The Integrin α9β1 Mediates Adhesion to Activated Endothelial Cells and Transendothelial Neutrophil Migration through Interaction with Vascular Cell Adhesion Molecule-1

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Abstract. The integrin α9β1 has been shown to be widely expressed on smooth muscle and epithelial cells, and to mediate adhesion to the extracellular matrix proteins osteopontin and tenascin-C. We have found that the peptide sequence this integrin recognizes in tenascin-C is highly homologous to the sequence recognized by the closely related integrin α4β1, in the inducible endothelial ligand, vascular cell adhesion molecule-1 (VCAM-1). We therefore sought to determine whether α9β1 also recognizes VCAM-1, and whether any such interaction would be biologically significant. In this report, we demonstrate that α9β1 mediates stable cell adhesion to recombinant VCAM-1 and to VCAM-1 induced on human umbilical vein endothelial cells by tumor necrosis factor-α. Furthermore, we show that α9β1 is highly and selectively expressed on neutrophils and is critical for neutrophil migration on VCAM-1 and tenascin-C. Finally, α9β1 and α4 integrins contribute to neutrophil chemotaxis across activated endothelial monolayers. These observations suggest a possible role for α9β1/VCAM-1 interactions in extravasation of neutrophils at sites of acute inflammation.

Key words: integrin • α9β1 • α4 • neutrophil migration • vascular cell adhesion molecule-1

INTGRINS are heterodimeric receptors for extracellular matrix and cell surface counter-receptors which play important roles in embryonic development, inflammation, wound healing, and tumorigenesis (Hynes, 1987, 1992; Rosolathi and Pierschbacher, 1987). Integrin ligand-binding specificity is determined by structural features of each subunit, but there is considerable ligand-binding overlap among integrin heterodimers. One clue to ligand-binding overlap has been the degree of sequence homology among integrin subunits. For example, the integrin α subunits α5, αv, αIIβ, and α8 are all closely related, and integrin heterodimers containing these α subunits recognize ligands containing the peptide sequence arginine-glycine-aspartic acid (Hynes, 1992; Schnapp et al., 1995). Similarly, the αm, αL, and α6 subunits are highly homologous to one another and recognize closely related immunoglobulin family members as ligands (Hynes, 1992). We previously cloned and sequenced the integrin α9 subunit, and have shown that it forms a single integrin heterodimer, α9β1 (Palmer et al., 1993). The α9 subunit cDNA sequence is 41% identical to the integrin α4 subunit sequence, but <27% identical to any other integrin subunit, identifying α9 and α4 as sole members of a subfamily of integrin α subunits.

In an effort to understand the structural basis of α9β1 ligand-binding in more detail, we recently mapped the α9β1 ligand-binding site in the extracellular matrix protein tenascin-C (Yokosaki et al., 1994). α9β1 binds to a single exposed loop in the third fibronectin type III repeat of tenascin-C (B–C loop) to a minimal sequence EIDGIEL (Schneider et al., 1998; Yokosaki et al., 1998). We noticed that a critical portion of this sequence (IDG) is homologous to the tripeptide sequence IDS present in the previously mapped ligand-binding site for the α4β1 ligand, vascular cell adhesion molecule-1 (VCAM-1; Clements et al., 1994; Yokosaki et al., 1998). Therefore, we undertook...
the current study to determine whether α9β1 recognizes VCA M-1 as a ligand and whether or not such interaction is biologically significant.

Materials and Methods

Reagents

BSA, formyl-methionyl-leucylphenylalanine (FMLP), and dextran were purchased from Sigma Chemical Co. Recombinant human tumor necrosis factor (TNF-α), recombinant human interferon (IFN-γ) (specific activity of 10⁷ U/mg), and recombiant interleukin 8 (IL-8) were obtained from R&D Systems, Inc. Fluorescent reagent, 2',7'-bis(carboxyethyl)-5,6-carboxy-fluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes, Inc. One recombinant form of the third fibronecton type III repeat of chicken tenasin-C (Prieto et al., 1993) containing alanine substitution mutations within the RGD site (TNfn3RAA), was obtained from A.nita Prieto and K athryn Crossin (Scrips Research Institute, La Jolla, CA) and prepared in E scherichia coli. A recombinant VCA M-1(gg chimera (Y ednock et al., 1995), was produced in baculovirus as previously described. Recombinant intercellular adhesion molecule-1 (ICAM M-1), fusion protein was a gift from B. I mho (Centre M edicaile U niversitaire, Geneva, Switzerland) to D. E rie (U niversity of California, San Francisco, C.A.). Ficoll-hypaque plus for isolation of neutrophils from venous blood was purchased from Pharmacia Biotech, Inc. and used according to the manufacturer’s specifications.

Antibodies, Cells, and Cell Culture

Mouse mAbs, Y9A2 against human α9β1 (Wang et al., 1996) and A N100226M (100226) against α4 (K ent et al., 1995), were prepared as previously described. Mouse mAbs, W6/32 against human MHC and IB4 against the integrin α2β1 subunit, were prepared from hybridomas obtained from A merican T ype Tissue Collection. Mouse monoclonal antihuman VCA M-1 (CD106) was purchased from R&D Systems. FITC-labeled mouse monoclonal anti-CD16 antibody was purchased from Caltag. Hu man umbilical vein endothelial (HUVE) cells were purchased from Clonetech and grown in endothelial cell growth media (EGM) containing 2% FBS, 10 ng/ml human recombinant EGF, 50 ng/ml gentamycin, 50 ng/ml amphotericin B, 12 μg/ml bovine brain extract, and 1 μg/ml hydrocortisone and were used between passage 3 and 10. VCAM-1 (CD106) was purchased from R&D Systems. FITC-labeled antibody, Y9A2 (10 μg/ml), 100226 (10 μg/ml), or combinations of these antibodies for 15 min on ice. In some experiments, HUVE cells were incubated with CD106 (5 μg/ml) for 15 min at 37°C. 50,000 cells in 200 μl of serum-free DMEM were added to each well, and plates were centrifuged at 20 g for 5 min, and covered with aluminum foil to prevent photobleaching. Plates were then incubated for 60 min at 37°C in 5% CO₂. A fer incubation, nonadherent cells were removed by washing twice with serum-free DMEM. Finally, 20 μl of the same medium was added to each well, and fluorescence was quantified with a fluorometer (Fluoroskan II; Lab systems) at excitation wavelength 485 nm and emission wavelength 538 nm. The adherent ratio (%) was calculated as follows: (fluorescence from experimental sample − fluorescence from negative control sample) / total fluorescence added to chamber. A ll determinations were carried out in triplicate.

Neutrophil Migration Assays

Neutrophils were purified from human peripheral venous blood containing 20 U/ml of heparin. Neutrophils were isolated by ficoll-hypaque density gradient centrifugation, followed by 3% dextran sedimentation (Gresham et al., 1986). Erythrocytes were subjected to hypotonic lysis, remaining neutrophils were washed and resuspended in PBS. The isolated neutrophils were >95% pure and >95% viable as assessed by Wright-Giemsa staining and trypan blue exclusion, respectively. Cell migration was analyzed essentially as described by M arks et al. (1991). In brief, glass coverslips were placed in 35-mm culture dishes and incubated with 100 μl serum-free media containing 10 μg/ml VCA M-1/g, 10 μg/ml TNf3RAA, and 5 μg/ml of ICA M-1 or 1% BSA for 60 min at 37°C, washed, and then incubated with 1% BSA for 30 min. Neutrophils were incubated with no antibody, Y9A2 (30 μg/ml), 100226 (10 μg/ml), IB4 (20 μg/ml), or combinations of these antibodies for 15 min at 37°C, and were then incubated for 10 min at 37°C with or without 10 nM FMLP. 10⁶ cells were plated onto the coverslip area of each well and allowed to attach at 37°C for 5 min. Dishes were then placed on a videomicroscope stage and individual fields (200 µm) were recorded for 3 min. Three different fields were examined in each chamber. To count the number of migrating cells in a given field, outlines were made of each cell. Cells were considered to have migrated when both the leading edge and tail of the cell moved >7 μm from their initial position. At least 40 neutrophils were analyzed per field and the ratio of migrating to total cells was calculated.
per et al. (1995). HUVE cells were plated onto polycarbonate inserts (Transwell, 6.5-mm diameter, 8-μm pore for 24-well plate; Costar Corp.) in 200 μl of serum-containing EGM, and allowed to grow to confluence over 72 h. 500 μl serum-free DMEM was added to the lower chamber of each well. 24 h before addition of neutrophils, upper chambers were washed twice with serum-free media and new medium with or without 3 ng/ml of TNF-α. Immediately before the addition of neutrophils, the upper chambers were washed twice with serum-free DMEM and medium in the lower chamber was replaced with 500 μl serum-free DMEM or serum-free DMEM with 10 nM FMLP or 50 ng/ml IL-8. In some experiments HUVE cells were incubated with CD106 (5 μg/ml) at 37°C for 15 min. Purified neutrophils were incubated with no antibody, Y9A2 (10 μg/ml), 100226 (10 μg/ml), IB4 (20 μg/ml), W6/32 (10 μg/ml), or combinations of antibodies for 15 min at 4°C, and 2 × 10^5 cells in 200 μl of media were added to each upper chamber. After 3 h at 37°C in 5% CO2, nonadherent cells in the upper chamber were removed. Medium, including migrated neutrophils from the lower chamber, was collected, the lower chamber was rinsed several times to collect all the neutrophils that had transmigrated, and the absence of additional adherent neutrophils was confirmed microscopically. The medium and all washes were pooled and resuspended, and cells were counted with a hemocytometer. All determinations were carried out in duplicate and repeated at least twice.

Results

α9β1 Mediates Static Adhesion of Resting α9-transfected SW480 Cells and CHO Cells to VCAM-1

To determine whether VCAM-1 could function as a ligand for α9β1, we performed cell adhesion assays with two different cell lines, SW480 and CHO, that had been stably transfected with either an α9-expression plasmid or empty vector. Both cell lines stably expressed α9β1 on the cell surface as demonstrated by flow cytometry with the anti-α9β1 antibody Y9A2 (Fig. 1, A and B). Adhesion assays were performed on plates coated with either the known α9β1 ligand, recombinant TNfn3RAA (Fig. 1, C and D), or recombinant VCA M-1/Ig (Fig. 1, E and F). For both cell lines, α9-transfectants adhered to both TNfn3 and to VCAM-1 in a concentration-dependent manner, whereas

![Figure 1](image-url)
mock-transfectants did not adhere to either substrate. Adhesion of each α9-transfected cell line was completely inhibited by the anti-α9β1 antibody, Y9A2, demonstrating that this effect was mediated by α9β1.

α9β1 Mediates Adhesion to TNF-α-activated, but not to IFN-γ-activated HUVE Cells, Via Interaction with Induced VCAM-1

To determine whether α9β1-mediated adhesion to VCAM-1 was biologically significant, we next examined the role of this integrin in adhesion of cells to resting HUVE cells, and to HUVE cells that had been activated by incubation with TNF-α (3 ng/ml), a well characterized inducer of VCAM-1 expression, or IFN-γ (3 ng/ml), a cytokine that does not induce VCAM-1 expression. The effects of each cytokine on VCAM-1 expression under the conditions used in these experiments were examined by flow cytometry with anti-VCAM-1 antibody CD106 (Fig. 2, B–D). As expected, resting HUVE cells (Fig. 2 B) and HUVE cells stimulated with IFN-γ (Fig. 2 D) did not express detectable levels of VCAM-1, but VCAM-1 was dramatically induced by TNF-α (Fig. 2 C). All cell lines examined demonstrated baseline adhesion to resting HUVE cells, and demonstrated a similar level of adhesion to HUVE activated by IFN-γ, and this baseline adhesion was unaffected by anti-α9β1 antibody (Fig. 2 A). However, only α9-transfected cells demonstrated enhanced adhesion to TNF-α-treated HUVE. This enhanced adhesion was returned completely to basal levels by antibody to either α9β1(Y9A2) or to VCAM-1 (CD106), demonstrating that it was due to an interaction between α9β1 and VCAM-1.

α9β1 Is Expressed on Neutrophils

We have previously demonstrated that α9β1 is widely expressed on epithelial and smooth muscle cells (Palmer et al., 1993), but expression on leukocytes has not been reported. To determine whether α9β1 is expressed on cells likely to encounter activated endothelial cells, we performed flow cytometry on whole blood leukocytes with the α9β1 antibody Y9A2. We evaluated expression on neutrophils, monocytes, and lymphocytes by gating on each population separately, based on differential light scattering. From a separate atopic donor we evaluated expression on eosinophils, which were separated from other leukocytes based on light scattering and the absence of surface expression of CD16. In parallel, we examined expression of the structurally related integrin subunit, α4. α9β1 was not detected on lymphocytes or eosinophils and was expressed at low levels on monocytes (Fig. 3 A). In contrast, α9β1 was highly and uniformly expressed on human neutrophils. As expected, α4 was highly expressed on lymphocytes, monocytes, and eosinophils, but was also detected on neutrophils, albeit at considerably lower levels.

Expression of α9 on neutrophils was further confirmed by immunoprecipitation with Y9A2 followed by Western blotting with an affinity-purified antiserum raised against

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Figure 2. Adhesion of α9- or mock-transfected SW480 cells to HUVE cells. (A) Confluent monolayers of HUVE cells were incubated for 24 h with medium alone (no activation), TNF-α (3 ng/ml), or IFN-γ (3 ng/ml). Fluorescently labeled α9- or mock-transfected SW480 cells were allowed to adhere to HUVE cell monolayers for 60 min in the presence or absence of the α9β1 blocking antibody Y9A2 (10 μg/ml) or the VCAM-1 blocking antibody CD106 (5 μg/ml). Nonadherent cells were removed by gentle washing and the percent of adherent cells was calculated based on fluorescence. Data are presented as the mean (+ SD) of triplicate measurements. Similar results were obtained in two separate experiments. Flow cytometric evaluation of cell surface expression of VCAM-1 on HUVE cells treated with medium alone (B), TNF-α (3 ng/ml; C), or IFN-γ (3 ng/ml; D). Open peaks represent fluorescence of unstained HUVE cells and shaded peaks represent fluorescence of cells stained with the anti-VCAM-1 antibody, CD106.
a unique portion of the α9 cytoplasmic domain. A band of 160 kD (appropriate molecular mass for α9) was detected in lysate of human neutrophils after immunoprecipitation with Y9A2, but not after immunoprecipitation with the control antibody R6G9 (Fig. 3 B).

α9β1 Mediates Migration of FMLP-activated Neutrophils on TNFα3 or VCAM-1

To determine whether α9β1 expression on neutrophils was biologically significant, we initially sought to examine static adhesion of neutrophils to dishes coated with either TNFα3AA or VCA M-1. However, in the absence of antibodies against β2 integrins, neutrophils avidly adhered to all surfaces examined, and in the presence of β2 integrin blocking antibodies, neutrophils could not be induced to adhere to either VCA M-1 or TNFα3AA by incubation with MnCl₂, FMLP, phorbol esters, or the β1 activating antibody TS2/16 (data not shown). Therefore, we examined the possible role of α9β1 in another important neu-
Neutrophil migration on VCAM-1 is an effective ligand for the integrin α9β1. This receptor-ligand interaction is sufficient to support adhesion of α9-transfected cell lines to VCAM-1 and to TNF-α-activated HUVE cells, an effect that is mediated by the binding of α9β1 to VCAM-1. Furthermore, α9β1 is uniformly and specifically expressed on normal resting human neutrophils, and mediates both neutrophil migration on a fragment of tenascin-C or VCAM-1 and transmigration of neutrophils across TNF-α-activated endothelial monolayers. Together, these data suggest a previously unsuspected role for α9β1 and VCA M-1 in extravasation of neutrophils at sites of acute inflammation.

In addition to α9β1, we found detectable, albeit low, levels of the structurally related integrin α4 subunit on resting human neutrophils. This finding is consistent with several previous reports of α4 expression on neutrophils from a variety of species (Iseki et al., 1996; Gao and Iseki, 1997; Davenpeck et al., 1998). Although the level of expression of α4 we detected on human neutrophils was one to two orders of magnitude lower than expression on eosinophils, monocytes, and lymphocytes, this low level expression appeared to be biologically significant, since antibody against α4 partially inhibited migration of neutrophils on VCA M-1 and migration across TNF-activated endothelial monolayers. Recently, α4β1 has been shown...
to mediate both neutrophil adhesion to VCA M-1 (Daven-
peck et al., 1998) and neutrophil transmigration across fi-
broblast monolayers (Gao and Issakutz, 1997). As ex-
pected, $\alpha_4$ integrins did not contribute to migration on
TNF-\(\alpha\) (3 ng/ml; B) for 24 h. DMEM con-
taining FMLP (10 nM) or DMEM alone was added to the bottom
chamber. After 3 h at 37°C in 5% CO\(_2\), neutrophils that had mi-
gated across the monolayer were collected from the bottom
chamber and counted. In additional chambers, untreated neutro-
phils were added to HUVE cells that had been preincubated
with (A) or without TNF-\(\alpha\) (10 ng/ml), $\beta_2$ (IB4, 20 \(\mu\)g/ml), a combination of these antibodies, or human MHC (W6/32, 10 \(\mu\)g/ml) were added
to the top chambers above microporous chambers containing
confluent monolayers of HUVE cells that had been incubated
with (A) or without TNF-\(\alpha\) (3 ng/ml; B) for 24 h. DMEM con-
taining FMLP (10 nM) or DMEM alone was added to the bottom
chamber. After 3 h at 37°C in 5% CO\(_2\), neutrophils that had mi-
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phils were added to HUVE cells that had been preincubated
for 15 min with antibody to VCAM-1 (CD106, 5 \(\mu\)g/ml). Data are ex-
pressed as the mean (\(\pm\) SD) of quadruplicate measurements
from two separate experiments.

A role for $\beta_2$ integrin-independent processes in neutro-
phil extravasation in vivo has been suggested by several sets of observations, including studies of neutrophil ex-
avtasation into the liver in response to endotoxin (Essani et al.,
1997) and neutrophil migration into the alveolar spaces of the lung in response to intratracheal instillation of live bacteria (Dierschuk et al., 1990). Recent studies
demonstrating neutrophil extravasation into the lungs and peritoneal cavity in $\beta_2$ integrin knockout mice also dem-
strate the importance of mechanisms independent of $\beta_2$
integrins (Mizgerd et al., 1997). The extent to which these
events are mediated by $\alpha_9$ integrin and/or $\alpha_4$ integrins needs to be
stratified across VCA M-1 expressing endothelial cells and shared ligands
such as osteopontin (Smith et al., 1996; Bayless et al.,
1998), and $\alpha_9$ integrins could be critical for migration across te-
nascin-C that is present outside the vasculature at sites of
inflammation (Erickson, 1993).

In addition to the expression on neutrophils described in
this report, $\alpha_9$ integrin is widely expressed on muscle cells, sur-
face epithelial cells, and hepatocytes (Palmer et al., 1993).
It is unclear what role, if any, interactions with VCA M-1
might have at these sites. VCA M-1 has also been reported
to be expressed on muscle cells under various conditions
(Rosen et al., 1992; Shepard et al., 1994), so it is conceiv-
able that $\alpha_9$ integrin/VCA M-1 interactions may be biologi-
ically significant in muscle as well. Such an effect could explain
the apparent contradiction between reports, based on anti-
body inhibition, that $\alpha_4$ integrin binding plays a criti-
cal role in myotube formation (Rosen et al., 1992) and the
normal muscle development of $\alpha_4$ knockout mice in chi-
meric mice (Yang et al., 1996), if the $\alpha_4$ knockout led to a
developmentally regulated increase in $\alpha_9$ integrin expression.

In summary, we have identified VCA M-1 as a novel and
biologically significant ligand for the integrin $\alpha_9$ integrin, have
demonstrated that this integrin is expressed on neutrophils
and mediates neutrophil migration on two relevant ligands
and neutrophil transmigration across activated endothelial
monolayers. These findings support a role for $\alpha_9$ integrin
VCA M-1 interactions in extravasation of neutrophils at sites of
inflammation.

Figure 5. Transmigration of neutrophils across activated HUVE
nerve cell monolayers. Purified human neutrophils that had been incu-
bated with no antibody or antibody to $\alpha_9$ integrin (Y 9A 2, 10 \(\mu\)g/ml), $\alpha_4$ integrin (100226, 10 \(\mu\)g/ml), $\beta_2$ (IB4, 20 \(\mu\)g/ml), a combination of these antibodies, or human MHC (W6/32, 10 \(\mu\)g/ml) were added
to the top chambers above microporous chambers containing
confluent monolayers of HUVE cells that had been incubated
with (A) or without TNF-\(\alpha\) (3 ng/ml; B) for 24 h. DMEM con-
taining FMLP (10 nM) or DMEM alone was added to the bottom
chamber. After 3 h at 37°C in 5% CO\(_2\), neutrophils that had mi-
gated across the monolayer were collected from the bottom
chamber and counted. In additional chambers, untreated neutro-
phils were added to HUVE cells that had been preincubated
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