Expression of vascular cell adhesion molecule-1 (CD 106) in normal and neoplastic human esophageal squamous epithelium

JAN HEIDEMANN[1], CHRISTIAN MAASER[1], ANDREAS LÜGERING[1], THOMAS W. SPAHN[1], KLAUS P. ZIMMER[2], HERMANN HERBST[3], PARVANEH RAFIEE[4], WOLFRAM DOMSCHKE[1], CHRISTIAN F. KRIEGLSTEIN[5], DAVID G. BINION[4] and TORSTEN F. KUCHARZIK[1]

Departments of [1]Medicine B and [2]Pediatrics, University of Münster, Münster; [3]Institute of Pathology, Klinikum am Urban, D-10967 Berlin, Germany; [4]Digestive Disease Center, Medical College of Wisconsin, Milwaukee, WI 53226, USA; [5]Department of Surgery, University of Münster, Münster, Germany

Received August 10, 2005; Accepted September 19, 2005

Abstract. Vascular cell adhesion molecule-1 (VCAM-1), a key receptor for the leukocyte-associated integrin (VLA4), is a crucial mediator of leukocyte adhesion and has co-stimulatory functions in inflammation at various organ sites. Specifically, VCAM-1/VLA4 interactions have been shown to play important roles in the setting of cutaneous immune responses, such as psoriatic lesions in humans and acute Graft-versus-Host-Disease in mice. VCAM-1 is generally expressed on activated endothelial cells in inflamed tissues, mediating endothelium-leukocyte interactions, leading to leukocyte diapedesis to the site of inflammation. We report novel and unexpected membrane expression of VCAM-1 in the basal squamous epithelial strata of the normal human esophagus and distinct patterns of epithelial expression in esophageal pathology. To further delineate the differential expression patterns of VCAM-1 in the esophageal epithelium, we examined specimens from squamous cell carcinoma (SCC), adenocarcinoma, and Barrett's columnar cell metaplasia. VCAM-1 was strongly expressed in squamous cell carcinoma, but not adenocarcinoma nor columnar epithelia in Barrett's esophagus. VCAM-1 expression was focally accentuated at sites characteristic of microscopic tumor invasion in SCC, pointing to a potential role of VCAM-1 in the development of metastasis. In addition, *in vitro* immunofluorescence studies using OE21 cells, an esophageal squamous epithelial cell line, displayed distinct VCAM-1 immunoreactivity confined to mitotic and dividing cells. Cell cycle arrest caused a significant decrease in VCAM-1 immunoreactivity in OE21 cells. These data suggest a previously unappreciated role for VCAM-1 in esophageal squamous epithelial homeostasis and pathology.

Correspondence to: Dr Jan Heidemann, Department of Medicine B, University of Münster, Albert-Schweitzer-Strasse 33, D-48129 Münster, Germany
E-mail: jan_heidemann@hotmail.com

Key words: esophagus, squamous epithelium, squamous cell carcinoma, VCAM-1, OE21

Introduction

Cell adhesion molecules (CAM) play an essential role in the development and homeostasis of all human tissues (1). This includes a physiologic role in contact inhibition and density-dependent cellular growth, as well as mediating leukocyte extravasation and tissue recruitment directed by endothelial cell-expressed CAM. CAM have been subdivided into four major groups based on their protein structure, which include members of the immunoglobulin superfamily, as well as the integrins, selectins, and cadherins (1-3). The immunoglobulin superfamily consists of a group of molecules that direct cell behavior and activity through mediation of cell-cell surface recognition (4). The intercellular cell adhesion molecules 1, 2, and 3 (ICAM-1,-2, and -3), platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31), and vascular cell adhesion molecule-1 (VCAM-1; CD106), form a group of functionally related CAMs that represent critical mediators in endothelial cell and leukocyte/connective tissue interactions (5). Generally detectable on activated endothelium in inflamed tissues and activated endothelial cells *in vitro*, these CAMs are known to control directed leukocyte migration across microvascular endothelial barriers (6). Further, ICAM-3 was reported to provide attachment points for developing endothelium during the process of angiogenesis (7).

The maintenance of intercellular junctions in normal human epithelial cells of most organs, including esophagus, is dependent on the expression of well-characterized epithelial CAMs, including E-cadherin (8). There is limited information on the expression of CAM in human esophageal tissues. During experiments characterizing microvascular expression of CAM in human esophageal inflammation (9), we detected novel and unexpected membrane expression of VCAM-1 in the basal squamous epithelial strata of normal human esophagus, pointing towards a previously unrecognized role of this cell adhesion molecule in esophageal epithelial pathobiology. To assess the possible functions of VCAM-1 in this setting, we examined pathologically altered tissue specimens

from human esophagus with respect to its possible functions in epithelial cell pathobiology and tumorigenesis.

Materials and methods

Tissue specimens. Normal, metaplastic and malignant esophageal mucosal specimens were obtained from patients undergoing scheduled resection for esophageal carcinoma. All studies involving human tissue were approved by the ethics committee of the University of Münster. Specimens were reviewed by one senior pathologist (Hermann Herbst).

Immunohistochemistry. Mucosal esophageal specimens were fixed in 4% (w/v) paraformaldehyde in PBS, saturated in 20% (w/v) sucrose in PBS overnight and embedded in OCT compound (Sakura, Japan). Endogenous peroxidase and biotin activities on esophageal tissue sections (5 μm) were blocked using hydrogen peroxide and the Avidin/Biotin blocking kit (Vector, Burlingame, CA), respectively. Tissue sections were immunostained using a murine monoclonal anti-VCAM-1 antibody (clone BBIG-V1; R&D Systems, Minneapolis, MN) and the Dako LSAB$^+$ tissue staining kit (Dako, Carpinteria, CA). Sections were counterstained with hematoxylin, mounted with gelatin and examined by light microscopy.

Immunofluorescence. For indirect immunofluorescence, mucosal esophageal specimens were fixed and sectioned as above. Sections were rehydrated in PBS, blocked in serum buffer [5% (v/v) normal donkey serum in PBS] and incubated with mouse monoclonal anti-VCAM-1 antibody (R&D Systems) diluted in serum buffer and incubated overnight at 4˚C. After extensive washing in PBS, immunodetection was performed using a polyclonal Cy3-labeled donkey anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) diluted in serum buffer, and nuclei were visualized by counterstaining with DAPI (Sigma-Aldrich, Steinheim, Germany). After mounting with Mowiol 4-88 (Hoechst KG, Frankfurt, Germany) containing 1,4-diazobicyclooctane (Sigma-Aldrich) as an anti-fading agent, immunofluorescence was assessed using a Leica DMLB microscope (Leica GmbH, Wetzlar, Germany). Similar epithelial immunoreactivity for VCAM-1 was detectable in fresh frozen acetone-fixed sections (not shown). As negative controls for immunofluorescence staining, preimmune mouse IgG at corresponding concentrations and preincubation of the primary monoclonal antibody with recombinant human VCAM-1 (R&D Systems; 20 μg per μg of monoclonal VCAM-1 antibody) were employed. For histological evaluation of esophageal carcinoma samples, formalin-fixed, embedded sections (4 μm) were deparaffinized in xylene, rehydrated in graded alcohols and washed briefly in PBS. Antigen retrieval was achieved by microwaving sections for 2x5 min in 10 mM citrate buffer (pH 6.0). Immunofluorescence staining was performed as described above.

Immunoelectron microscopy. Specimens were fixed in 4% (w/v) paraformaldehyde buffered in 50 mM HEPES and cryoprotected by infiltration with PVP/sucrose. Sectioning and labeling of ultrathin frozen sections was performed as previously described (10). Thin frozen sections (50 nm) were prepared with an ultracryomicrotome (Leica EM FCS, Vienna, Austria) at -110˚C and placed onto mesh nickel grids. After blocking with 10% (v/v) fetal bovine serum, sections were incubated with the monoclonal VCAM-1 antibody (dilution, 1:10) for 45 min, rinsed in PBS, and incubated with the goat anti-mouse gold-conjugated secondary antibody (particle size, 12 nm). Grids were contrasted and embedded in 2% (w/v) methylcellulose solution containing 0.3% (w/v) uranyl-acetate and examined with an EM 208 S electron microscope (Philips, Kassel, Germany).

Immunoblot analysis. Squamous epithelial sheets were readily separated from the underlying mucosa by dispase digestion as described earlier (11). Epithelial sheets were pulverized in liquid nitrogen, and total cellular protein was harvested by lysis in modified RIPA buffer [50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.25% (w/v) sodium deoxycholate, 1% (v/v) NP-40, 0.1% (w/v) sodium dodecylsulfate, 1% (v/v)] Triton X-100 (all from Sigma-Aldrich) containing Protease Inhibitor Cocktail III (Calbiochem, San Diego, CA) on ice. Lysates were cleared by ultracentrifugation, and total protein concentration was determined using a Bradford assay (Bio-Rad, Hercules, CA). Total cellular protein (100 μg) was immunoprecipitated using anti-human VCAM-1 mAb (R&D Systems) and Protein G-plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4˚C overnight. Immunoprecipitates were washed four times with PBS, collected in a microcentrifuge and denatured in Laemmli buffer. Immunoprecipitates and native protein samples were size-separated by 10% SDS-PAGE, blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Arlington Heights, IL) and blocked with blocking buffer [PBS containing 0.05% (v/v) Tween-20, 5% (w/v) non-fat dry milk]. Blots were incubated with polyclonal goat anti-VCAM-1 antibody (Clone C19; Santa Cruz) overnight at 4˚C. Immunodetection was performed using an HRP-conjugated rabbit anti-goat antibody (Sigma-Aldrich) and enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). Whole cell lysates from human intestinal microvascular endothelial cells (HIMEC; reviewed in ref. 9) stimulated for 16 h with TNF-α (20 ng/ml; Promega, Madison, WI) and LPS (1 μg/ml; *E. coli* O111:B4, Sigma-Aldrich) were used as positive control for expression of VCAM-1. Epithelial lysates were negative for the endothelial cell marker, von Willebrand Factor (vWF), as assessed by immunoblot analysis using polyclonal rabbit anti-human vWF antibody (data not shown).

Immunocytochemistry. OE21 cells, an epithelial cell line derived from a squamous esophageal carcinoma were a kind gift of Dr T. Pohle, University of Münster, Germany. Cells were grown in RPMI-1640 medium containing 5% (v/v) of fetal calf serum and antibiotics in a humidified atmosphere of 5% CO_2/95% air. Subconfluent monolayers grown on glass chamber slides (LabTek II, Nunc, Naperville, IL) were washed with PBS, fixed with paraformaldehyde (4% w/v in PBS) and permeabilized using 0.1% (v/v) of Triton X-100 (Sigma) in PBS. After washing, the specimens were blocked and stained for expression of VCAM-1 as described above. For inhibition experiments, subconfluent OE21 monolayers were treated

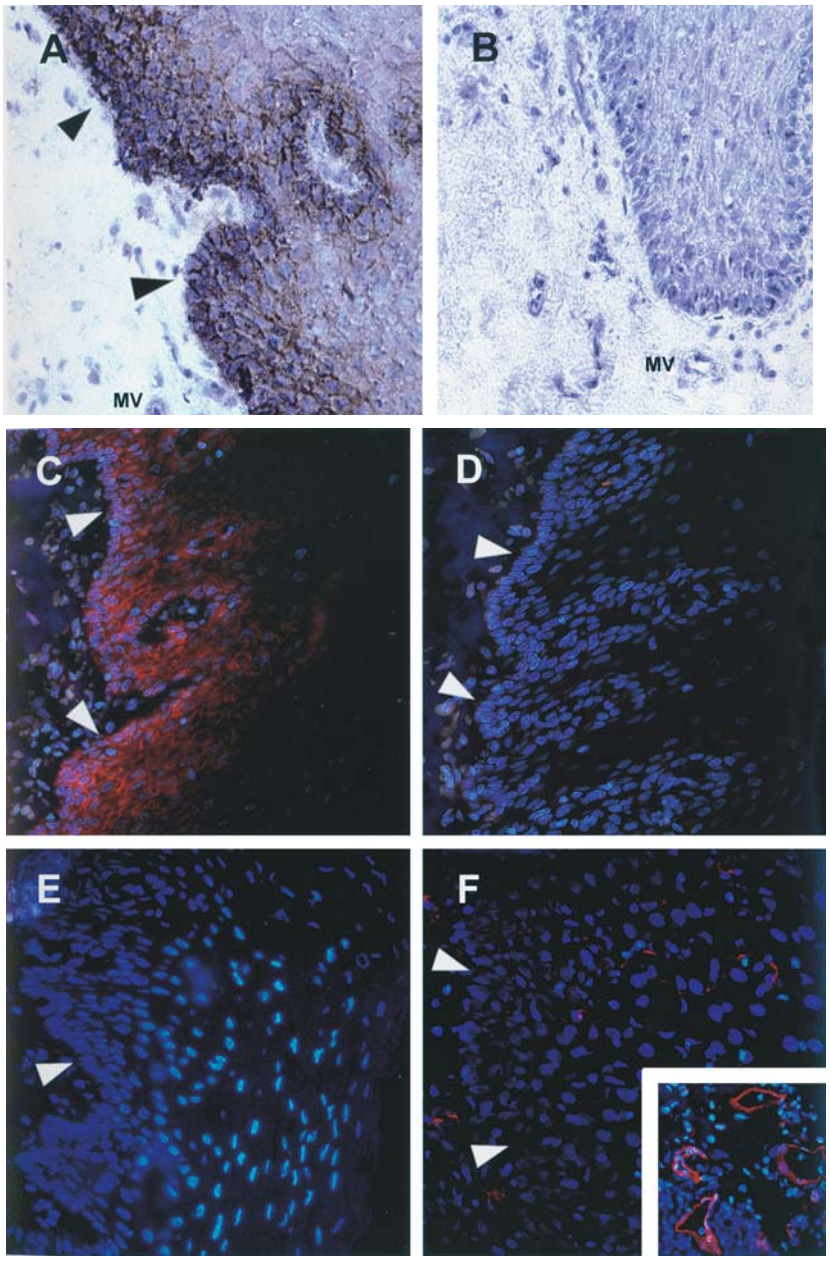

Figure 1. Immunohistochemical patterns of VCAM-1 expression by normal human esophageal squamous epithelium. A, Strong VCAM-1 immunoreactivity in basal and suprabasal squamous epithelial cell layers of normal human esophageal mucosa (A, chromagen DAB). Minor immunoreactivity of submucosal microvessels (MV) for VCAM-1. B, Negative control using murine isotype IgG at corresponding concentration. C-F, Indirect immunofluorescence using Cy3-labeled secondary antibodies. C, Marked membrane-associated VCAM-1 reactivity corresponding to the staining pattern observed in A. D and E, Negative controls lacking VCAM-1 immunoreactivity using murine isotype IgG at corresponding concentration (D) and by preincubation of the monoclonal VCAM-1 antibody with a 20-fold excess of recombinant human VCAM-1 (E). F, Discretely scattered ICAM-1 immunoreactivity (red) of infiltrating leukocytes. Epithelial immunoreactivity for ICAM-1, a closely-related immunoglobulin-type cell adhesion molecule is absent. F, inset, Strong constitutive reactivity for ICAM-1 in submucosal esophageal microvessels (positive control). Original magnification x40. Arrowheads, basal epithelial stratum. Representative results of at least three independent experiments are shown.

with 0-5 μM of Mitomycin C (Sigma) for 24 h, a bifunctional alkylating agent known to inhibit DNA synthesis by cross-linking mechanisms. Its action is reported to be most prominent during the late G1 and early S phases of the cell cycle, causing a cell cycle arrest in a dose-dependent fashion. After treatment, cells were immunostained as indicated above, and VCAM-1 positive cells were counted in 20 random high power fields displaying comparable overall cellular densities (HPF; magnification x40). Results are stated as mean cell number ± standard deviation, and the P-value was calculated using unpaired t-test. P-values <0.05 were considered significant.

Results

Squamous epithelial cells from normal human esophagus express VCAM-1. Initial staining experiments employing anti-human VCAM-1 mAb and HRP/DAB based immunohistochemistry revealed novel and unexpected expression of VCAM-1 in the basal and suprabasal epithelial layers of the normal human esophagus. The staining pattern was membrane-associated with only little cytoplasmic immunoreactivity of VCAM-1 in these cells (Fig. 1A). To rule out non-specific staining due to heterogeneous DAB/HRP reaction, immuno-

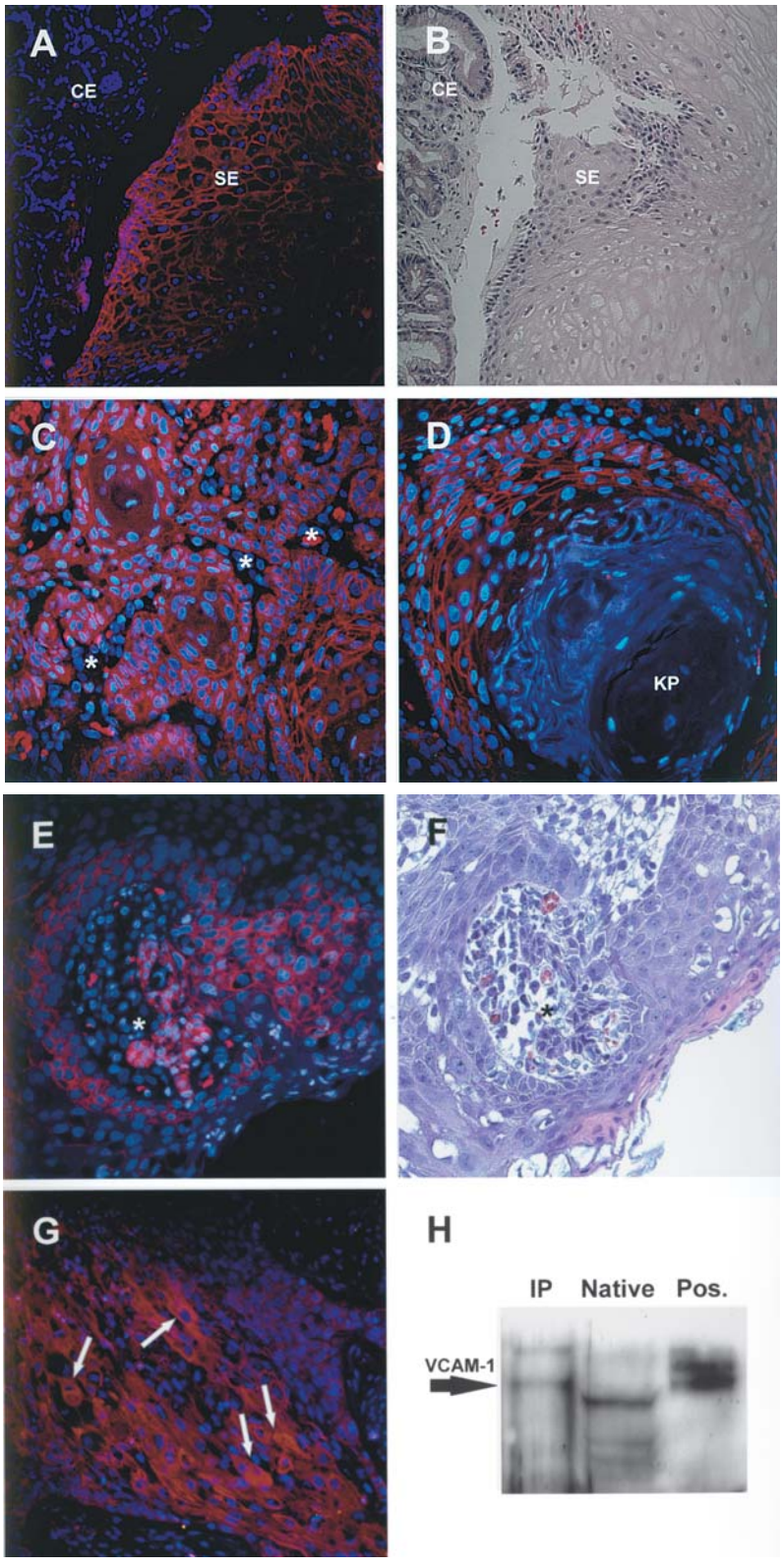

Figure 2. VCAM-1 expression patterns in Barrett's esophagitis and squamous cell carcinoma (SCC) of the esophagus. Indirect immunofluorescence staining (A) for VCAM-1 (red) and corresponding H&E section of Barrett's esophagitis (B). A Strong membrane-associated immunoreactivity for VCAM-1 is notable in squamous epithelium (SE) but absent in columnar epithelium (CE). C and D, Immunofluorescence staining pattern in squamous cell carcinoma of the esophagus. C, Strongly membrane-associated staining pattern in moderately differentiated SCC. Note the focally accentuated cytosplasmic staining pattern. VCAM-1 immunoreactivity is not detectable in the invaded submucosal stroma (C, asterisks) and keratin pearls (KP) of highly differentiated SCC of the esophagus (D). E, VCAM-1 immunoreactivity is focally accentuated at sites characteristic of microscopic invasion (asterisks) in SCC of the esophagus (F, H&E section corresponding to E). G, Distribution of VCAM-1 in poorly differentiated SCC of the esophagus. VCAM-1 immunoreactivity is irregularly distributed with cytoplasmic expression of VCAM-1 in a fraction of tumor cells (arrows). Original magnification x40. H, Immunoblot analysis of esophageal squamous epithelial VCAM-1. To verify the detection specificity of the monoclonal VCAM-1 antibody, we performed immunoprecipitation of VCAM-1 from native squamous epithelial whole cell lysate from human esophagus. Immunodetection with a polyclonal VCAM-1 antibody yields a band of 100-110 kDa, the putative molecular weight of VCAM-1 (IP). Immunoblotting of native squamous epithelial lysate (Native) was lacking a VCAM-1-specific band. Total cellular lysates of human endothelial cells stimulated with TNF-α and LPS for 16 h served as a positive control (Pos.), showing a marked dual band, likely corresponding to different VCAM-1 isoforms. Representative results of at least three independent experiments are shown.

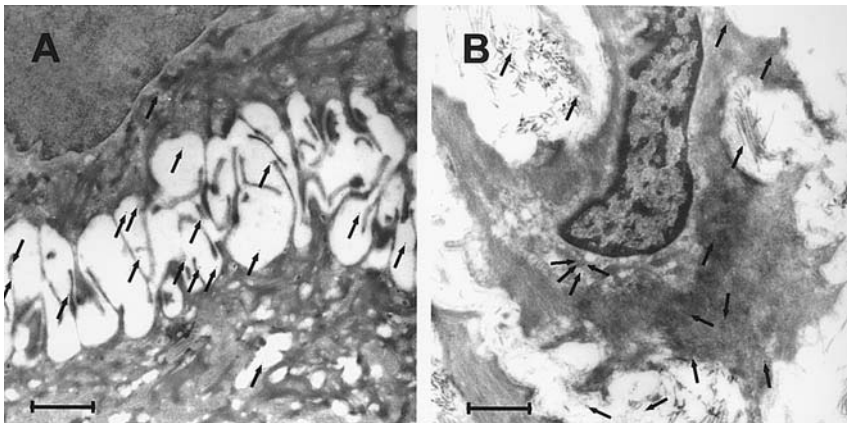

Figure 3. Altered ultrastructural distribution of VCAM-1 in SCC of the esophagus as assessed by immunogold electron microscopy. A, Distribution of VCAM-1 expression is confined mainly to the intercellular space and outer cellular membranes in squamous epithelial cells of normal human esophageal mucosa (arrows denote distribution of immunogold particles). B, Ultrastructural analysis of VCAM-1 in moderately differentiated SCC of the esophagus revealing enhanced cytoplasmic deposition of immunogold particles. VCAM-1 expression in the intercellular space appears to be reduced. Original magnification x13.000. Immunogold particle size, 12 nm. Bars indicate 1 μm. Representative results of three independent experiments are shown.

detection of VCAM-1 was additionally performed by indirect immunofluorescence using Cy3-labeled secondary antibodies (Fig. 1C). In both staining techniques, specific epithelial immunoreactivity was absent using murine isotype control IgG (Fig. 1B and D). To further test the specificity of this staining pattern, the VCAM-1 mAb was preincubated with saturating concentrations (20-fold excess) of recombinant human VCAM-1. As expected, the staining intensity was significantly reduced in a dose-dependent fashion, leading to near complete abolishment at saturating concentrations (Fig. 1E). Interestingly, no epithelial immunoreactivity for a closely related immunoglobulin-type adhesion molecule, intercellular cell adhesion molecule-1 (ICAM-1) was detectable (Fig. 1F), whereas immunoreactivity for ICAM-1 was ubiquitously present in microvascular endothelial cells of the esophageal mucosa (Fig. 1F, inset). Epithelial expression of VCAM-1 was further assessed by immunoblot analysis to verify the detection specificity of the monoclonal VCAM-1 antibody. Esophageal epithelium of freshly resected specimens was gently separated from the underlying mucosa by dispase digestion as described earlier (11). Epithelial sheets were lysed, and the lysate was pre-cleared and subjected to immunoprecipitation using the anti-VCAM-1 monoclonal antibody. Immunodetection with a polyclonal anti-human VCAM-1 antibody yields a band of 100-110 kDa, the putative molecular weight of VCAM-1 (Fig. 2H). Native epithelial lysates, however, were lacking a specific band, potentially due to a low detection capacity of this assay. Total cellular protein from human endothelial cells stimulated with TNF-α and bacterial LPS served as positive control, showing a marked dual band, likely corresponding to different VCAM-1 isoforms.

Disturbed subcellular VCAM-1 immunoreactivity in neoplastic esophageal epithelium. After having shown physiological expression of VCAM-1 in normal esophageal epithelium we next sought to define the VCAM-1 expression patterns exhibited in pathologically altered esophageal epithelia. We performed indirect immunofluorescence staining for VCAM-1 on sections of columnar cell carcinoma (n=3), Barrett's metaplasia (n=3), and squamous cell carcinoma (SCC; n=7) specimens. Of interest, VCAM-1 immunoreactivity was not detectable in esophageal columnar epithelial cells of Barrett's esophagitis (Fig. 2A and B) and esophageal adenocarcinoma (not shown), whereas it was highly and consistently expressed in neoplastic epithelial cells of esophageal squamous cell carcinoma (Fig. 2C-G). In highly differentiated SCC, VCAM-1 immunoreactivity was generally membrane-associated, as similarly seen in normal epithelial cells from control samples (Fig. 2C and D; asterisks: stroma devoid of tumor cells). VCAM-1 immunoreactivity in esophageal SCC appeared to be focally accentuated at sites characteristic of microscopic invasion, displaying cytoplasmic distribution of the staining pattern (Fig. 2E and F). In contrast to these observations, VCAM-1 staining was redistributed to intracellular compartments in poorly differentiated SCC specimens with pronounced nuclear polymorphy, displaying marked cytosolic immunofluorescence (Fig. 2G, arrows).

Esophageal squamous epithelial expression of VCAM-1 is confined to distinct ultrastructural compartments. Having detected VCAM-1 in the basal and suprabasal squamous epithelial strata of normal esophagus, we next sought to determine the ultrastructural localization of VCAM-1 in these cells by immunoelectron microscopy. Immunogold particles (12 nm) indicating VCAM-1 localization were present in the intercellular space and along the plasma membrane linings of basal and suprabasal squamous epithelial cells (Fig. 3A, arrows). In strong contrast, cytosolic compartments were virtually devoid of VCAM-1 reactivity. In epithelial cells of esophageal SCC, however, VCAM-1 was distributed throughout the cytosol, with minor VCAM-1 expression along the plasma membrane and within the intercellular space (Fig. 3B, arrows).

VCAM-1 immunoreactivity is restricted to dividing OE21 squamous epithelial esophageal carcinoma cells in vitro. We next aimed to assess the expression of VCAM-1 in OE21

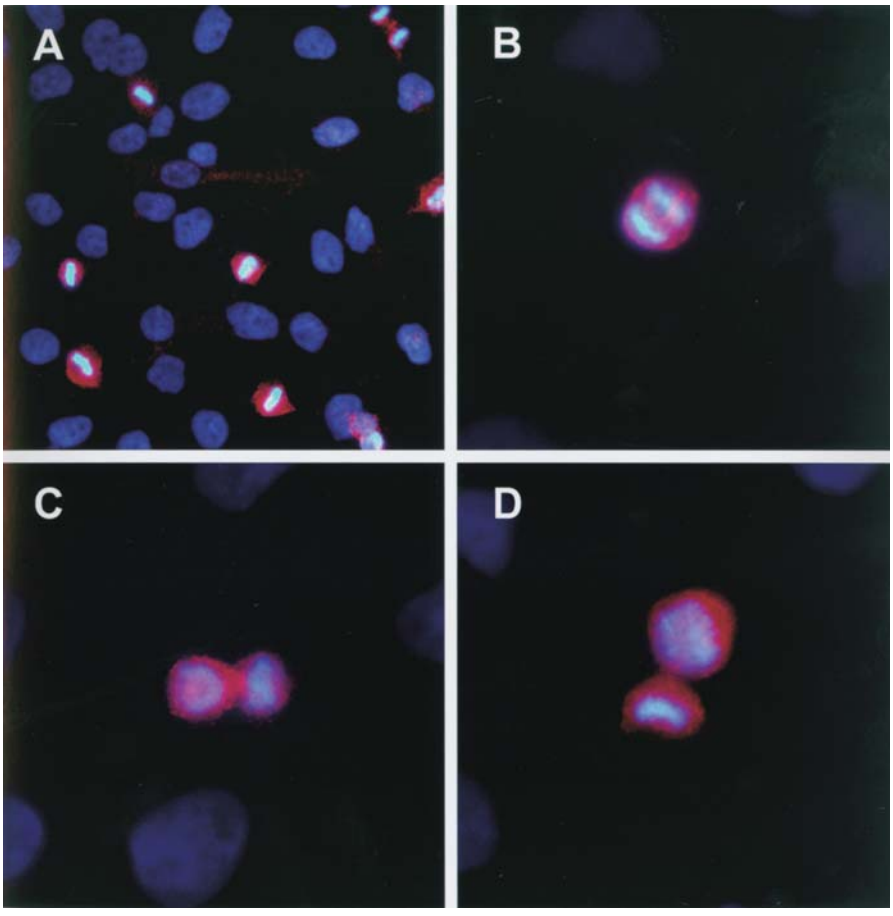

Figure 4. VCAM-1 immunofluorescence of resting and dividing OE21 squamous epithelial esophageal carcinoma cells. VCAM-1 immunoreactivity is restricted to dividing and mitotic OE21 epithelial cells, while resting cells were lacking detectable immunoreactivity for VCAM-1 (A, original magnification x40). B-D display subsequent stages of OE21 cell division, starting with mitosis (B) (original magnification x100). Representative results of five independent experiments are shown.

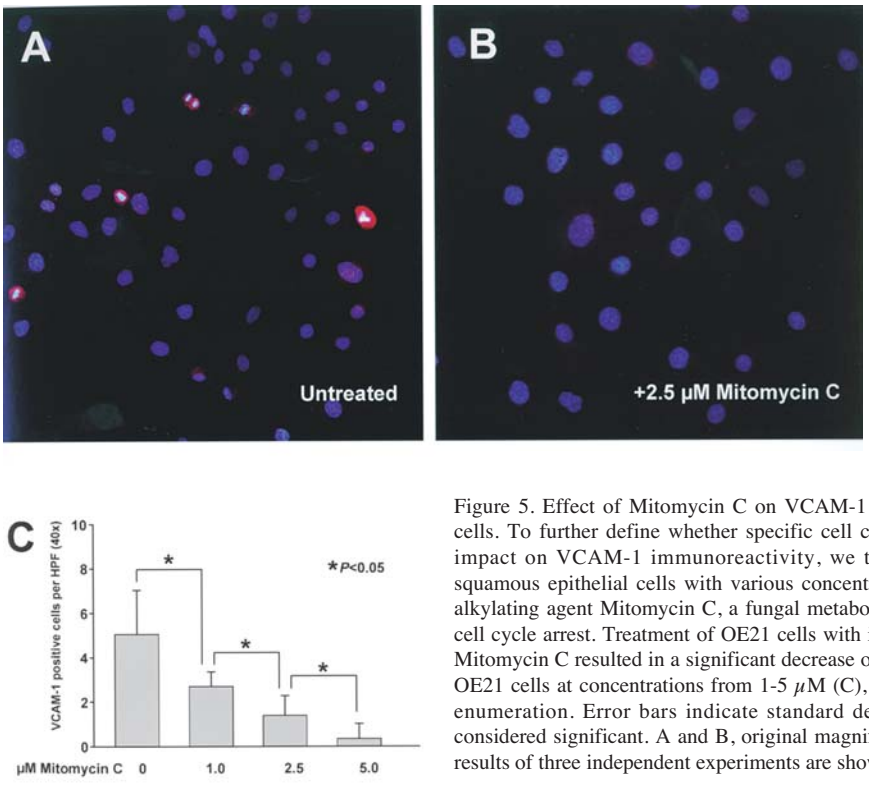

Figure 5. Effect of Mitomycin C on VCAM-1 immunoreactivity in OE21 cells. To further define whether specific cell cycle phases would have an impact on VCAM-1 immunoreactivity, we treated OE21 oesophageal squamous epithelial cells with various concentrations (1.0-5.0 μM) of the alkylating agent Mitomycin C, a fungal metabolite causing dose-dependent cell cycle arrest. Treatment of OE21 cells with increasing concentrations of Mitomycin C resulted in a significant decrease of VCAM-1 immunoreactive OE21 cells at concentrations from 1-5 μM (C), as assessed by microscopic enumeration. Error bars indicate standard deviations, and $P<0.05$ was considered significant. A and B, original magnification x40. Representative results of three independent experiments are shown.

squamous epithelial esophageal carcinoma cells *in vitro*. Using subconfluent cell monolayers cultured in growth medium, VCAM-1 immunoreactivity appeared to be restricted to dividing and mitotic OE21 epithelial cells, while resting cells were devoid of detectable immunoreactivity for VCAM-1 (Fig. 4). After accomplishing cell division, cells appeared to gradually loose VCAM-1 immunoreactivity (not shown). To further define whether specific cell cycle phases would have an impact on VCAM-1 immunoreactivity, we treated OE21 cells with various doses of the alkylating agent Mitomycin C, a fungal agent causing dose-dependent cell cycle arrest in various human cancer cells. Accordingly, treatment of OE21 cells with increasing concentrations of Mitomycin C resulted in a significant decrease of VCAM-1 immunoreactive OE21 cells at concentrations from 1 to 5 μM (Fig. 5), as assessed by microscopic enumeration.

Discussion

The expression of cell adhesion molecules in human esophageal epithelium has been the subject of limited studies. Dobson and coworkers have reported on the differential epithelial expression of cell-matrix (integrins) and cell-cell adhesion molecules (cadherins) and their tissue distribution in human squamous epithelium from esophagus. According to their findings, these adhesion molecules display a well defined spatial pattern of immunoreactivity in the esophageal mucosa, playing a potential role in the maintenance of normal tissue architecture and physiological homeostasis (12). In this study, we report that VCAM-1, an adhesion molecule generally found on activated endothelial cells, is constitutively and highly expressed on human squamous epithelial cells of the esophageal basal and suprabasal strata. No such immunoreactivity could be detected on columnar epithelial cells in Barrett's metaplasia or adenocarcinoma, indicating differentiation specificity. Additionally, expression patterns for VCAM-1 were obviously specifically altered in SCC, pointing towards a previously unrecognized role of this prototypic endothelial cell adhesion molecule in esophageal epithelial pathobiology. These findings were reflected by ultrastructural expression patterns, indicating a cytosolic translocation of VCAM-1 immunoreactivity in squamous cell carcinoma.

Assessment of OE21 squamous epithelial esophageal carcinoma cells *in vitro* revealed marked and exclusive VCAM-1 immunoreactivity in cells undergoing cell division. Resting epithelial cells, however, appeared to be devoid of any specific VCAM-1 immunoreactivity. This parallels our *in vivo* data, which indicate that VCAM-1 immunoreactivity is confined to the germinative layer of normal human squamous esophageal epithelium, gradually decreasing towards the suprabasal layers. These findings indicate that VCAM-1 immunoreactivity in squamous esophageal epithelial cells might be regulated by cell cycle phase and appears to be inversely correlated with squamous epithelial differentiation.

In previous studies VCAM-1 expression was also reported, among others, in activated neurons (13), dendritic cells (14), smooth muscle cells (15), Sertoli cells (16), oocytes (17), and fibroblasts (18). Soluble forms of VCAM-1 have been identified in blood (19,20) and tissue culture supernatants (21), showing elevated levels in immune-mediated disorders, such as acute myelomonocytic leukemia (19), bronchial asthma (20), multiple sclerosis (22), and sepsis (23). The expression of VCAM-1 in experimental systems is dependent on multiple cytokine pathways involving tumor necrosis factor (TNF)-α (24), interleukins (IL)-1 and -4 (25), as well as pathogenic stimuli including bacterial lipopolysaccharide (LPS) (26) and viral infections (27,28). The VCAM-1 gene includes NF-κB binding sites (29). Recently, dermal squamous epithelial expression of VCAM-1 was observed by Kim and coworkers in murine aGvHD. The authors hypothesized that epithelially expressed VCAM-1 might serve as a potential ligand for infiltrating T-cells in this pathophysiological setting (11).

Epithelial VCAM-1 expression has been reported in several other previous studies. Melendez *et al* have found VCAM-1 and ICAM-1 expression in dermal keratoacanthoma and cutaneous squamous cell carcinoma. Similar to the present study, highest epithelial expression of VCAM-1 was found in poorly different carcinomas (30). In human gastric carcinoma, epithelial overexpression of VCAM-1 has been associated with oncogenesis, tumor angiogenesis and metastasis. Furthermore, serum levels of soluble VCAM-1 appeared to be of diagnostic and prognostic significance in these gastric carcinoma patients (31). In addition, Ali and coworkers have shown constitutive and regulated expression of VCAM-1 in human breast adenocarcinoma cell lines, where stimulation with TNF-α augmented VCAM-1 expression in a time-dependent manner (32). These findings suggest that VCAM-1 could serve as a diagnostic marker which might be an important tool in predicting the biologic behavior and pathogenesis of poorly differentiated carcinomas.

Reported ligands for VCAM-1 are the heterodimers α4ß1 (very late antigen-4; VLA-4; CD49d) and α4ß7 of the integrin family (33,34), with VLA-4 being the principal ligand or co-receptor for VCAM-1 (33). In addition, VLA-4 is known to bind the extracellular matrix protein fibronectin (35). Cells expressing VLA-4 include eosinophils (36), basophils (37), NK cells (38), monocytes, T cells, and B cells (39), among others. Interestingly, neutrophils were not shown to express VLA-4 (40). Given this notion, it can be hypothesized that epithelial VCAM-1 expression may support adhesive interactions with infiltrating cells, thus facilitating tumor infiltration by leukocytes.

At present, little is known about the intracellular signaling properties of immunoglobulin-type cell adhesion molecules, including VCAM-1. In endothelial cells, VCAM-1 was shown to transduce the activation of endothelial cell NADPH oxidase, a signal required for lymphocyte migration (41). In addition to their well-recognized 'passive' adhesive properties, there is emerging evidence that immunoglobulin-type cell adhesion molecules, such as ICAM-1 and VCAM-1, exert much more complex outside, in signaling functions upon ligation of their respective extracellular ligands. Activation of ICAM-1 by cross-linking using specific ICAM-1 antibodies or by its ligand, ß2 integrin, resulted in rapid stress fiber reorganization by activation of heterotrimeric G-proteins (42), expression induction of VCAM-1 (43), as well as intracellular responses including protein phosphorylation and subsequent activation of transcription factors (44). More specifically, activation of ICAM-1 led to phosphorylation of

the cyclin-dependent protein kinase cdc2, a cell cycle regulator (45), and of the mitogen activated protein kinases ERK1/2 (46), which is a well-established mitogenic stimulus leading to enhanced proliferation in effector cells (47).

These findings leave room for speculation that expression of VCAM-1 on human squamous esophageal epithelial cells might serve functions other than chemoattraction and adhesion of VLA-4 bearing lymphocytes in target tissues. Expressed in normal human esophageal squamous epithelium, VCAM-1 plays a potential role in regulation of density-dependent epithelial cell growth and maintenance of a physiological mucosal barrier, whereas a disturbed subcellular distribution, as indicated by cytosolic immunoreactivity of squamous epithelial cells at sites of invasion and in poorly differentiated squamous cell carcinoma might point to another previously unrecognized function of VCAM-1 in this setting. A reduction in cell-cell adhesion of cancer cells is considered to be an essential step in the progression from localized tumor growth to metastatic disease. The expression of E-cadherin, a well-characterized component of epithelial cell-cell contacts, was reported to play a pivotal role in tumor invasion and metastasis. The reduced membrane-expression of E-cadherin in poorly differentiated SCC is correlated with its high propensity for lymph node metastasis (48), as well as with hematogenous recurrence and poor prognosis in patients with esophageal SCC (49). Similarly, cytosolic expression patterns of E-cadherin in low-grade esophageal SCC were associated with lymph node metastasis, invasive growth and lower survival rates (48).

The loss of E-cadherin expression in esophageal squamous epithelial cancer cells is most likely caused by CpG island hypermethylation of the E-cadherin 5' promoter region (50). Interestingly, treatment of esophageal squamous epithelial cancer cells with the differentiation-inducing agent all-trans retinoic acid (ATRA) led to a re-induction of epithelial E-cadherin *in vitro* (8). E-cadherin has therefore been proposed as a squamous cell differentiation marker. Similar findings were reported for desmoglein, an adhesion molecule of the cadherin family, which expression was cell-membrane associated in normal epithelial cells and redistributed throughout the cytoplasm in SCC cells (51). Our findings on VCAM-1 being expressed in esophageal squamous epithelium potentially reflect the concept obtained from studies on E-cadherin expression in human esophageal SCC.

In conclusion, the altered cellular expression of VCAM-1 at sites of invasive growth and in low-grade differentiated esophageal carcinomas might hint at a previously unrecognized signaling pathway involved in the control of squamous epithelial cell growth. Our *in vitro* findings corroborate the hypothesis that VCAM-1 might be involved in cell cycle regulation and epidermal differentiation of squamous esophageal epithelial cells. Further studies are warranted to assess the role of VCAM-1 expression in esophageal squamous epithelium.

Acknowledgements

The authors wish to thank Mrs. C. Westermann for conducting the immunoelectron microscopy studies and Dr C. Müller-Tidow for helpful discussion.

References

1. Hynes RO: Integrins. Versatility, modulation, and signaling in cell adhesion. Cell 69: 11-25, 1992.
2. Hynes RO: Cell adhesion: old and new questions. Trends Cell Biol 9: 33-37, 1999.
3. Joseph-Silverstein J and Silverstein RL: Cell adhesion molecules: an overview. Cancer Invest 16: 176-182, 1998.
4. Williams AF and Barclay AN: The immunoglobulin superfamily - domains for cell surface recognition. Annu Rev Immunol 6: 381-405, 1988.
5. Vestweber D: Regulation of endothelial cell contacts during leukocyte extravasation. Curr Opin Cell Biol 14: 587-593, 2002.
6. Kanda K, Hayman GT, Silverman MD and Lelkes PI: Comparison of ICAM-1 and VCAM-1 expression in various human endothelial cell types and smooth muscle cells. Endothelium 6: 33-44, 1998.
7. Patey N, Vazeux R, Canioni D, Potter T, Gallatin WM and Brousse N: Intercellular adhesion molecule-3 on endothelial cells: expression in tumors but not in inflammatory responses. Am J Pathol 148: 465-472, 1996.
8. Wu H, Lotan R, Menter D, Lippman SM and Xu XC: Expression of E-cadherin is associated with squamous differentiation in squamous cell carcinomas. Anticancer Res 20: 1385-1390, 2000.
9. Rafiee P, Ogawa H, Heidemann J, Li MS, Aslam M, Lamirand TH, Fisher PJ, Graewin SJ, Dwinell MB, Johnson CP, Shaker R and Binion DG: Isolation and characterization of human esophageal microvascular endothelial cells: mechanisms of inflammatory activation. Am J Physiol Gastrointest Liver Physiol 285: G1277-G1292, 2003.
10. Kersting S, Bruewer M, Schuermann G, Klotz A, Utech M, Hansmerten M, Krieglstein CF, Senninger N, Schulzke JD, Naim HY and Zimmer KP: Antigen transport and cytoskeletal characteristics of a distinct enterocyte population in inflammatory bowel diseases. Am J Pathol 165: 425-437, 2004.
11. Kim JC, Whitaker-Menezes D, Deguchi M, Adair BS, Korngold R and Murphy GF: Novel expression of vascular cell adhesion molecule-1 (CD106) by squamous epithelium in experimental acute graft-versus-host disease. Am J Pathol 161: 763-770, 2002.
12. Dobson H, Pignatelli M, Hopwood D and D'Arrigo C: Cell adhesion molecules in oesophageal epithelium. Gut 35: 1343-1347, 1994.
13. Birdsall HH, Lane C, Ramser MN and Anderson DC: Induction of VCAM-1 and ICAM-1 on human neural cells and mechanisms of mononuclear leukocyte adherence. J Immunol 148: 2717-2723, 1992.
14. Huang MJ, Osborn L, Svahn J, Schiffer SB, Eliseo L, Zhou LJ, Rhynhart K, Benjamin CD and Freedman AS: Expression of vascular cell adhesion molecule-1 by follicular dendritic cells. Leuk Lymphoma 18: 259-264, 1995.
15. Ardehali A, Laks H, Drinkwater DC, Ziv E and Drake TA: Vascular cell adhesion molecule-1 is induced on vascular endothelia and medial smooth muscle cells in experimental cardiac allograft vasculopathy. Circulation 92: 450-456, 1995.
16. Riccioli A, Filippini A, De Cesaris P, Barbacci E, Stefanini M, Starace G and Ziparo E: Inflammatory mediators increase surface expression of integrin ligands, adhesion to lymphocytes, and secretion of interleukin 6 in mouse Sertoli cells. Proc Natl Acad Sci USA 92: 5808-5812, 1995.
17. Campbell S, Swann HR, Seif MW, Kimber SJ and Aplin JD: Cell adhesion molecules on the oocyte and preimplantation human embryo. Hum Reprod 10: 1571-1578, 1995.
18. Meng H, Marchese MJ, Garlick JA, Jelaska A, Korn JH, Gailit J, Clark RA and Gruber BL: Mast cells induce T-cell adhesion to human fibroblasts by regulating intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression. J Invest Dermatol 105: 789-796, 1995.
19. Sudhoff T, Wehmeier A, Kliche KO, Aul C, Schlomer P, Bauser U and Schneider W: Levels of circulating endothelial adhesion molecules (sE-selectin and sVCAM-1) in adult patients with acute leukemia. Leukemia 10: 682-686, 1996.
20. Koizumi A, Hashimoto S, Kobayashi T, Imai K, Yachi A and Horie T: Elevation of serum soluble vascular cell adhesion molecule-1 (sVCAM-1) levels in bronchial asthma. Clin Exp Immunol 101: 468-473, 1995.
21. Pigott R, Dillon LP, Hemingway IH and Gearing AJ: Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells. Biochem Biophys Res Commun 187: 584-589, 1992.

22. Matsuda M, Tsukada N, Miyagi K and Yanagisawa N: Increased levels of soluble vascular cell adhesion molecule-1 (VCAM-1) in the cerebrospinal fluid and sera of patients with multiple sclerosis and human T lymphotropic virus type-1-associated myelopathy. J Neuroimmunol 59: 35-40, 1995.
23. Boldt J, Muller M, Heesen M, Neumann K and Hempelmann GG: Influence of different volume therapies and pentoxifylline infusion on circulating soluble adhesion molecules in critically ill patients. Crit Care Med 24: 385-391, 1996.
24. Croft D, McIntyre P, Wibulswas A and Kramer I: Sustained elevated levels of VCAM-1 in cultured fibroblast-like synoviocytes can be achieved by TNF-alpha in combination with either IL-4 or IL-13 through increased mRNA stability. Am J Pathol 154: 1149-1158, 1999.
25. Atsuta J, Sterbinsky SA, Plitt J, Schwiebert LM, Bochner BS and Schleimer RP: Phenotyping and cytokine regulation of the BEAS-2B human bronchial epithelial cell: demonstration of inducible expression of the adhesion molecules VCAM-1 and ICAM-1. Am J Respir Cell Mol Biol 17: 571-582, 1997.
26. Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosa M, Osborn L, Chi-Rosso G, Newman B, Lobb R and Rosa M: Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. Blood 76: 965-970, 1990.
27. Wang SZ, Hallsworth PG, Dowling KD, Alpers JH, Bowden JJ and Forsyth KD: Adhesion molecule expression on epithelial cells infected with respiratory syncytial virus. Eur Respir J 15: 358-366, 2000.
28. Papi A and Johnston SL: Respiratory epithelial cell expression of vascular cell adhesion molecule-1 and its up-regulation by rhinovirus infection via NF-kappaB and GATA transcription factors. J Biol Chem 274: 30041-30051, 1999.
29. Neish AS, Williams AJ, Palmer HJ, Whitley MZ and Collins T: Functional analysis of the human vascular cell adhesion molecule 1 promoter. J Exp Med 176: 1583-1593, 1992.
30. Melendez ND, Smoller BR and Morgan M: VCAM (CD-106) and ICAM (CD-54) adhesion molecules distinguish keratoacanthomas from cutaneous squamous cell carcinomas. Mod Pathol 16: 8-13, 2003.
31. Ding YB, Chen GY, Xia JG, Zang XW, Yang HY and Yang L: Association of VCAM-1 overexpression with oncogenesis, tumor angiogenesis and metastasis of gastric carcinoma. World J Gastroenterol 9: 1409-1414, 2003.
32. Ali S, Kaur J and Patel KD: Intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and regulated on activation normal T cell expressed and secreted are expressed by human breast carcinoma cells and support eosinophil adhesion and activation. Am J Pathol 157: 313-321, 2000.
33. Chan BM, Elices MJ, Murphy E and Hemler ME: Adhesion to vascular cell adhesion molecule 1 and fibronectin: comparison of alpha 4 beta 1 (VLA-4) and alpha 4 beta 7 on the human B cell line JY. J Biol Chem 267: 8366-8370, 1992.
34. Ruegg C, Postigo AA, Sikorski EE, Butcher EC, Pytela R and Erle DJ: Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. J Cell Biol 117: 179-189, 1992.
35. Irie A, Kamata T, Puzon-McLaughlin W and Takada Y: Critical amino acid residues for ligand binding are clustered in a predicted beta-turn of the third N-terminal repeat in the integrin alpha 4 and alpha 5 subunits. EMBO J 14: 5550-5556, 1995.
36. Walsh GM, Symon FA, Lazarovils AL and Wardlaw AJ: Integrin alpha 4 beta 7 mediates human eosinophil interaction with MAdCAM-1, VCAM-1 and fibronectin. Immunology 89: 112-119, 1996.
37. Bochner BS, Sterbinsky SA, Briskin M, Saini SS and MacGlashan DW Jr: Counter-receptors on human basophils for endothelial cell adhesion molecules. J Immunol 157: 844-850, 1996.
38. Rott LS, Briskin MJ, Andrew DP, Berg EL and Butcher EC: A fundamental subdivision of circulating lymphocytes defined by adhesion to mucosal addressin cell adhesion molecule-1. Comparison with vascular cell adhesion molecule-1 and correlation with beta 7 integrins and memory differentiation. J Immunol 156: 3727-3736, 1996.
39. Hemler ME, Elices MJ, Parker C and Takada Y: Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. Immunol Rev 114: 45-65, 1990.
40. Bochner BS, Luscinskas FW, Gimbrone MA Jr, Newman W, Sterbinsky SA, Derse-Anthony CP, Klunk D and Schleimer RP: Adhesion of human basophils, eosinophils, and neutrophils to interleukin 1-activated human vascular endothelial cells: contributions of endothelial cell adhesion molecules. J Exp Med 173: 1553-1557, 1991.
41. Matheny HE, Deem TL and Cook-Mills JM: Lymphocyte migration through monolayers of endothelial cell lines involves VCAM-1 signaling via endothelial cell NADPH oxidase. J Immunol 164: 6550-6559, 2000.
42. Van Wetering S, van den Berk N, van Buul JD, Mul FP, Lommerse I, Mous R, ten Klooster JP, Zwaginga JJ and Hordijk PL: VCAM-1-mediated Rac signaling controls endothelial cell-cell contacts and leukocyte transmigration. Am J Physiol Cell Physiol 285: C343-C352, 2003.
43. Lawson C, Ainsworth M, Yacoub M and Rose M: Ligation of ICAM-1 on endothelial cells leads to expression of VCAM-1 via a nuclear factor-kappa B-independent mechanism. J Immunol 162: 2990-2996, 1999.
44. Durieu-Trautmann O, Chaverot N, Cazaubon S, Strosberg AD and Couraud PO: Intercellular adhesion molecule 1 activation induces tyrosine phosphorylation of the cytoskeleton-associated protein cortactin in brain microvessel endothelial cells. J Biol Chem 269: 12536-12540, 1994.
45. Chirathaworn C, Tibbetts SA, Chan MA and Benedict SH: Cross-linking of ICAM-1 on T cells induces transient tyrosine phosphorylation and inactivation of cdc2 kinase. J Immunol 155: 5479-5482, 1995.
46. Sano H, Nakagawa N, Chiba R, Kurasawa K, Saito Y and Iwamoto I: Cross-linking of intercellular adhesion molecule-1 induces interleukin-8 and RANTES production through the activation of MAP kinases in human vascular endothelial cells. Biochem Biophys Res Commun 250: 694-698, 1998.
47. Gardiner EE and D'Souza SE: Sequences within fibrinogen and intercellular adhesion molecule-1 (ICAM-1) modulate signals required for mitogenesis. J Biol Chem 274: 11930-11936, 1999.
48. Kaihara T, Kusaka T, Kawamata H, Oda Y, Fujii S, Morita K, Imura J and Fujimori T: Decreased expression of E-cadherin and Yamamoto-Kohama's mode of invasion highly correlates with lymph node metastasis in esophageal squamous cell carcinoma. Pathobiology 69: 172-178, 2001.
49. Tamura S, Shiozaki H, Miyata M, Kadowaki T, Inoue M, Matsui S, Iwazawa T, Takayama T, Takeichi M and Monden M: Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. Br J Surg 83: 1608-1614, 1996.
50. Si HX, Tsao SW, Lam KY, Srivastava G, Liu Y, Wong YC, Shen ZY and Cheung AL: E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. Cancer Lett 173: 71-78, 2001.
51. Pittella F, Katsube K, Takemura T, Hashimoto T, Kawano T, Garrod D and Takagi M: Perinuclear and cytoplasmic distribution of desmoglein in esophageal squamous cell carcinomas. Pathol Res Pract 197: 85-91, 2001.