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Endothelial Expression of VCAM-1 in Experimental Crescentic Nephritis and Effect of Antibodies to Very Late Antigen-4 or VCAM-1 on Glomerular Injury

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The migration of leukocytes into glomeruli in crescentic glomerulonephritis is fundamental to pathogenesis, and offers important therapeutic opportunities. We addressed the importance of VCAM-1, and its leukocyte ligand very late antigen-4 (VLA-4), in such leukocyte migration. In a rat model of nephrotoxic nephritis, glomerular expression of VCAM-1, studied by immunohistochemistry, was up-regulated by day 6 of nephritis. To quantify kidney endothelial VCAM-1 expression, a differential radiolabeled mAb technique was used, which demonstrated that protein expression was not up-regulated by day 2 of nephritis, but rose threefold between days 2 and 5, and remained elevated until at least day 28. An in vivo study was then performed, using blocking mAbs to either VCAM-1 or VLA-4, starting mAb treatment on the day prior to disease induction, and continuing until animals were sacrificed at day 7. mAbs to VLA-4 significantly attenuated renal injury (albuminuria, glomerular fibrinoid necrosis, and crescent formation), but mAbs to VCAM-1 had no significant effect. Surprisingly, the number of leukocytes within glomeruli was unaffected by anti-VLA-4 mAb therapy, despite the reduction in renal injury. Paradoxically, classical markers of macrophage activation were increased in the anti-VLA-4- and anti-VCAM-1-treated animals. This study demonstrates that kidney endothelial VCAM-1, in contrast to ICAM-1, is not up-regulated by day 2 of nephrotoxic nephritis, and plays little part in early leukocyte influx into glomeruli. However, VLA-4 is an important mediator of glomerular injury, operating after transendothelial leukocyte migration, and presumably binding to alternate ligands within the kidney.

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eukocyte migration into glomeruli is a typical feature of human glomerulonephritis (GN), and leukocytes are key mediators of kidney damage (1). A similar leukocyte influx is also seen in several animal models of GN (2), allowing detailed study of the control of leukocyte trafficking into the kidney. It is widely assumed that leukocytes leave the glomerular capillaries to enter the extracapillary space under the influence of classical cell adhesion molecules. Such molecules are known to be key to leukocyte emigration from postcapillary venules in artificially inflamed microvascular beds, such as the mesentery and cremaster, where vessels can be directly stimulated (e.g., by cytokine application) and leukocyte movement visualized by intravital microscopy (3–6). These classical adhesion molecules include selectins with their carbohydrate ligands, and the leukocyte integrins, LFA-1 and very late Ag-4 (VLA-4), and their respective endothelial cell ligands, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both members of the Ig superfamily (7–9).

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3 Abbreviations used in this paper: GN, glomerulonephritis; NTN, nephrotoxic nephritis; WKY rat, Wistar-Kyoto rat; GBM, glomerular basement membrane; FN, fibronectin; Mac-1, monocyte cell lineage membrane; NTS, nephrotoxic serum; %ID/g, percentage of injected dose per gram of tissue; VLA-4, very late Ag-4, PMN, neutrophil.
monocyte migration into glomeruli has not been entirely elucidated. In an early study examining PMN influx in the first 6 h of NTN in the Long-Evans rat, using various mAb that block integrins, Mulligan et al. (10) showed a partial role for VLA-4. PMN from rats express VLA-4 at levels sufficient to play a role in PMN recruitment (26), which explains this effect. In a model of NTN in Lewis rats, Wu et al. (27) duplicated the effect on glomerular PMN migration (20% reduction in the first 24 h) using the same anti-VLA-4 mAb (TA-2). However, the mAb did not attenuate proteinuria or monocyte migration into glomeruli up to 72 h after administration of anti-GBM Ab. It should be noted that TA-2 binds to the α7 integrin chain and also inhibits function of the αβ3 integrin, a leukocyte receptor for the mucosal addressin cell adhesion molecule (MadCAM-1). To date, however, there is no evidence of either MadCAM-1 expression in the kidney, or recruitment of αβ3-expressing leukocytes to this organ. The only other study to examine directly the role of VLA-4 in renal disease was in the mercuric chloride model of interstitial nephritis, and it demonstrated a reduction in interstitial lymphocyte infiltration in animals receiving anti-VLA-4 mAb (28, 29). A very recent study in the model suggested that anti-VCAM-1 mAb also inhibits leukocyte recruitment into the renal interstitium (29). Significantly, leukocyte influx into glomeruli is not a feature of the mercuric chloride model.

We wished to clarify the importance of VCAM-1 and VLA-4 in monocyte migration into glomeruli in inflammatory GN, beyond the first wave of PMN influx. We first quantified and examined the time course of expression of VCAM-1 on endothelial cells in a model of NTN in the WKY rat. We achieved this by immunohistochemistry and by using a dual radiolabeled mAb technique. We then compared the roles of VLA-4 and VCAM-1 in the development of GN by giving blocking mAb up to day 7 of nephritis, by which time severe crescentic glomerular injury has commenced.

Materials and Methods

Animals

Male WKY rats aged between 6 and 8 wk (weight 200–250 g) were purchased from Charles River Laboratories (Margate, U.K.) and fed standard rat chow and water ad libitum. They were housed individually for 24 h in metabolic cages for urine collection prior to sacrifice. All procedures were performed according to United Kingdom Home Office regulations.

Antibodies

Blocking murine IgG1 anti-rat α5 integrin mAb TA-2 (30, 31) was purchased from PharMingen (San Diego, CA). Blocking murine IgG2a anti-rat VCAM-1 mAb (5F10) (32, 33) and isotype-matched controls P1.17 (IgG2a) and 1E6 (IgG1) were purified as described (30, 33) and contained <5.3 EU/mg protein.

Nephrotoxic nephritis model

Heterologous nephrotoxic serum (NTS) was prepared by standard methods. Briefly, rat glomeruli were isolated, sonicated, and lyophilized to provide a rat GBM preparation with minimal (<5%) tubular contamination. Albino rabbits were immunized with rat GBM in CFA, then boosted at monthly intervals with GBM in IFA until high titer anti-GBM antiserum was obtained at test bleeds. A total of 0.1 ml of NTS was then injected i.v. into WKY rats, leading to the development of NTN. The rats were sacrificed at various stages of nephritis up to day 28, according to the experimental protocols.

Assessment of renal disease

Urine was collected in metabolic cages for 24 h prior to harvest (days 6–7). Urinary albumin concentration was determined by rocket immunoelectrophoresis, and total albumin excretion over 24 h was calculated (34). Peripheral blood was taken at harvest for determination of serum creatinine concentration using Olympus reagents and an Olympus AU600 analyzer (Olympus, Eastleigh, U.K.). Urinary creatinine concentration was also measured (Bayer RA-XT, Newbury, U.K.) to permit calculation of creatinine clearance.

At harvest, rats were perfused with 50 ml PBS by aortic cannulation and the kidneys removed. One portion of kidney was fixed in formaldehyde and embedded in paraffin. Sections were cut, stained by hematoxylin-eosin and periodic acid-Schiff, and examined in blinded fashion by an experienced renal histopathologist (H.T. Cook). The percentage of glomeruli containing crescents was determined by examining 50 consecutive glomeruli per section. Similarly, the mean number of apoptotic bodies contained within glomeruli (excluding areas of necrosis) was calculated from examination of 50 consecutive glomeruli per section.

Computer-aided image analysis of glomerular fibrinoid necrosis

To quantify fibrinoid necrosis within glomeruli, paraffin-embedded kidney sections were stained using a standard MSB trichrome technique (Martius Yellow, Brilliant Crystal Scarlet, and Aniline Blue) which stains fibrin a red/orange color. Sections were then examined under ×200 magnification using an Olympus BX45 microscope (Olympus Optical, London, U.K.) mounted with a Photonic Science Colour Coolview digital camera (Photon Science, East Sussex, U.K.). Images were captured and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD), and color segmentation was performed for each slide individually, defining pixels that contained appropriate red/orange coloration. For each slide, 50 consecutive glomeruli were defined as areas of interest, and the percentage of each glomerular cross-sectional area comprising fibrin was calculated. The final value for each slide was derived by taking the mean of the results from the 50 glomeruli.

Immunohistochemistry

The mAb used were 5F10, ED1 (anti-CD68-like molecule in cytoplasm of rat monocytes/macrophages), ED3 (anti-sialoadhesin), HIS19 (anti-rat class II MHC, RT1b), PC10 (anti-proliferating cell nuclear Ag) and clone 6 (anti-inducible nitric oxide synthase (iNOS), Transduction Laboratories, Lexington, KY). Apart from 5F10 and clone 6, all mAbs were purchased from Serotec (Oxford, U.K.). Kidney tissue was snap frozen in isopentane and subsequently stored at −70°C. Five-micrometer sections were cut on a cryostat and endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol. Sections were blocked with normal rabbit serum and incubated overnight at 4°C with 5F10, ED3, or HIS19. Sections were washed and bound Ab was detected using the avidin-biotin complex method (Dako, Glostrup, Denmark) with the chromogenic substrate 3,3′-diaminobenzidine (DAB). Sections were counterstained with hematoxylin and mounted. For ED3 and HIS19, positive cells were counted in at least 20 glomeruli by HTC in blinded fashion.

For both ED1 and anti-iNOS Abs, paraffin-embedded sections were first heated in 10% citrate buffer in a microwave oven. Sections were then treated as for frozen tissue with blocking of endogenous peroxidase, blocking with normal sheep serum, and incubation overnight with primary mAb at 4°C. Sections were washed, incubated with peroxidase-conjugated sheep anti-mouse Ig Fe (Amersham, Buckinghamshire, U.K.), and bound Ab was detected with 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin, and mounted and positive cells were counted in at least 50 consecutive glomeruli by HTC in blinded fashion.

FACS analysis of glomerular leukocytes

Glomeruli were isolated from kidneys by sequential sieving, counted, then enzymatically digested with trypsin, collagenase, and DNase, as previously described (35), to yield a single cell suspension. Cells were washed in HBSS (Sigma, St. Louis, MO) with 5% FCS (HBSS-FCS), suspended at a concentration of 5 × 10^6/ml, then incubated with primary Ab at 4°C for 45 min. The Abs used were: MRC OX1 (anti-CD45, rat leukocyte common Ag), MRC OX8 (anti-CD8), MRC OX42 (anti-CD11b/c), and MRC OX23 (anti-human complement factor H) as control (all from Serotec), plus BSA3 (anti-CD11a) and BSA4 (anti-CD18), (both from British Biotechnology, Oxford, U.K.). After washing, cells were incubated with fluorescein-conjugated rabbit F(ab′)2 anti-mouse Ig (Dako) with 5% normal rat serum for 30 min at 4°C. Cells were then washed again in HBSS-FCS and underwent FACS analysis (Coulter EPICS, Luton, U.K.) to quantify positively-stained cells after subtraction of background and control mAb binding. The number of positively stained cells per glomerulus was then calculated for each of the animal and mAb conditions. Mice receiving NTS alone in previous experiments gave comparable glomerular cell numbers to the contemporaneous controls of this experiment, assuring reproducibility of results.
Radiolabeled Abs

Anti-VCAM-1 (5F10) and isotype-matched control mAb (P1.17, IgG2a) were radiolabeled with 125Iodine (125I) and 111Indium (111In), respectively (Amersham), as previously described (36, 37). To quantify endothelial VCAM-1 expression, a mixture of approximately 25 μg each of radiolabeled 5F10 and P1.17 was injected i.v. into control rats (n = 5) or rats with NTN of variable duration (days 1, 2, 5, 7, 14, 21, and 28, n = 5 per group). The activity of injected material was calculated precisely by weighing syringes before and after injection. After exactly 5 min of circulation time, rats were perfused out via aortic cannulation and both kidneys and liver harvested. This short circulation time minimizes the quantity of mAb able to cross the endothelial barrier to bind subendothelial ligand. A short circulation time also reduces the possibility of VCAM-1 shedding from the endothelial cell surface with subsequent redistribution to the spleen, as observed by Harrison et al. (37). Thus organ uptake of anti-adhesion molecule mAb, after subtraction of control mAb binding, reflects endothelial Ag expression in a quantitative manner (37). The organs were weighed, then placed in an automated gamma counter along with isotope standards (to calculate channel crossover) and a known volume of injected mAb mixture (to calculate injected dose). Correction was made for background and crossover between channels, then data were expressed as counts per minute per gram of tissue, and as percentage of injected dose (%ID) per gram of tissue. The %ID/g for control mAb 111In-P1.17 was subtracted from the %ID/g for 125I-5F10 to yield the final corrected %ID/g.

Treatment protocols

The day of induction of NTN by NTS injection was designated day 0. Animals were killed on day 7 by being bled out while under isoflurane anesthesia. Rats received mAb by i.p. injection on days −1, 1, 3, and 6. TA-2 and its control 1E6 were used at a dose of 5 mg/kg, after a pilot study demonstrated little effect at a dose of 2.5 mg/kg. Studies consisted of six animals receiving anti-VCAM-1 or anti-VLA-4 and five animals receiving the IgG1 or IgG2a control mAb. To ensure that the effects of mAb were not mediated by leukocyte depletion, peripheral blood was taken from animals at harvest into EDTA-tubes and cell counts performed on a Sysmex SE 9000 analyzer (Sysmex, Milton Keynes, U.K.).

Statistics

Data are expressed as mean ± SEM. Comparison between test and control groups for in vivo blocking mAb experiments was by two-tailed Mann-Whitney U test. For radiolabeled mAb uptake data, comparison between groups was by analysis of variance with Kruskal-Wallis test (nonparametric), with multiple comparisons between control and other time points made by Dunnett posttest. Differences were considered significant if p < 0.05. Statistical calculations were performed using Prism software (GraphPad Software, San Diego, CA).

Results

Kidney VCAM-1 expression by immunohistochemistry

NTN was induced in WKY rats, and kidneys were harvested at various time points for analysis of VCAM-1 protein expression by immunohistochemistry with the mAb 5F10. Normal WKY rat kidney expressed little VCAM-1, although the parietal epithelial cells of Bowman’s capsule were noted to express some VCAM-1 constitutively (Fig. 1). VCAM-1 was not seen on the luminal surface of normal glomerular endothelial cells. Occasional interstitial cells were positive for VCAM-1, as were some peritubular capillaries and very occasional basolateral surfaces of tubular epithelial cells. Larger venules and arterioles expressed low levels of VCAM-1 along the endothelial surface. At day 4 of nephritis, little difference was seen in the expression pattern of VCAM-1 compared with baseline, although cellular proliferation was seen in glomeruli at this time point. From previous work (both FACS analysis and immunohistochemistry), glomerular leukocytes peak in number at around day 3–4 in this model. By day 6, however, up-regulation of VCAM-1 was evident, with substantial amounts of protein present within glomeruli, particularly on cells within crescents and cells within the tuft. Glomerular endothelial cell VCAM-1 expression was noted at this time point and beyond. VCAM-1 was also expressed more densely on basolateral surfaces of tubular epithelial cells, and in some peritubular capillaries, particularly by day 11 of nephritis (Fig. 1). No VCAM-1 staining was seen on apical surface of tubular epithelial cells at any time point.

Endothelial VCAM-1 expression by radiolabeled mAb uptake

Immunohistochemistry is an insensitive method of detecting endothelial cell surface proteins. Quantitative comparisons between different sections/animals are also unreliable. To quantify kidney endothelial VCAM-1 expression through the course of nephritis, we chose to use the technique of dual mAb radiolabeling, as has been used in pig and mouse models of cutaneous inflammation and
of systemic endothelial activation (36–38). Anti-VCAM-1 mAb SF10 was labeled with 125I, control mAb P1.17 was labeled with 111In, and a mixture of the two mAbs was injected i.v. into normal and nephritic rats. Uptake of both mAbs onto endothelium was measured for kidneys and liver after a short circulation time of 5 min, and subtraction of control mAb counts from anti-VCAM-1 counts allowed reproducible quantification of endothelial Ag expression. By this technique, normal rat kidneys expressed low levels of endothelial VCAM-1, which was unchanged at day 2 of nephritis (Fig. 2). Between days 2 and 5, kidney endothelial VCAM-1 was significantly up-regulated by approximately 3-fold \( (p < 0.01 \text{ for all time points after day 5 inclusive}) \) and remained elevated until day 28. For comparison, liver was examined as an unaffected organ, and there was no significant change in endothelial VCAM-1 at any time point of nephritis compared with the control. It should be noted that this technique does not distinguish between glomerular and interstitial vascular endothelial VCAM-1 expression. To ensure that we were detecting glomerular VCAM-1, rats at day 6 of NTN were injected with 0.5 mg of cold mAb (control or anti-VCAM-1) and perfused after a 5-min circulation time, as for radiolabeled mAb studies. Indirect immunohistochemistry for mouse Ig on frozen kidney sections from these rats demonstrated minimal specific binding of control mAb but extensive glomerular binding of anti-VCAM-1 mAb (Fig. 1).

**The role of VCAM-1 and VLA-4 in glomerular leukocyte recruitment**

Having demonstrated substantial up-regulation of VCAM-1 within the kidney during NTN, the effects of preventive therapy with blocking mAb to either VCAM-1 or to its ligand, VLA-4, were determined. A 7-day study was performed, with mAb being given on days \(-1, 1, 3, \text{ and } 6.\) The effects of mAb therapy on leukocyte migration into glomeruli was examined both by immunohistochemistry and by FACS analysis of glomerular leukocytes. Macrophage infiltration was first examined by ED1 immunohistochemistry and the results are shown in Figs. 3A and 4; neither anti-VLA-4 nor anti-VCAM-1 therapy led to any reduction in glomerular macrophages. Similarly, FACS analysis of glomerular leukocytes (Fig. 3B) showed that treatment with either anti-VLA-4 or anti-VCAM-1 led to no reduction in glomerular leukocyte numbers, including the important CD8 +ve population of cells, as compared with controls. Of note, mAb therapy was not associated with any significant change in numbers of circulating neutrophils, lymphocytes, or monocytes (data not shown).

**Effects of anti-VCAM-1 or anti-VLA-4 mAb therapy on renal injury**

The effects of mAb therapy on renal injury as reflected by albuuminuria are shown in Figure 5A. An 82% reduction in albuminuria was seen after treatment with anti-VLA-4 compared with the control \( (p < 0.01) \). Anti-VCAM-1 mAb, by contrast, reduced albuminuria by only 15% (not significant). In terms of glomerular fibrinoid necrosis, an important precursor to crescent formation (39, 40), anti-VLA-4 therapy led to a 74% reduction \( (p < 0.05) \), whereas anti-VCAM-1 had no significant effect (Figs. 4 and 5B). Anti-VLA-4 therapy also reduced glomerular crescent formation by 58% \( (p < 0.01) \), and anti-VCAM-1 had a small effect (21% reduction, \( p < 0.05) \) (Fig. 5C). By day 7 of the NTN model, renal failure had not yet developed, and therefore the results for serum creatinine and creatinine clearance for all four groups of animals were within normal limits, with no differences observed between groups (data not shown).

**Mechanism of action of mAb therapy**

Simple counting of glomerular macrophage numbers does not allow firm conclusions to be drawn regarding monocyte migration since it has recently been shown that macrophages can proliferate within the kidney (41), and emigration or apoptosis are other potential fates awaiting glomerular leukocytes (42). We therefore examined intraglomerular apoptosis by counting intraglomerular apoptotic bodies (excluding necrotic areas). This technique correlates...
well with results obtained by TUNEL or propidium iodide staining (42), and demonstrated (Fig. 6A) that no difference in the extent of intraglomerular apoptosis was evident at day 7 between any of the experimental groups. Proliferation of leukocytes was assessed by immunohistochemistry for intraglomerular expression of the proliferating cell nuclear Ag (Fig. 6B), which demonstrated no significant difference between groups.

In view of the possibility that leukocyte activation may have been inhibited by the anti-VLA-4 mAb therapy, several classical macrophage activation markers were examined by immunohistochemistry: namely sialoadhesin, class II MHC, and iNOS. Surprisingly, treatment with either anti-VCAM-1 or anti-VLA-4 led to significantly more glomerular cells expressing class II MHC and sialoadhesin, with a nonsignificant rise in iNOS staining cells in the group receiving anti-VLA-4 (Fig. 7).

**Discussion**

This study aimed to clarify the relative importance of VLA-4 and VCAM-1 in the development of glomerular inflammation in a model of NTN in the WKY rat in which monocytes/macrophages predominate. This is an important area since monocytes are involved not only in acute inflammation, but also in glomerulosclerosis, a feature common to both immune and nonimmune forms of progressive renal disease (16).

**FIGURE 4.** Glomerular fibrinoid necrosis (arrow) and crescent formation (arrowhead) was widely seen in the hypercellular glomeruli of animals receiving control mAb (A). Similar hypercellularity was seen in the glomeruli of animals receiving anti-VLA-4 (B), but fibrinoid necrosis and crescent formation was significantly reduced. Glomerular macrophages were detected by immunohistochemistry with ED1 mAb, and similar numbers were seen in the glomeruli of animals receiving control mAb (C) or anti-VLA-4 (D).

**FIGURE 5.** Renal injury and glomerular inflammation in animals receiving anti-VLA-4 (TA-2), anti-VCAM-1 (5F10) or isotype-matched control mAbs (1E6, IgG1 and P1.17, IgG2a). Albuminuria (A) was measured as milligrams/day. Fibrinoid necrosis within glomeruli (B) was assessed by computer-aided image analysis of MSB-stained sections, and for each of 50 consecutive glomeruli, the percentage of glomerular cross-sectional area comprising fibrin was determined and an overall mean calculated. Glomerular crescents (C) were counted in 50 consecutive glomeruli per animal using periodic acid-Schiff-stained sections and the percentage containing crescents was calculated. Anti-VLA-4 mAb significantly reduced albuminuria, glomerular crescent formation, and fibrinoid necrosis within glomeruli (*, p < 0.05, **, p < 0.01).
mAb was not zero (percentage of injected dose localizing to kidney for anti-VCAM-1 incomplete organ perfusion. dothelium (particularly if the endothelium is injured/leaking) and subtracted to correct for mAb penetration into tissues beyond en-
lial adhesion molecule expression (36, 38, 47). In this study, 
been accepted as a useful way of quantifying changes in endothe-
la short circulation time, and then counting isotopic activity has 
proached this issue by measuring the binding to endothelium of 
tifying endothelial adhesion molecule expression, and we ap-
showing substantial staining with the anti-VCAM-1 mAb. 
flamed glomeruli, with crescent cells and probably mesangial cells 
model. Of note, VCAM-1 was also expressed elsewhere in in-
thelial VCAM-1 is up-regulated during GN, in both humans and 
ichemistry. However, as others have observed, glomerular endo-
thelial VCAM-1 is up-regulated during GN, in both humans and 
imal model. Previous examination of the role of VLA-4 in NTN has 
expressed ICAM-1 dependent, and the absence of VCAM-1 up-regulation 
with the appropriate time course is consistent with this.

In the only study to address the role of VCAM-1 in nephritis, Escudero et al. gave 5F10 to rats with mercuric chloride-induced nephritis and observed inhibition of interstitial cellular infiltrates (29), glomerular leukocyte recruitment not being a feature of this model. Previous examination of the role of VLA-4 in NTN has been limited to the very early stages of nephritis (10, 27). Mono-
cyte migration into glomeruli, studied up to day 3 in NTN by Wu et al. (27), was found to be unaffected by blocking mAb to VLA-4. They also observed no reduction in proteinuria in animals receiving anti-VLA-4. Our data on VCAM-1 expression would suggest that looking only at the first 3 days of nephritis might miss any VCAM-1/VLA-4-dependent leukocyte migration since endothelial VCAM-1 is not up-regulated until day 3 at the earliest in our model of NTN.

Therefore, our study is the first to analyze the pathophysiological role of both VCAM-1 and VLA-4 in experimental GN beyond the first wave of PMN-dominated leukocyte migration. To study these adhesion molecules, we used the well-characterized adhesion blocking murine mAb 5F10 (which binds an epitope on domains 1 and 2 of rat VCAM-1) (33) and TA-2 (which binds the rat α4 integrin chain) (30). Both immunohistochemistry of glomeruli and FACS analysis of isolated glomerular cells demonstrated that neither mAb reduced leukocyte recruitment into glomeruli. It is unlikely that higher doses of 5F10 would be effective at reducing recruitment for three reasons. First, no dose-response effect was observed when the 5F10 dose was doubled from 2.5 to 5 mg/kg (pilot study, data not shown). Second, 5F10 at lower doses than 5 mg/kg has proved highly effective at blocking leukocyte migration in vivo in other studies, including migration into kidney intersti-
tium (29, 33). Third, 5F10 administration led to a significant effect
on macrophage activation markers (see below) suggesting that the dose used was biologically effective. Therefore, we can suggest that VCAM-1 has little role in early leukocyte recruitment into glomeruli in this model, and the significance of its up-regulation is unknown. We have not formally excluded the possibility that anti-VCAM-1 mAb may itself function as a proinflammatory ligand by presenting mAb Fc domain to adjacent cells. In vivo studies with anti-VCAM-1 Ab caused an even greater increase in class II and iNOS-expressing cells, whereas 5F10 had no significant effect at 5 mg/kg. The discrepancy between the results with anti-VA-4 and anti-VCAM-1 is interesting. Although VCAM-1 and VLA-4 mAbs show similar efficacy in some in vivo systems of leukocyte recruitment (29, 33), in others anti-VLA-4 is an effective inhibitor whereas anti-VCAM-1 is not (54). Presumably alternative VLA-4 ligands are operative within glomeruli in NTN, and the most obvious candidate is the N-terminal V region of FN, the rat homologue of the CS1 peptide of the CSIII domain of FN (24). FN is present within the mesangium of normal glomeruli (55), and during GN different isoforms are up-regulated, with different patterns in acute leukocyte-rich and chronic fibrotic stages of disease (56–58). In particular, Alonso et al. (57) showed that the V120 protein isoform of FN, containing the rat VLA-4 ligand, was up-regulated in glomeruli in an immune complex model of crescentic GN in rats. We have confirmed, by RT-PCR, that mRNA encoding V120 FN is present in normal and nephritic glomeruli in the NTN model of WKY rats (data not shown). There are other, weaker, VLA-4 ligands such as thrombospondin, and other domains within FN, but their functional relevance is unclear since they bind only to a conformation of the VLA-4 integrin present on highly activated cells (25). It remains possible that there are unidentified VLA-4 ligands within glomeruli. Finally, there is no evidence that kidney endothelium expresses MadCAM-1, which would be another possible explanation for the efficacy of anti-α4 integrin, but not anti-VCAM-1 (by blocking α4β1, integrin).

Together with the effects on albuminuria, anti-VLA-4 mAbs, but not anti-VCAM-1 mAbs, were effective at reducing glomerular crescent formation and fibrinoid necrosis. Our observations suggest that VLA-4 is implicated in tissue injury, mediated by non-VCAM-1 ligands. The dominant glomerular leukocyte in NTN in the WKY rat is the monococyte/macrophage, which expresses VLA-4 (25). Our hypothesis, therefore, is that VLA-4 expressed on macrophages binds a ligand within glomeruli, which signals to the macrophage. This signal results in the macrophages developing a toxic phenotype, leading to tissue damage, fibrin deposition, and crescent formation. Classical T lymphocytes (expressing CD3 and TCR αβ or γδ) are not present in the WKY rat NTN model in significant numbers within the first week, a point of relevance since activated T lymphocytes express VLA-4, and mAb effects in NTN could theoretically be mediated via such a cell population. CD8+ leukocytes are critical to renal injury in this model, as shown by depletion studies, but these cells are not classical T cells, do not express NK cell markers (49), and a subset of them coexpresses the macrophage marker ED1, suggesting that at least some are macrophages. Others have recently demonstrated CD8 expression by rat alveolar and peritoneal macrophages, which differs from T cell CD8 in the ligand-binding domain of the α-chain, but has a signaling function (59, 60).

To examine the state of macrophage activation within glomeruli, we performed immunohistochemistry for several markers of macrophage activation, including sigaloadhesin, class II MHC, and iNOS. It was surprising that, despite the significant reduction of injury, anti-VLA-4 was associated with a significant increase in glomerular cells expressing class II MHC and sialoadhesin. A non-significant rise in iNOS-expressing cells was seen as well. The anti-VCAM-1 Ab caused an even greater increase in class II and sialoadhesin expression within glomeruli, suggesting that although

**FIGURE 7.** Markers of macrophage activation expressed within glomeruli were assessed using immunohistochemistry for sialoadhesin (A), class II MHC (B), and iNOS (C). There was a significant increase in ED3+ cells and MHC class II+ cells within glomeruli in animals receiving anti-VLA-4 (*, p < 0.05) and anti-VCAM-1 (***, p < 0.01).
it has no role in disease pathogenesis, glomerular VCAM-1 is functional. The possibility of mAb Fc-mediated effects on tissue activation has not been formally excluded but, as noted above, such a proinflammatory phenomenon has not been described in previous anti-adhesion molecule studies using intact mAb (10, 18, 21, 29, 33, 50). It is clear that there is dissociation between “activation markers” and glomerular inflammation in this model. It is also becoming apparent that macrophages do not simply operate in binary states of “quiescent” or “activated.” For example, recent work on LPS-priming of murine macrophages suggests that there are two states inducible by LPS, with reciprocal regulation of TNF-α and nitric oxide production according to cytokines expressed during priming (61). It is, therefore, not possible simply to correlate the presence of “activation markers” with macrophage-mediated injury—a greater understanding of the various macrophage phenotypes is required before we can meaningfully interpret such data.

In conclusion, we have demonstrated that VCAM-1 is up-regulated in the glomeruli of rats with experimental GN, but with a delayed time course compared with ICAM-1. The expression of VCAM-1 is probably not critical to monocyte migration into glomeruli. Mononuclear Abs to VLA-4 significantly attenuate renal injury, although without reduction in either leukocyte numbers or classical markers of macrophage activation. Alternative ligands for VLA-4 are implicated in mediating glomerular injury at a point after transendothelial migration of monocytes. Future studies will address the role of VLA-4 in the processes underlying progressive glomerulosclerosis and chronic renal failure.

References


