Clin Exp Immunol 1996; **103**:259–267

Antibodies to proteinase 3 mediate expression of vascular cell adhesion molecule-1 (VCAM-1)

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(Accepted for publication 24 October 1995)

SUMMARY

VCAM-1 was first identified as an adhesion molecule induced on human endothelial cells (HEC) by inflammatory cytokines such as IL-1, tumour necrosis factor (TNF), and lipopolysaccharide (LPS). The molecule binds to a variety of leucocytes, including B cells, T cells, basophils, eosinophils and monocytes. Vascular expression of VCAM-1 has been associated with a number of disease states, including rheumatoid arthritis and vasculitis. The detection of antineutrophil cytoplasmic antibodies (ANCA), especially to proteinase 3 (PR3), has become important in the diagnosis of Wegener's granulomatosis (WG) and related vasculitides. Recently we were able to demonstrate a direct effect of anti-PR3 antibodies on neutrophil–endothelial interactions (Blood 1993; **82**:1221). Binding of anti-PR3 antibodies to their antigen translocated into the membrane of HEC leads to an enhanced adhesion of neutrophils via induction of E-selectin (Clin Exp Immunol 1993; **94**:440). The aim of this study was to investigate the effect of anti-PR3 antibodies on the expression of VCAM-1. HEC was isolated from umbilical vein and cultured on microtitre plates. After preincubatiion with purified anti-PR3 antibody, purified control antibodies (SS-A, SS-B, RNP) (IgG and $F(ab')_2$ fragments) or different cytokines (controls), VCAM-1 was detected on the surface of unfixed HEC by cyto-ELISA and polymerase chain reaction analysis. Incubation of HEC with anti-PR3 antibodies led to a marked increase of endothelial VCAM-1 expression with a peak after 8 h. Incubation with TNF-α also led to maximal VCAM-1 expression after 4–6 h (control). Increased adhesion of T lymphocytes to HEC after binding of anti-PR3 antibodies to their antigen could be confirmed by performing adherence assays. This effect could be inhibited by antibodies to VLA-4. In conclusion, we have been able to show that cytokine-like effects of anti-PR3 antibodies on HEC are not limited to induction of neutrophil adhesion. Anti-PR3 antibodies may thus contribute to the regulation of T lymphocyte migration from the blood by HEC in ANCA-related vasculitides.

Keywords ANCA proteinase 3 vascular cell adhesion molecule-1 endothelial cells vasculitis

INTRODUCTION

The association of anti-neutrophil cytoplasmic autoantibodies (ANCA) with systemic necrotizing vasculitis, including Wegener's granulomatosis (WG) and glomerulonephritis, is well established. C-ANCA (cytoplasmic pattern) are usually reactive with an azurophilic granule constituent known as proteinase 3 (PR3). Several *in vitro* studies support the hypothesis that ANCA are more than an epiphenomenon and are intimately involved in the pathogenesis of these disorders.

Recently we were able to demonstrate that the target antigen of anti-PR3 antibodies is expressed in the membrane of cytokine-primed human endothelial cells (HEC) [1]. In addition, we have described a direct effect of anti-PR3 antibodies on neutrophil–endothelial interactions [2]. Binding of anti-PR3 antibodies to their antigen which is translocated into the membrane HEC leads to an enhanced adhesion of neutrophils via induction of E-selectin.

VCAM-1 was originally identified as an adhesion molecule expressed on cytokine-induced human umbilical vein EC.

VCAM-1 is expressed by endothelium, dendritic cells, macrophages and renal proximal tubules [3,4]. VCAM-1 mediates binding to a variety of leucocytes, including B cells, T cells, basophils, eosinophils and monocytes. VCAM-1 is a counter-receptor for the integrin very late antigen-4 (VLA-4) and has been shown to act as a T cell costimulatory molecule [5]. Vascular expression of VCAM-1 has been associated with a number of disease states, including rheumatoid arthritis and vasculitis. The

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aim of this study was to investigate the effect of anti-PR3 antibodies on the expression of VCAM-1.

PATIENTS AND METHODS

Serum samples

Serum samples were obtained from 185 donors, 50 of whom suffered from clinically active WG. The diagnosis was established on the basis of classical symptoms and the typical histological findings in biopsy specimens as described earlier [6]. Sera of 25 patients with systemic lupus erythematosus (SLE), five patients with Sharp syndrome, five patients with Sjögren's syndrome and 100 healthy blood donors (HBD) served as controls.

Antibody testing

All WG sera were tested for anti-PR3 antibodies by indirect immunofluorescence test (IFT) on fixed neutrophils. ELISA and Western blot. Several antigen preparations served as antigens: alpha-extract of human neutrophils, purified PR3, myeloperoxidase, cathepsin G and elastase [7]. ELISAs were performed as described earlier [8]. PAGE and Western blotting were performed as described previously [9]. In addition, all sera were screened with routine methods for other antibody specificities. Rheumatoid factor (RF) was determined by a latex fixation test (Behring Werke, Marburg, Germany).

Purification of anti-PR3 antibodies

IgG was prepared from five monospecific anti-PR3 antibody-positive WG sera (WG 1, 3 and 4, patients clinically active; WG 2 and 3, patients in remission; C-ANCA titres 1 : 80–1 : 1640) by ammonium sulphate precipitation and ion exchange chromatography on DEAE-Sephadex (Pharmacia, Uppsala, Sweden). Anti-PR3 antibodies were affinity-purified as described earlier for other antibody specificities [10] using purified PR3. $F(ab')_2$ fragments were prepared as previously described [6]. An anti-PR3-specific B cell clone was established and characterized earlier [11]. Antibody preparations were tested for endotoxin using a commercially available E-toxate assay (Sigma, Deisenhofen, Germany).

Purification of anti-Ro (SS-A), anti-La (SS-B), anti-Sm, and anti-RNP antibodies

Antibodies to Ro (SS-A), La (SS-B), Sm and RNP were purified and characterized as described previously [12].

Preparation of cell extracts

An extract (alpha-fraction) of human neutrophils was prepared as described by Rasmussen *et al.* [13] and Savage *et al.* ('acid' extract) [14]. An extract of Hep2 cells was prepared as described earlier [12].

Total extracts of cytokine-treated HEC were prepared according to Elbeda *et al.* [5]. Only cell cultures free of fibroblasts or monocytes obtained after several passages were used for these experiments.

Purification of PR3

PR3 was purified as described by Kao *et al.* [16] and affinity-purified as described by Lüdemann *et al.* [7] using an extract of granulocytes. For inhibition experiments, serum antibodies were mixed with purified PR3 v/v diluted to 0·1 mg/ml protein concentration in PBS, and incubated on a rotator for 1 h at 37°C and 12 h at 4°C. The mixture was centrifuged at 30 000 ***g*** for 15 min at 4°C and the supernatants kept as absorbed material.

Isolation and culture of human endothelial cells

HEC were isolated according to the method of Jaffe *et al.* [18] and cultured under standard conditions. These cells were used for further experiments between passages 4 and 6. Cells of 10 donors were pooled to exclude the influence of blood group antigens. Morphology was confirmed by phase contrast light microscopy showing the typical cobblestone monolayer appearance of cells. Purity of culture was tested with antibodies to factor VIII antigen and Ulex lectine. HEC were passaged on gelatin-coated culture slides (Lab-Tek; Miles Scientific, Naperville, IL) or Primaria culture dishes (Falcon, NJ) [1]. Tumour necrosis factor-alpha (TNF-α; 3 ng/ml; Boehringer, Mannheim, Germany) was added to the medium to test the influence of this cytokine on VCAM-1 expression. Appropriate cytokine concentrations were determined by testing dilutions from 0·001 to 100 ng/ml. Cells on Primaria culture dishes were left unfixed.

Testing of VCAM-1 expression on HEC

HEC were incubated with purified IgF/$F(ab')_2$ fragments (5 μg/ml), purified control antibodies (anti-SS-A, anti-SS-B, anti-Sm, anti-RNP, pol of HBD) or the supernatant of the PR3-specific B cell clone Ho3 diluted 1 : 40 in PBS for 1–120 min in a humid chamber at room temperature or 4°C. After extensive washing with PBS the cells were incubated with an anti-VCAM-1 antibody (no. 1244, Klon 1G11, mouse IgG; Dianova, Hamburg, Germany; dilution 1 : 50) or purified antiRNP antibody (controls). After another washing step the second antibody, anti-mouse horseradish peroxidase (HRP; P16102; Dako, Hamburg, Germany; dilution 1 : 1000) or FITC-conjugated anti-mouse IgG (F313; Dako; dilution 1 : 1000) was added. For detecting surface expression of antigens, all experiments were done with unfixed live cells as described earlier (Mayet *et al.* [7]). Cyto-ELISAs with unfixed cytokine-treated HEC were performed as described by Frampton *et al.* [19] with minor modifications using the same antibodies as described for IFT. To determine specific antibody binding, experiments were performed with HEC preincubated with heat-aggregated human IgG to block Fc receptors. All tests were performed in triplicate.

RNA extraction and semiquantitative polymerase chain reaction

Total cellular RNA was extracted from cells at passages 3–5, from cells after stimulation with recombinant human TNF-α for 8 h (1 ng/ml) and after culture of cells in presence of PR3-specific antibodies using a modification of the technique of Chomaczynski *et al.* [20]. RNA was diluted in double distilled (dd) water (1 μg/14 μl) and heated at 65°C for 5–10 min. Reverse transcription (RT) mix (16 μl/μg sample RNA) consisting of 3 μl oligo $(dT)_{16}$, 0·5 mg/ml (Sigma, St Louis, MO), 150 U M-MLV reverse transcriptase (BRL, Gaithersburg, MD) and 40 U Rnasin (Promega, Madison, WI) in 50 mM Tris–HCl pH 8·3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 125 μM each dATP, dTTP, dGTP, dCTP (Boehringer, Indianapolis, IN) was then added to achieve a final volume of 30 μl. RT was carried out at 39°C for 1 h, after which samples were heated at 65°C for 5–10 min to terminate reverse transcription.

For polymerase chain reaction (PCR) amplification c-DNA (5 μl) was added to PCR mixture (45 μl) to obtain a final volume of 50 μl. The PCR mixture consisted of buffer (10 mM Tris–HCl pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·01% (w/v) gelatin), 50 pmol of 5′ and 3′ primers, 0·1 mM dTAP, dTTP, dGTP, dCTP (Boehringer, Mannheim, Germany), 1·25 U Taq polymerase (Boehringer). The 50 μl reactions were covered with one drop of mineral oil and incubated in a Perkin Elmer Cetus thermal cycler using 30 cycles at 94°C (20 s), 60°C (40 s), 72°C (60 s). Twenty microlitres of each RT-PCR reaction were electrophoresed on Seakem GTG 3% agarose gels (FMC Bioproducts, Rockland, MD) and specific bands were visualized using 1 μg/ml ethidium bromide. The following primers were used in these studies. VCAM-1a: 5′ GGAACCTTGCAGCTTACAGTGACAGC-TCCC3′; VCAM-1a; 5′ CAAGT CTACATATCACCCAAG3′ [21], VCAM-1b; 5′ TGTCAGG CTAAGTTACATATTGAT3′; VCAM-1b; 5′ AACATCAA GTGTTAAACTTCTTAA3′.

For semiquantitative determination of VCAM mRNA expression the amount of sample cDNA was normalized in a first PCR run using GAPDH as a standard. Dilutions of sample cDNAs that yielded equal amounts of GAPDH PCR products were then subjected to a second PCR run using primers specific for VCAM-1.

T cell adhesion assay

T cells were prepared as described by Gmelig-Meyling & Ballieux [22].

HEC were isolated and cultured as described above. Cells (1×10^5) were passaged on Primaria culture dishes (Falcon). After 1–2 days cells were confluent.

The endothelial monolayer was left unactivated (control) or pretreated for 8 h with anti-PR3 antibodies (3 μg/ml). To remove the antibodies cells were washed three times for 15 min with culture medium. The washing procedure was also performed with untreated HEC (controls) to exclude any non-specific activation. T cells (2×10^6) were layered onto the endothelial monolayer and adhered at room temperature or at 4°C. All tests were performed in triplicate on three different days. In addition, some control tests were included in the presence of clone HP 2/1 (Dianova; dilution 1 : 100) to block VLA-4. Non-attached cells were removed by washing five times with culture media and the dishes were examined by phase contrast microscopy to quantify the number of rosettes according to Delneste *et al.* [23]. A rosette was taken into account when more than five T cells were fixed around HEC.

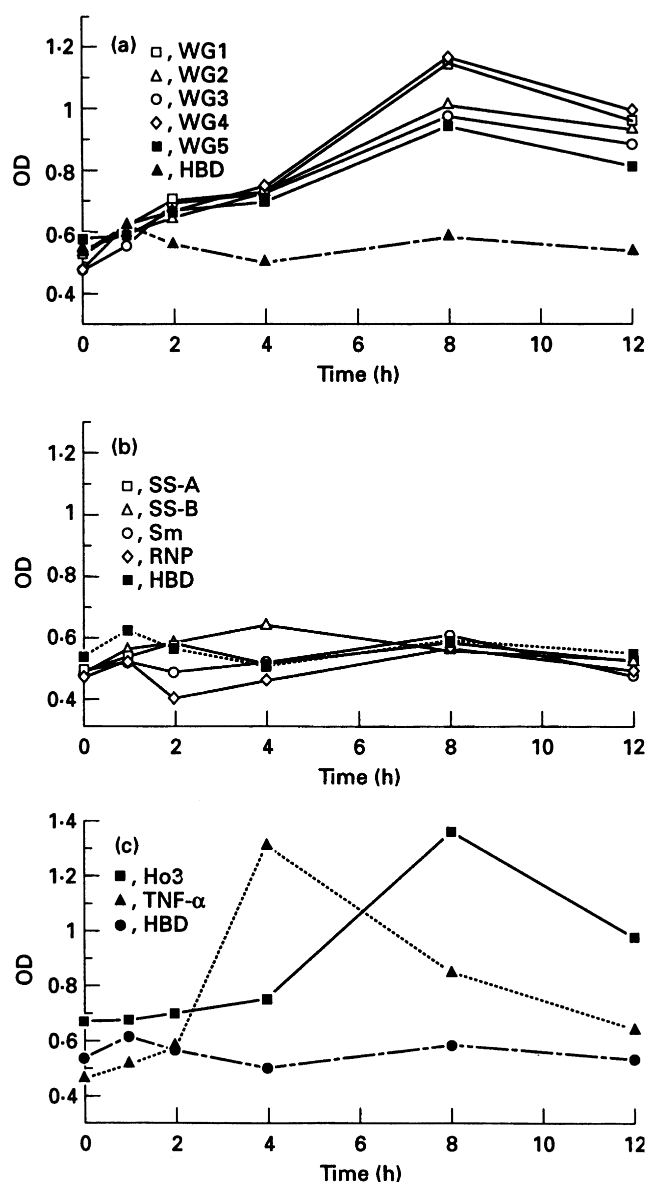

Fig. 1. Kinetics of anti-proteinase 3 (PR3) antibody-induced VCAM-1 expression on human endothelial cells. Cyto-ELISA with unfixed cells. Measurements at 0, 1, 2, 8 and 12 h of incubation with purified antibodies or tumour necrosis factor-alpha (TNF-α). Each point represents the mean of three measurements (data including mean ± s.d. are given in Table 1). Wegener's granulomatosis (WG) 1–5 $F(ab')_2$ fragments of purified anti-PR3 antibodies (five different patients); Ho3, anti-PR3 antibody Ho3; TNF-α, effect of TNF-α; SS-A, SS-B, Sm, RNP, purified control antibodies; HBD, pool of healthy blood donors; OD, optical density.

RESULTS

Specificity of antibodies

Five out of 50 selected WG sera were monospecifically positive for anti-PR3 antibodies as determined by IFT on human neutrophils (C-ANCA$^+$; titres 1 : 80–1 : 640), by ELISAs with alpha-fraction and 'acid' extract of human neutrophils and total extract of cytokine-treated HEC, by Western blot (reaction at 29 kD) and other routine methods [9] (data not shown). $F(ab')_2$ fragments of the affinity-purified antibodies reacted positively in an ELISA and in Western blot (data published earlier) [1,2]. Antibody reactivity could be blocked by incubation with purified PR3 antigen (affinity-purified as well as Kao preparation) as measured by ELISA and determined by Western blot [1]. Antibody reactivity could not be inhibited by preincubation with extracts of Hep2 cells. In addition, four sera of patients with Sjögren's syndrome and one serum of a patient with Sharp syndrome were found to be positive for anti-Ro (SS-A), anti-La (SS-B) and anti-RNP antibodies. Antibodies of these sera were purified and served as controls.

Antibody preparations diluted to the highest concentrations used in the experiments were free of endotoxin as determined by a Limulus amoebocyte lysate assay.

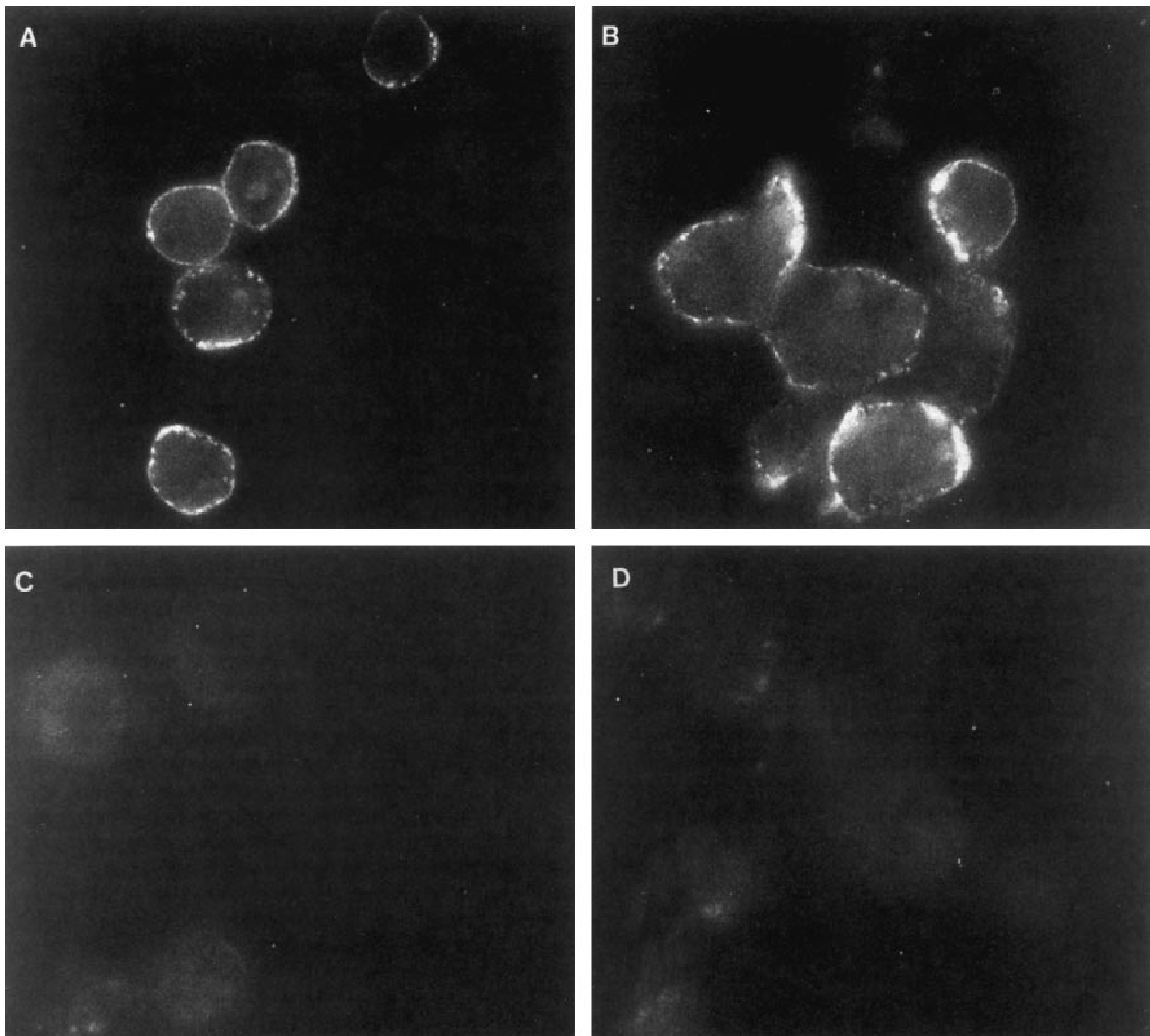

Fig. 2. Detection of increased expression of VCAM-1 in the membrane of human endothelial cells (HEC) (IFT, cells in suspension; original mag. $\times$ 100). (A,B) Unfixed HEC preincubated for 8 h with $F(ab')_2$ fragments of purified anti-proteinase 3 (PR3) antibodies. Bright surface staining. (C) Unfixed HEC preincubated for 8 h with purified anti-RNP antibodies (control). Only faint surface staining. (D) Negative control (only second antibodies).

Induction of VCAM-1

Incubation of HEC with purified PR3 antibodies or the PR3-specific B cell but not purified anti-Ro, anti-La, anti-Sm and anti-RNP antibodies or a pool of HBD led to a marked increase of VCAM-1 expression, with a peak after 8 h (Figs 1–3). These results could be confirmed by an IFT with unfixed HEC. Stimulation of HEC with $F(ab')_2$ fragments of anti-PR3 antibodies led to an increased membrane expression of VCAM-1 (Fig. 2). Incubation of HEC with TNF-α led to a marked increase of VCAM-1 expression, with a peak after 4 h (Fig. 1). Figure 2a, b show a bright surface staining due to VCAM-1 expression of unfixed HEC preactivated by anti-PR3 antibodies. The rim pattern of Fig. 2a could be converted to a bright pattern of diffuse membrane staining by altering the focus (data not shown). In contrast, preactivation of HEC with purified anti-RNP antibodies (control) led to a faint membrane staining which could almost not be discriminated from background fluorescence (Fig. 2c).

Activation of HEC with TNF-α and coincubation with purified PR3 antibodies led to a prolonged VCAM-1 expression, with a peak after 4 h and a slow decrease after 8 h. Preincubation of anti-PR3 antibodies with purified PR3 (controls) inhibited the effect of PR3 antibodies on HEC (Fig. 3). Blocking of Fc receptors on HEC with heat-aggregated human IgG did not inhibit the effect of anti-PR3 antibodies on HEC (data not shown). Induction of VCAM-1 mRNA expression by anti-PR3 antibodies was demonstrated by semiquantitative PCR analysis (Fig. 5).

Adhesion of T cells

Increased adhesion of T lymphocytes to anti-PR3 antibody-primed HEC could be visualized by phase contrast microscopy (90% HEC

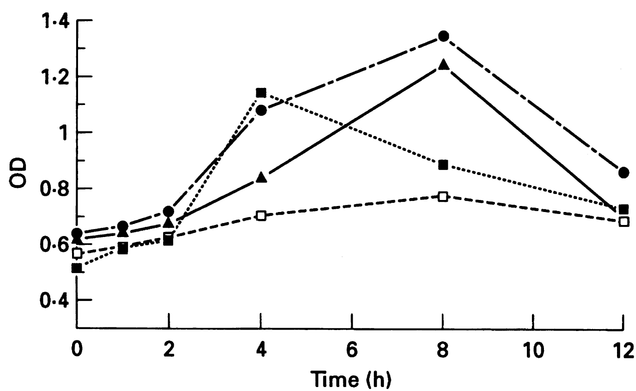

Fig. 3. Kinetics of anti-proteinase 3 (PR3) antibody-induced VCAM-1 expression on human endothelial cells. Cyto-ELISA with unfixed cells. Measurements at 0, 1, 2, 8 and 12 h of incubation with purified antibodies or tumour necrosis factor-alpha (TNF-α). Each point represents the mean of three measurements (data including mean $\pm$ s.d. are given in Table 1).WG4 (▲), $F(ab')_2$ fragments of purified anti-PR3 antibodies; TNF-α (■), effect of TNF-α, TNF-α + WG4 (●), effect of TNF-α and $F(ab')_2$ fragments of purified anti-PR3 antibodies; WG4-a (□), reaction of $F(ab')_2$ fragments of purified anti-PR3 anti-bodies after coincubation with purified PR3 (control); no reaction.

with rosettes). Adhesion could be inhibited by an antibody to VLA-4 (clone HP2/1) (10% HEC with rosettes) (Fig. 4). The results were reproducible on different days using different HEC preparations. Comparing adherence conditions (i.e. room temperature or 4°C), no significant difference could be observed.

DISCUSSION

Until now, only sparse data concerning cellular immune response during the course of C-ANCA-related vasculitides have been available. Immunohistochemical examinations of nasal biopsies of patients with active WG revealed a marked increase of T lymphocytes in the cellular infiltrates [14]. T lymphocyte proliferation was selectively induced by *in vitro* stimulation with an extract of human neutrophil alpha granules containing PR3 in patients with C-ANCA and active disease [25].

Elevated levels of soluble IL-2 receptors (sIL-2R) and increased levels of sIL-2R preceding relapses of disease suggest involvement of activated T cells [26].

Lymphocytes express a variety of adhesion molecules which enable them to recognize HEC via complementary adhesion molecules on these cells. The level of expression and activity of individual adhesion molecules varies on both the lymphocyte and HEC according to cell type and state of activation [27]. Although HEC are known to express class I and class II MHC antigens, it is unclear whether antigen-specific lymphocytes can be selected for extravasation by peptide/MHC complexes at the luminal surface of HEC. Lymphocyte extravasation is a complex event which involves several distinct stages, including binding to the luminal surface of HEC and migration across the endothelial lining and the basal lamina which constitute the vessel wall [28]. *In vitro* models have been used to identify adhesion molecules on lymphocytes (and on other inflammatory cells such as neutrophils, monocytes and eosinophils) that mediate interactions with HEC (for review, see [29]).

Primary adhesion is mediated by lectin–carbohydrate interactions involving selectins and their oligosaccharide ligands [29].

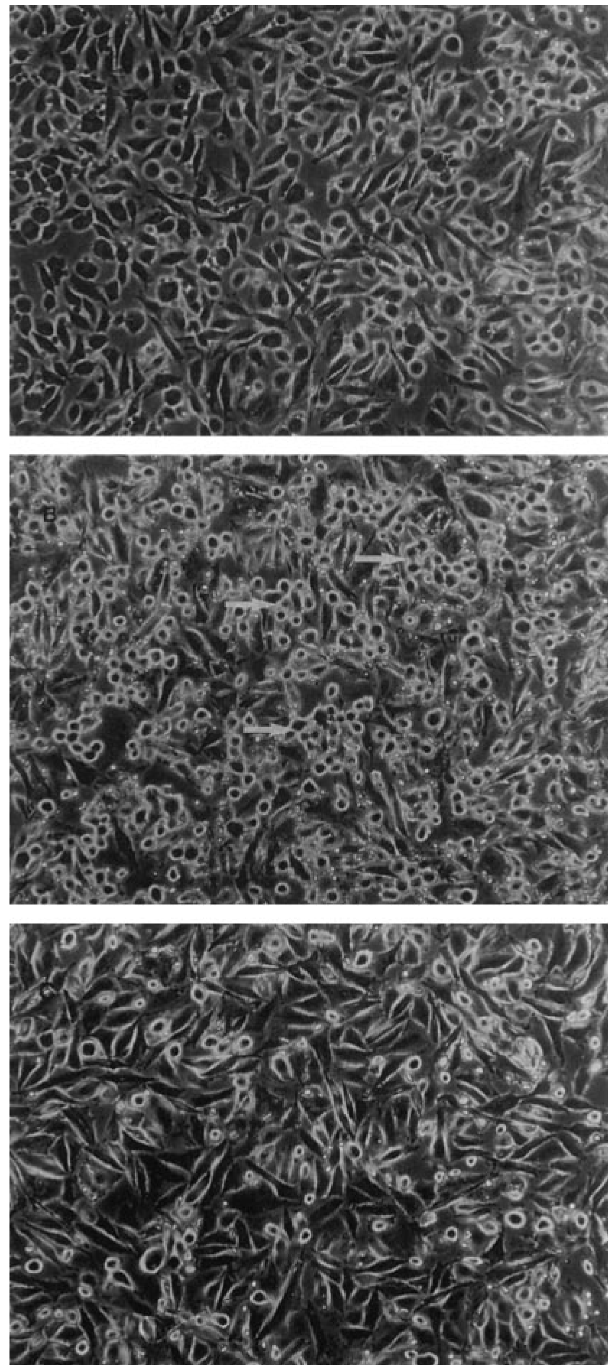

Fig. 4. Adhesion of T lymphocytes to anti-proteinase 3 (PR3) antibody-primed human endothelial cells (HEC) via VCAM-1. (A) Spontaneous adhesion of T cells to untreated HEC. (A) HEC activated by anti-PR3 antibodies. Enhanced adhesion of T cells (arrows: rosette forming). (C) Blocking of VCAM-1-mediated T lymphocyte adhesion to anti-PR3 antibody-primed HEC in the presence of clone HP2/1 (anti-VLA-4).

Secondary adhesion molecules belonging to the $\beta1$ and $\beta2$ integrin families achieve a strong attachment and extravasation of leucocytes [3,30]. VCAM-1 and its ligand VLA-4 play an important role in the *in vitro* adherence of monocytes and activated T cells [31,32] to endothelium. A recent report [33] described glomerular VCAM-1 expression in vasculitis. It is suggested

Table 1. Data of Fig. 1

a.

Time (h)	WG1	WG2	WG3	WG4	WG5	SS-A	SS-B	Sm	RNP	Ho3	TNF-α	HBD	
0	0·536	0·533	0·544	0·478	0·588	0·550	0·522	0·466	0·477	0·632	0·458	0·553	OD
	0·492	0·587	0·438	0·432	0·593	0·439	0·501	0·493	0·430	0·674	0·433	0·499	OD
	0·549	0·510	0·447	0·504	0·542	0·499	0·438	0·502	0·498	0·699	0·492	0·549	OD
	0·526	0·543	0·476	0·471	0·574	0·496	0·487	0·487	0·468	0·668	0·461	0·534	M
	0·024	0·032	0·048	0·030	0·023	0·045	0·036	0·015	0·028	0·028	0·024	0·025	s.d.
1	0·596	0·589	0·568	0·566	0·550	0·568	0·574	0·544	0·521	0·721	0·497	0·675	OD
	0·612	0·574	0·532	0·682	0·599	0·547	0·571	0·510	0·533	0·638	0·523	0·633	OD
	0·653	0·610	0·551	0·631	0·612	0·483	0·532	0·503	0·517	0·664	0·522	0·549	OD
	0·620	0·591	0·550	0·626	0·587	0·533	0·559	0·519	0·524	0·674	0·514	0·619	M
	0·024	0·015	0·015	0·047	0·027	0·036	0·019	0·018	0·007	0·035	0·012	0·052	s.d.
2	0·688	0·632	0·685	0·679	0·637	0·612	0·580	0·497	0·377	0·687	0·599	0·521	OD
	0·697	0·641	0·633	0·699	0·692	0·597	0·632	0·455	0·402	0·699	0·538	0·637	OD
	0·715	0·657	0·743	0·603	0·663	0·533	0·533	0·493	0·408	0·710	0·584	0·519	OD
	0·700	0·643	0·687	0·660	0·664	0·581	0·582	0·482	0·396	0·699	0·574	0·559	M
	0·011	0·010	0·045	0·041	0·022	0·034	0·040	0·019	0·013	0·009	0·026	0·055	s.d.
4	0·739	0·714	0·699	0·745	0·699	0·457	0·677	0·501	0·457	0·755	0·988	0·478	OD
	0·710	0·699	0·783	0·773	0·697	0·521	0·631	0·523	0·422	0·731	1·453	0·425	OD
	0·748	0·725	0·685	0·715	0·685	0·538	0·594	0·501	0·483	0·766	1·475	0·593	OD
	0·732	0·713	0·722	0·744	0·694	0·505	0·634	0·508	0·454	0·751	1·305	0·499	M
	0·016	0·011	0·043	0·024	0·006	0·035	0·034	0·010	0·025	0·015	0·225	0·070	s.d.
8	1·229	1·034	0·964	1·323	0·945	0·559	0·541	0·587	0·532	1·358	0·864	0·587	OD
	1·032	1·002	0·988	1·027	0·987	0·594	0·572	0·632	0·566	1·243	0·859	0·563	OD
	1·174	0·994	0·971	1·145	0·893	0·561	0·538	0·566	0·571	1·483	0·816	0·598	OD
	1·145	1·010	0·974	1·165	0·942	0·571	0·550	0·595	0·556	1·361	0·846	0·583	M
	0·083	0·017	0·010	0·122	0·038	0·016	0·015	0·028	0·017	0·098	0·022	0·015	s.d.
12	0·988	0·985	0·901	1·011	0·883	0·498	0·541	0·471	0·488	0·965	0·657	0·541	OD
	0·965	0·910	0·845	0·973	0·812	0·501	0·502	0·498	0·432	0·993	0·647	0·529	OD
	0·934	0·900	0·899	0·993	0·744	0·543	0·487	0·433	0·533	0·984	0·633	0·533	OD
	0·962	0·932	0·882	0·992	0·813	0·514	0·510	0·467	0·484	0·981	0·646	0·534	M
	0·022	0·038	0·026	0·016	0·057	0·021	0·023	0·027	0·041	0·012	0·010	0·005	s.d.

OD, Optical density; M, mean.

that interactions between adhesion molecules like VCAM-1 on HEC may play an important role in the perpetuation of vasculitis.

Renal lesions in WG are characterized by intraglomerular and interstitial infiltration of neutrophils, monocytes and lymphocytes [34]. The authors assume that VCAM-1 is involved in the recruitment of monocytes in WG. Serum levels of soluble VCAM-1 and ICAM-1 were significantly elevated in WG patients compared with normal controls. Assuming that plasma levels of soluble VCAM-1 reflect expression of VCAM-1 as a result of endothelial activation, a rise in levels of this soluble isoform suggests increased expression of VCAM-1 in the tissue [35].

At present the exact mechanism of a direct pathogenic effect of anti-PR3 antibodies remains unknown. There is no definitive proof as yet for a causative role of ANCA in ANCA-related diseases. Recent data, however, generated from *in vitro* experiments point to a possible pathophysiological role of ANCA (for review see [36]).

Endothelium has often been considered a possible target of immune-mediated aggression during vasculitic processes due to its functional characteristics and its constant contact with circulating humoral and cellular immune effectors [37]. During the last years our research has been focused on the interaction between ANCA and HEC. Recently we detected PR3 in HEC, and we were able to demonstrate that PR3 is translocated into the membrane of HEC under the influence of cytokines such as TNF-α or IL-1, and that is accessible to the respective antibodies [1]. Anti-PR3 antibodies can act on vascular endothelium to alter its fundamental surface properties related to neutrophil adhesion [2]. Furthermore, we were able to show a cytotoxic effect of anti-PR3 antibodies towards HEC [38]. The exact mechanism of anti-PR3 antibody-induced induction of VCAM-1 expression if not known. During our previous studies of membrane expression of PR3 it became evident that activation of HEC with cytokines is not the sole prerequisite for translocation of PR3 into the cell membrane [1]. A low level of PR3 can also be detected on the surface of 'untreated' cells. Activation of HEC, however, during isolation can not be ruled out. Cytokines such as TNF-α or interferon-gamma (IFN-γ) are known inductors of endothelial VCAM-1 and PR3. Coincubation of HEC with TNF-α and anti-PR3 antibodies results in an enhancement of this effect. Surface expression of VCAM-1 is prolonged, with a slow decline after 8 h. As described earlier [2], we consider it likely that anti-PR3

Table 1. Data of Fig. 3.

b.

Time (h)	WG4	TNF-α	WG4-a	TNF-α+ WG4	
0	0·631	0·562	0·538	0·633	OD
	0·622	0·521	0·559	0·621	OD
	0·598	0·468	0·596	0·649	OD
	0·617	0·517	0·564	0·634	M
	0·014	0·038	0·024	0·011	s.d.
1	0·667	0·593	0·631	0·670	OD
	0·612	0·598	0·582	0·633	OD
	0·643	0·573	0·564	0·684	OD
	0·641	0·588	0·592	0·662	M
	0·023	0·011	0·028	0·022	s.d.
2	0·639	0·630	0·693	0·699	OD
	0·688	0·608	0·601	0·734	OD
	0·690	0·599	0·586	0·718	OD
	0·672	0·612	0·627	0·717	M
	0·024	0·013	0·047	0·014	s.d.
4	0·799	0·986	0·706	0·961	OD
	0·838	1·198	0·716	1·087	OD
	0·864	1·254	0·699	1·198	OD
	0·834	1·146	0·707	1·083	M
	0·027	0·115	0·007	0·096	s.d.
8	1·103	0·835	0·798	1·345	OD
	1·345	0·961	0·754	1·388	OD
	1·283	0·877	0·783	1·306	OD
	1·244	0·891	0·778	1·356	M
	0·103	0·052	0·018	0·033	s.d.
12	0·638	0·695	0·681	0·897	OD
	0·756	0·721	0·657	0·837	OD
	0·712	0·784	0·718	0·864	OD
	0·702	0·733	0·685	0·866	M
	0·049	0·037	0·025	0·025	s.d.

antibodies binding to membrane-expressed PR3 on HEC might induce an activation change, followed by some form of signal transduction within the endothelial cell.

In this study we were able to show that binding of anti-PR3 antibodies on HEC induces membrane expression of VCAM-1. Different kinetics of membrane expression suggest that regulation of this effect is different from TNF-α induced VCAM-1 expression on HEC. Differences in the regulation of anti-PR3-and TNF-induced VCAM-1 expression were also suggested by the fact that anti-PR3 was a similar potent inductor of VCAM surface expression, but a less potent inductor of VCAM mRNA expression compared with TNF. This suggests that anti-PR3 is a more potent inductor of post-transcriptional mechanisms of VCAM induction.

Enhanced expression of VCAM-1 on the endothelium may facilitate the transmigration of monocytes and $CD4^+$ memory/effector cells, as these cells express VLA-4, the ligand for VCAM-1 [39]. Infiltration with PMN and monocytes was detected in inflammatory lesions of ANCA-associated vasculitis and glomerulonephritis [40]. These effector mononuclear cells may enhance or sustain the expression of VCAM-1 as a result of local cytokine production [41].

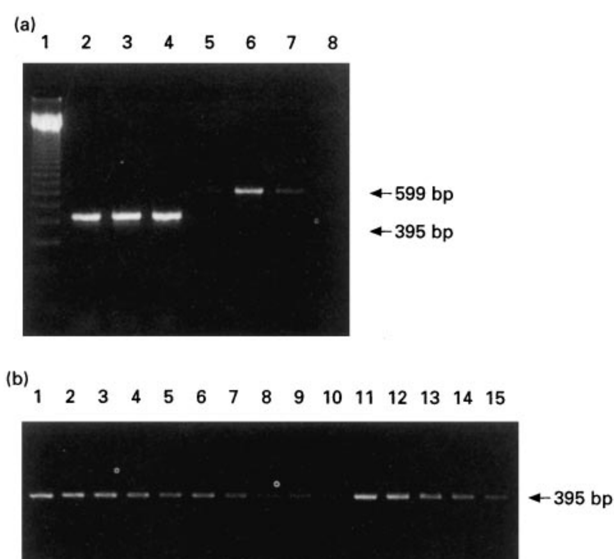

Fig. 5. VCAM-1 mRNA induction in human endothelial cells (HEC) after stimulation with tumour necrosis factor-alpha (TNF-α) or proteinase 3 (PR3)-specific antibodies. (a) VCAM-1 mRNA expression in unstimulated HEC (lane 5) and HEC stimulated with TNF-α (lane 6) or PR3-specific antibodies (lane 7) was determined by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR). To show that equal amounts of cDNA were used from every source, PCR for GAPDH was performed in parallel (lane 2, unstimulated HEC using dilution 5 from (b); lane 3, HEC-stimulated with TNF-α using dilution 6 from (b); lane 4, HEC stimulated with PR3-specific antibodies using dilution 15 from (b)). Lane 1, 123 bp ladder; lane 8, H_2O. (b) Two-fold dilutions of cDNA derived from untreated HEC (lanes 1–5), HEC stimulated with TNF-α (lanes 60–10) and HEC stimulated with PR3-specific antibodies (lanes 11–15) were subjected to RT-PCR using GADPH-specific primers.

Our data demonstrate that cytokine-like effects of anti-PR3 antibodies on HEC are not limited to induction of neutrophil adhesion, and that ANCA are able to modulate the interaction of endothelium and T cells in ANCA-related vasculitides. One important aspect in this context is the detection of endogenous PR3 in HEC by our group [1]. HEC could present this antigen to cytotoxic $CD8^+$ T cells [42].

In conclusion, our data support the hypothesis that ANCA, especially anti-PR3 antibodies, may play an important role in the pathogenesis of ANCA-related vasculitides.

ACKNOWLEDGMENTS

We are grateful for the expert technical assistance of Ms Sabine Kloß. We thank Professor Dr Peters and the staff of the St Hildegardis Hospital, Mainz, for collecting umbilical cords. We also thank Mrs Dr Ilka Helmreich-Becker for reviewing the manuscript.

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