatory cell infiltration). (**B**) Same artery as A. VCAM-1 mRNA protection in endothelial cells is evident by discrete localization of grains indicative of hybridized probe. (**C**) Same artery as B, hybridization with a control sense probe to VCAM-1 mRNA. No discrete hybridization is detected. (**D**) Small muscular artery with features of acute vascular rejection, again showing prominent endothelial production of VCAM-1 mRNA. (**E**) Allograft kidney with prominent VCAM-1 mRNA production by glomerular parietal epithelial cells (arrows) and tubular epithelial cells (arrowheads). No specific hybridization was detected using control sense probes to VCAM-1. A, 60×. B-D, 600×. E, 360×. Reproduction of this figure in color was made possible through support from Biogen, Inc., Cambridge, Massachusetts, USA.

dendritic cells and interdigitating cells in T cell dependent areas of lymph nodes [23, 27, 46, 47]. Further, we have demonstrated that prominent numbers of VCAM-1 expressing dendritic cells can be identified within lymphoid aggregates in rejecting liver and pancreas allografts [23]. Others have found similar cells in lymphoid aggregates occasionally encountered in inflamed synovial tissue [48]. The lymphoid aggregates identified in this study were indistinguishable morphologically from those encountered and illustrated in our prior studies of liver and pancreas rejection [23]. Immunohistochemical characterization of the VCAM-1 expressing dendritic cells in kidneys showed they also typically express the p75 nerve growth factor receptor and C3b complement receptor (CD35) similar to follicular dendritic cells encountered in other lymphoid tissues [31, 32, 49]. Finally, our studies of normal and non-rejecting allograft biopsies failed to detect a stable population of VCAM-1 expressing dendritic cells within the kidney parenchyma, which is in agreement with others [27, 50]. Therefore, such cells must either migrate from host lymphoid tissues to the kidney allograft at times of rejection, or a constitutive population of such cells

must undergo phenotypic modulation from VCAM-1$^-$ to VCAM-1$^+$ in the course of active rejection. We emphasize that this last possibility is at present theoretical; we know of no direct evidence that such modulation may occur in dendritic cells.

Given the infrequency with which lymphoid aggregates are encountered in routine kidney allograft biopsies demonstrating rejection, the role of dendritic cells and these aggregates in mediating rejection may be open to question. However, evidence that dendritic cells may have biologic functions important to the rejection response come from studies that VCAM-1 expression by follicular dendritic cells promotes adhesion of lymphoid cells, and hence may help further localize immunocompetent cells to sites of immune injury [46, 49]. In addition, studies by several groups of investigators have demonstrated that VCAM-1 interactions with lymphocytes extend beyond enhanced leukocyte adhesion. These studies suggest the VCAM-1/VLA4 binding process provides additional co-stimulatory activation of resting T cells beyond that provided by antigen and MHC class II molecules, thus providing a means to amplify or promote immune reactions mediating allograft rejection [51–53]. Based on previous studies of the biology of dendritic cells, it is also likely that VCAM-1 expressing dendritic cells directly present foreign antigens to alloreactive immune cells. Taken together with the localization data presented in this study, there is now sufficient evidence to suggest VCAM-1 expressing dendritic cells may participate in local recruitment and activation of lymphocytes in rejecting kidney allografts.

In agreement with others, basolateral tubular expression of VCAM-1 was focally identified in normal kidneys, and was up-regulated in rejecting kidneys as determined both by number of involved tubular segments and by a subjective assessment of increased staining intensity [25, 26]. Differences between our findings and those illustrated by Briscoe et al were that we usually did not find evidence of VCAM-1 expression by a majority of tubular segments in rejecting allografts [25]. The somewhat lesser extent of tubular VCAM expression encountered in our study may have been due to the use of fixed tissues, with some resultant loss of antigenicity, in contrast to the unfixed frozen tissue sections utilized by those investigators and others [25, 26].

In addition to transplant rejection, enhanced tubular expression of VCAM-1 has been described in human drug-induced tubulointerstitial nephritis, in experimental models of lupus nephritis, and in cultured cells [54–56]. Although evidence is somewhat scanty at present, it has been proposed that renal tubular epithelial cells may present antigen to immunocompetent cells and that furthermore, by their expression of leukocytic adhesion molecules, may further promote and localize immune/inflammatory injury in the kidney [57, 58]. This study lends support to this scenario being an important mechanism in renal transplant rejection.

In agreement with others, glomerular expression of VCAM-1 by parietal epithelial cells and induced expression by mesangial cells was also identified in this study [25, 27, 50]. The mesangial cell expression was noteworthy because it occurred in conjunction with up-regulated mesangial expression of α-smooth muscle actin, a cytoskeletal protein previously shown to be up-regulated in the course of mesangial injury or activation [41, 42]. Up-regulated mesangial cell expression of VCAM-1 *in vitro* has been shown in response to cytokines and inflammatory stimuli [59]. Although the mesangial cell is not commonly thought to have a major role in mediating transplant rejection, it is possible the up-regulated VCAM-1 expression we and others have identified may help explain how monocytes/macrophages often become concentrated in glomeruli during severe rejection [1, 39].

In summary, we have provided evidence that: (1) expression of VCAM-1 by endothelial cells and other renal cell types may define sites of acute inflammation in renal transplant rejection; (2) smooth muscle cells in injured muscular arteries may express VCAM-1, and (3) there is probably a population of VCAM-1 expressing dendritic cells that migrates into host kidneys and participates in the cellular rejection process. Due to the nature of a static morphology study, our findings can not distinquish between the possibility that the VCAM-1 expression identified is a primary mechanistic process for recruiting and activating leukocytes within the rejecting kidney versus the alternate possibility that VCAM-1 expression is a secondary response induced by a population of activated leukocytes already present within the parenchyma. Some evidence in experimental models of transplantation indicate a primary role for VCAM-1. Interruption of the binding of endothelial VCAM-1 with integrin counter receptors on leukocytes in these models appears to ameliorate the inflammatory response [60, 61]. The relative importance of VCAM-1 expression by various renal cell types in comparison to expression of other molecules such as ICAM-1 was also not addressed by our study. We believe these questions in humans may be resolved by protocol biopsy studies, in which early expression of VCAM-1 or related leukocyte adhesion molecules may predict subsequent occurrences of clinically detectable rejection. Such studies are currently in progress.

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