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Light and electron microscopic immunolocalization of endothelial leucocyte adhesion molecule-1 in inflammatory bowel disease

Morphological evidence of active synthesis and secretion into vascular lumen

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Summary. Endothelial leucocyte adhesion molecule-1 (ELAM-1) is a rapidly inducible adhesion molecule for neutrophils in vascular endothelial cells. We investigated its immunohistochemical localization in 17 cases of inflammatory bowel disease. ELAM-1 was preferentially expressed in venules in actively inflamed mucosa and granulation tissue. Most capillaries were negative for ELAM-1. In areas with mild inflammation its expression diminished markedly and in normal mucosa of the colon and small intestine its expression was sparse. Electron microscopically, venules in active inflammation had swollen endothelial cells with well-developed rough endoplasmic reticulum. Immunoelectron microscopy revealed ELAM-1 localization along the luminal plasma membrane and in rough endoplasmic reticulum and round granules, findings suggestive of active production in endothelial cells. Furthermore, exocytosis of immunoreactive substance into the lumen was confirmed. Our study suggests that venules in actively inflamed area play an important role in eliciting and/or maintaining acute inflammatory processes by active permeation of neutrophils from the blood stream into the tissue, and that ELAM-1 may be a secretory protein as well as a transmembrane receptor protein.

Key words: Inflammatory bowel disease – ELAM-1 – Endothelial cells – Immunoelectron microscopy – Neutrophils

Introduction

Inflammation and neoplasia are two major pathological conditions associated with angiogenesis. Although extensively studied in experimental models, morphological details associated with angiogenesis have remained poorly understood in human tissues. Endothelial cells not only constitute the wall of blood vessels but play an important roles in many metabolic aspects. They express MHC class II antigens, interleukin-1 (IL-1) and other adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), suggesting a close interaction between endothelial cells and leucocytes (Pober 1988; Beilke 1989). The present authors have studied phenotypical and morphological changes of microvasculature in human cancer stroma and inflammatory diseases (Ohtani and Sasano 1987; Ohtani and Nagura 1990; Matsumoto et al. 1989). We have found that endothelial cells not only make a barrier between the blood stream and tissues but are also closely related to the control of changes in the microenvironment. We have emphasized the importance of the study of morphological changes in the microvasculature in disease.

Ulcerative colitis and Crohn's disease are two important forms of inflammatory bowel disease (IBD) and we have studied adhesion molecules in these diseases in view of their importance in the microvasculature. Adhesion molecules are classified into the integrin family, the immunoglobulin superfamily and the lectin-type molecules (LECAM) (Yong and Khwaja 1990). Endothelial leucocyte adhesion molecule (ELAM)-1 belongs to the LECAM family together with GMP-140 and MEL-14 with a molecular weight of 115 kDa. ELAM-1 is rapidly induced in endothelial cells by inflammatory cytokines [IL-1, tumour necrosis factor (TNF), gamma-interferon (INF-γ)] (Bevilacqua et al. 1987, 1989) and plays an important role in permeation of neutrophils in inflammatory lesions. Its ligand has been identified as sialyl Le^x (sialosyl-X hapten) sugar chain (NeuAcα2→3-Galβ1→4[Fucα1→3]GlcNAc) (Lowe et al. 1990; Phillips et al. 1990). Sialyl Le^x is expressed in neutrophils and HL-60 myeloid leukaemia cells (Symington et al. 1985) as well as adenocarcinoma cells (Fukushi et al. 1984). In the present study, we have studied immunolocalization of ELAM-1 in IBD using prefixed frozen sections. We discuss its importance in the pathogenesis of IBD at both light and electron microscopic levels.

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Materials and methods

We used surgically resected cases of IBD obtained at Tohoku University Hospital, Sendai, including eight patients with ulcerative colitis and six with Crohn's disease. Biopsy specimens were used in three other cases of ulcerative colitis. The average age of the patients was 31 years. Immediately after resection, small slices of the specimens were fixed in periodate-lysine-paraformaldehyde (PLP) for 4–8 h. After washing in 10% sucrose in phosphate-buffered saline (PBS), 15% sucrose in PBS and 20% sucrose in PBS, the specimens were embedded in OCT compound (Miles) and rapidly frozen in acetone-dry ice. They were stored at −70° C until use. For control, normal-appearing mucosal tissues were obtained from the colon and ileum near the surgical resection margin of patients with colorectal cancer (four cases). Peyer's patch and lymph nodes were also used (both two cases). All the control tissues were fixed and processed by the same method (Table 1).

Serial, frozen sections, 6 μm in thickness, were cut on a cryostat. They were mounted on ovalbumin-coated glass slides and air-dried. After immersion in non-immunized rabbit serum, the primary antibodies were applied (monoclonal anti-ELAM-1 antibody and OKM5; Table 2) and incubated overnight at 4° C. We adopted the biotin-streptavidin-peroxidase method with the Histofine kit (Nichirei, Tokyo, Japan) with diaminobenzidine (DAB; Dojin, Kumamoto, Japan) as a chromogen. Endogenous peroxidase activity was abolished by immersing specimens in 0.3% H_2O_2 in methanol for 15 min after incubation with the primary antibodies and by adding sodium azide in DAB (65 mg/100 ml). For better preservation of morphological details, we fixed specimens in 1% glutaraldehyde for 1 min before incubation in DAB for both light and electron microscopic observation. The number of granulocytes was evaluated by endogenous peroxidase activity. For negative controls, the primary antibodies were replaced with PBS or indifferent antibodies including CD68 (Dako anti-macrophage) and CD11c (LeuM5). No specific reactivity was observed in venules with these negative controls in any case.

Specimens of ten cases of ulcerative colitis observed in our previous studies were reviewed for morphological changes in microvasculature by electron microscopy (Ohtani and Sasano 1983). These were obtained by biopsy or at surgical resection and fixed in 2.5% glutaraldehyde-2% paraformaldehyde for 2 h. After fixation in 1% osmium tetroxide for 2 h, they were dehydrated and embedded in Epon.

For immunoelectron microscopy, the staining procedure was the same as for light microscopic immunohistochemistry, but omitting the immersion of specimens in 0.3% H_2O_2/methanol. In some cases, the Histofine staining kit was replaced by peroxidase-labelled $F(ab')_2$ fragments of anti-mouse IgG (Amersham, diluted at 1:150), the enzyme-linked indirect method. The incubation time was 14–16 h. Penetration of the antibody into cell organelles was the same with both staining methods. After DAB reaction, the specimens were fixed in 1% osmium tetroxide for 1 h, dehydrated in graded ethanols and embedded in Epon. Ultrathin sections of silver-gold interference colour were stained with lead citrate for 1–2 min and observed with a Jeol JEM 100B electron microscope.

Table 1. List of specimens used (all the tissues for immunostaining were fixed in PLP and frozen)

	Number of cases examined
Ulcerative colitis	11 (3)[b] 8[c]
Crohn's disease	6[a] (3)[b]
Control colonic mucosa	2
Control ileal or jejunal mucosa	2
Peyer's patches	2
Lymph nodes	2

[a] Including one ileitis-type, three ileocolitis type and two colitis type
[b] Number examined by immunoelectron microscopy
[c] Number examined by conventional electron microscopy

Table 2. List of monoclonal antibodies used

Name	Working dilution	Source
Anti-ELAM-1	1:6000 (1:2000)[a]	British Biotechnology, Oxford, UK
OKM5 (CD36)	1:600	Ortho Diagnostic System, Raritan, N.J.

[a] Dilution for the peroxidase-labelled indirect method

Results

Light microscopy of the normal mucosal tissues showed that all capillaries were negative for ELAM-1, and venules were sporadically positive for ELAM-1 (Fig. 1a, arrow). Most of the capillaries were positive for CD36 (OKM5). Infiltration of granulocytes was sparse. In Peyer's patches and lymph nodes, some high endothelial venules (HEV) in the T-zone were positive for ELAM-1 (Fig. 1b) with capillaries uniformly negative.

In active inflammatory areas of IBD, venules with swollen endothelial cells were positive for ELAM-1. The

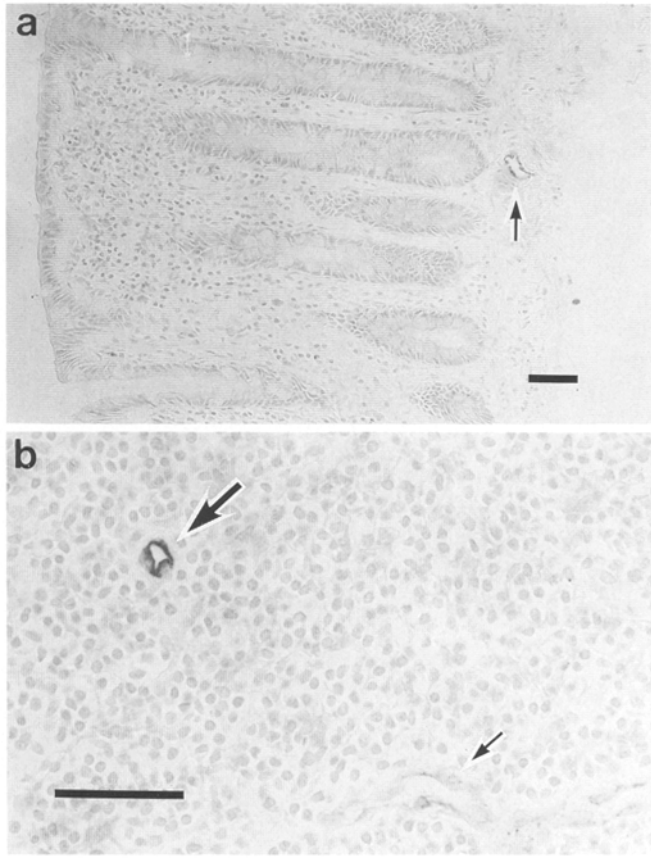
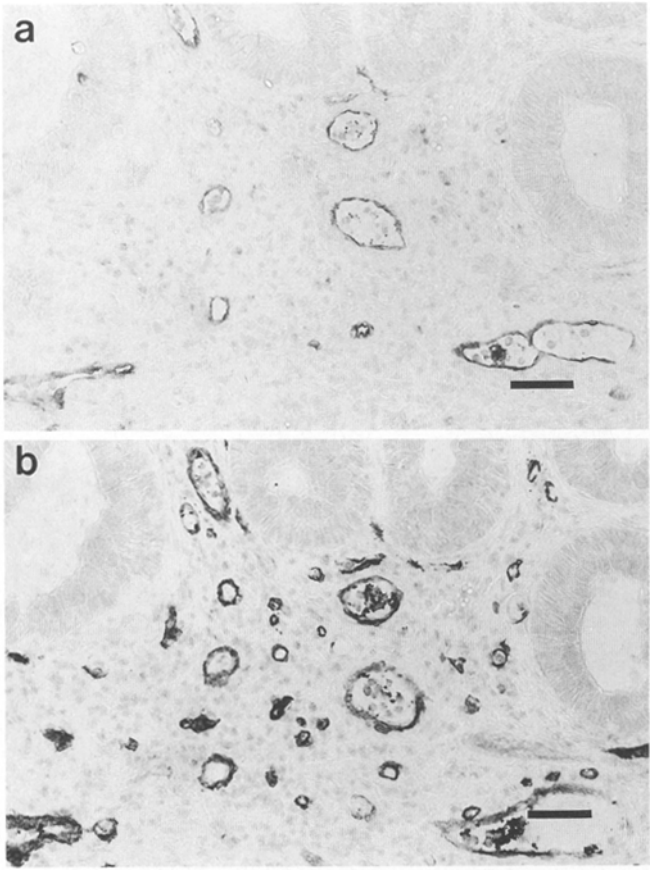


Fig. 1a, b. Normal control tissues. **a** ELAM-1 reactivity in the normal colonic mucosa. Note rarity of ELAM-1 positive vessels. An *arrow* indicates a venule positive for ELAM-1. ×130. *Bar* = 50 μm. **b** Normal Peyer's patch. Some of high endothelial venules (HEV) are positive (*arrows*). ×340. Bar = 50 μm



Figs. 2a, b. Serial sections in ulcerative colitis in active inflammatory stage. **a** ELAM-1. Immunoreactivity is present mainly in venules. ×170. *Bar* = 50 μm. **b** CD36. Capillaries and venules are positive. ×170. *Bar* = 50 μm

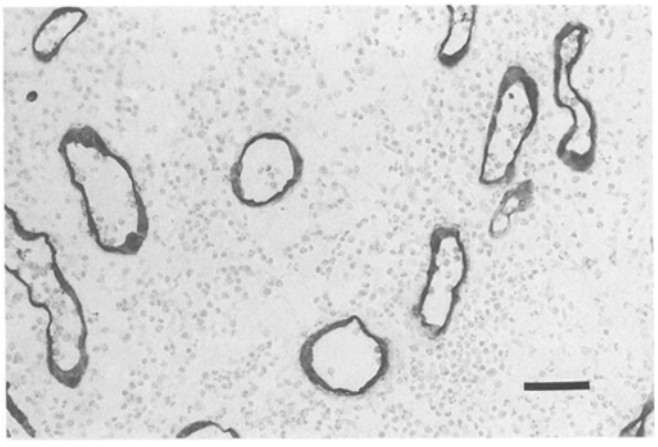


Fig. 3. ELAM-1 immunoreactivity in granulation tissue in the base of an ulcer in Crohn's disease. Note the immunoreactive venules. ×170. *Bar* = 50 μm

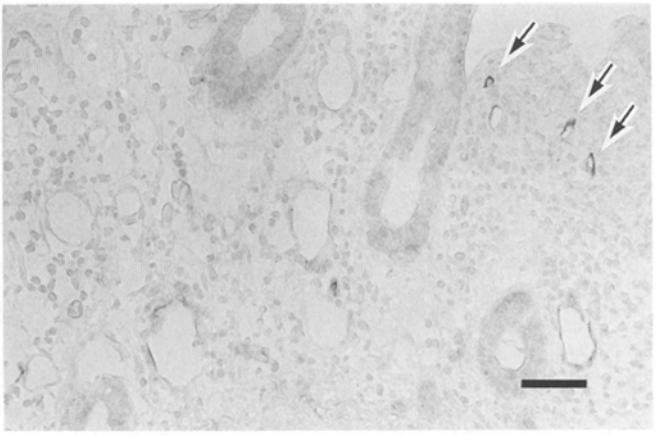


Fig. 4. Mild inflammatory stage of ulcerative colitis. ELAM-1-positive vessels are very much fewer. *Arrows* indicate immunoreactive vessels. ×170. *Bar* = 50 μm

origin of the specimen did not affect this result. Figure 2a and b shows a set of serial sections. CD36 stained most capillaries and venules, while ELAM-1 was expressed mainly in venules. The active inflammatory lesions were massively infiltrated by granulocytes as revealed by endogenous peroxidase activity (not shown). The immunoreactivity for ELAM-1 was most striking in granulation tissue at the base of ulcers in both ulcerative colitis and Crohn's disease (Fig. 3). In Crohn's disease, venules positive for ELAM-1 appeared also in the muscularis propria and the subserosa. In the area of mild inflammation (resolving phase), vessels positive for ELAM-1 diminished markedly in number (Fig. 4).

On conventional electron microscopy of ulcerative colitis in active inflammatory areas, there were capillaries of continuous type, capillary tips, and venules among the numerous inflammatory infiltrates. These had swollen endothelial cells with an abundance of rough endoplasmic reticulum and Golgi apparatus, showing endothelial activation (Fig. 5a). Capillary tips had large round endothelial cells with slit-like lumen and multilayered basal lamina (Fig. 5b). Occasionally, neutrophils were present between endothelial cells and pericytes (Fig. 5a, asterisks) and permeation of neutrophils through endothelial cells was confirmed (Fig. 5a, arrows).

Immunoelectron microscopy for ELAM-1 revealed, both in the lamina propria and granulation tissue, that ELAM-1 was localized along the luminal plasma membrane of endothelial cells of venules in active inflammatory areas (Figs. 6, 7a–d), and this was its basic localization pattern. Immunoreactivity in capillaries was infrequent (arrow in Fig. 6). Higher magnification in venules showed that immunoreactive substance was present as a flocculent substance on the outer surface of the plasma membrane as well as along the cell membrane (Fig. 7a, b, d). Corresponding to the cytoplasmic staining by light microscopy, ELAM-1 was observed in well-developed rough endoplasmic reticulum (Fig. 7a), perinuclear spaces (Fig. 7a, c), and in round granules, 200–450 nm in diameter (Fig. 7b). Figure 7c and d depicts exocytosis of immunoreactive substance into the lumen, which was seen in three of six cases.

Discussion

This is the first report on the subcellular localization of ELAM-1 in endothelial cells. We have shown that

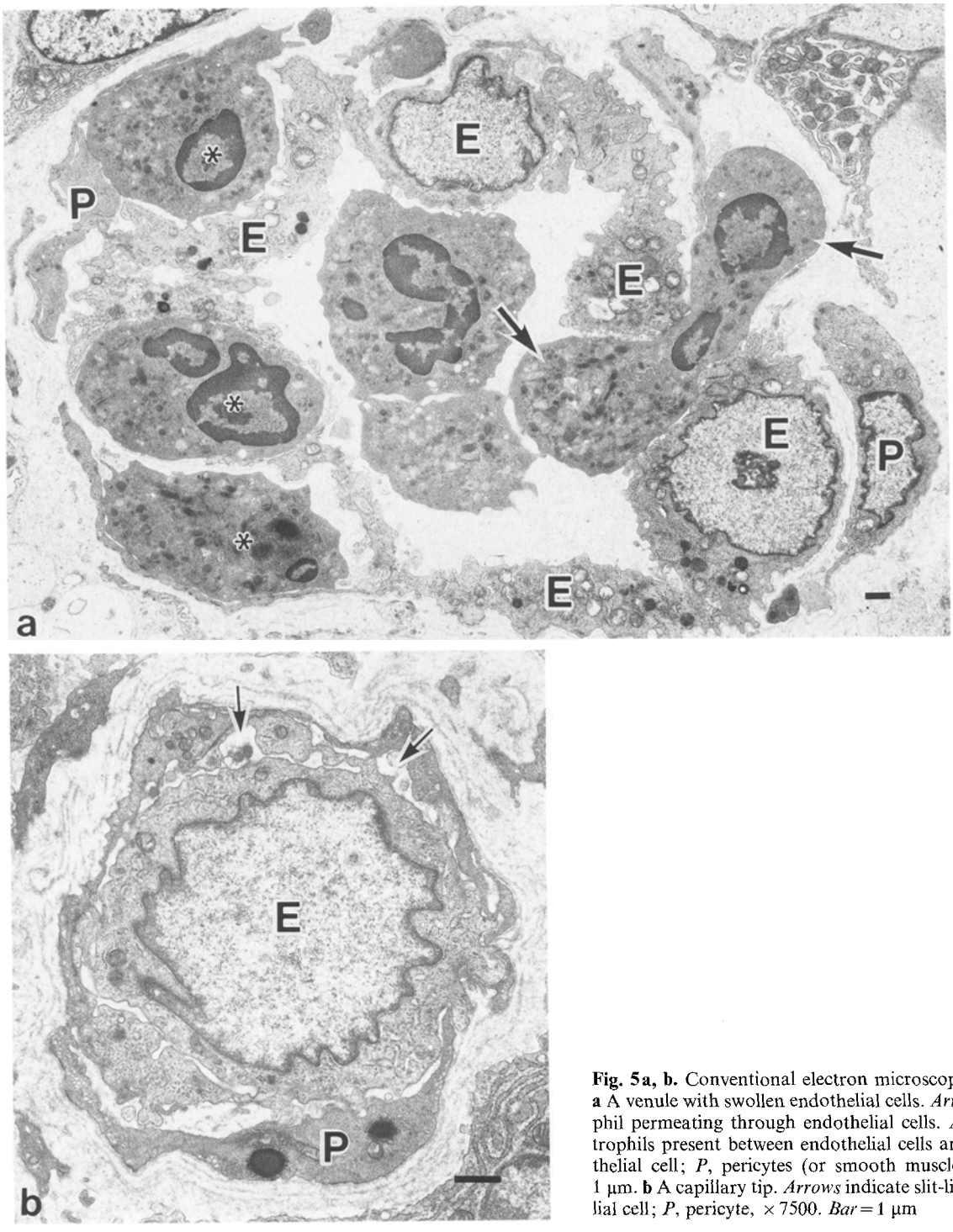


Fig. 5a, b. Conventional electron microscopy in ulcerative colitis. **a** A venule with swollen endothelial cells. *Arrows* indicate a neutrophil permeating through endothelial cells. *Asterisks* indicate neutrophils present between endothelial cells and pericytes. *E*, Endothelial cell; *P*, pericytes (or smooth muscle cell). ×3400. *Bar* = 1 μm. **b** A capillary tip. *Arrows* indicate slit-like lumen. *E*, Endothelial cell; *P*, pericyte, ×7500. *Bar* = 1 μm

ELAM-1 is expressed in venules with swollen endothelial cells in IBD, contrasted by rarity of its expression in the normal mucosa. Its ultrastructural localization is consistent with its nature as a transmembrane receptor, but we have further suggested that it is also a secretory protein.

Overt expression of ELAM-1 was strictly confined to the area of active inflammation. This expression pattern is consistent with the fact that ELAM-1 is rapidly induced by *IL-1*, TNFα and INF-γ, and rapidly diminished after the cessation of stimulus (Bevilacqua et al. 1987) in a time course longer in in vivo studies than in in vitro (Groves et al. 1991). ELAM-1 is also induced in microvessels in septic shock by TNF and IL-1 (Redl et al. 1991). Furthermore, substance P-nerve fibres may contribute to its induction (Matis et al. 1990). Active inflammatory tissues are infiltrated with granulocytes which bear sialyl Le^x antigen, the ligand of ELAM-1. Similar relationships between expression of ELAM-1 and number of neutrophils have already been reported

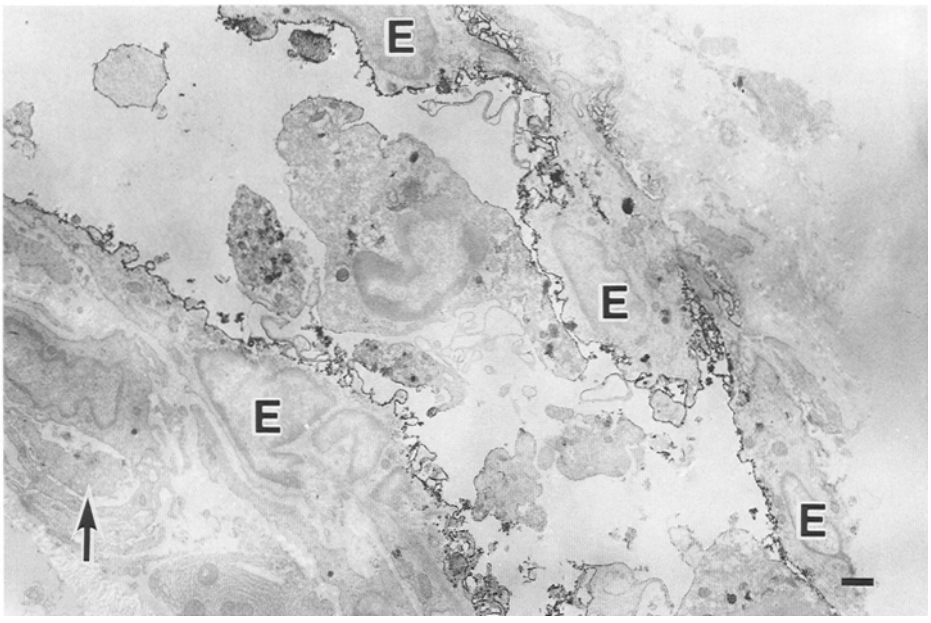
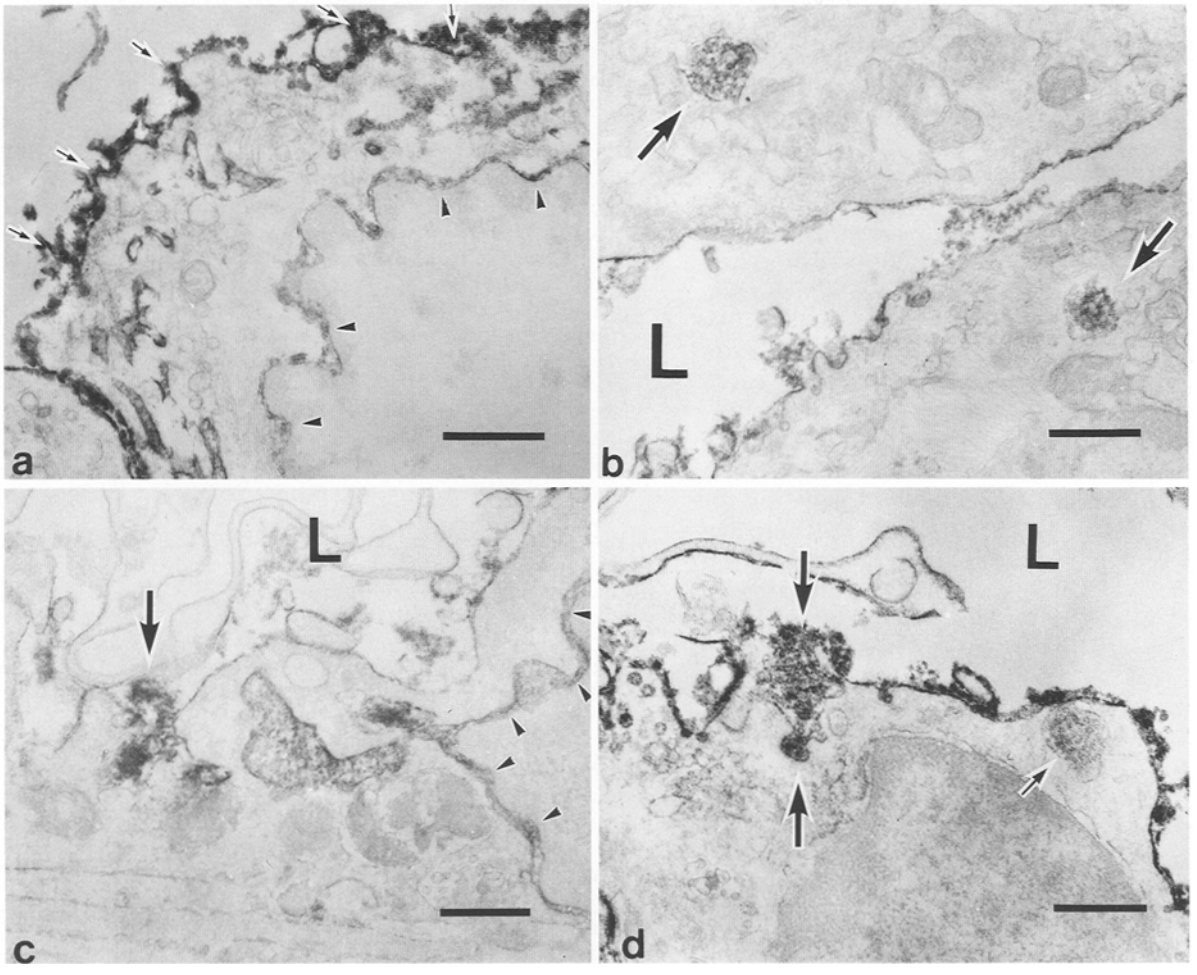


Fig. 6. Immunoelectron microscopy for ELAM-1 in Crohn's disease. Immunoreactivity was observed mainly along the luminal cell membrane of a venule. *Arrow*, a capillary without immunoreactivity for ELAM-1; *E*, endothelial cell; *P*, pericyte. ×4300. *Bar* = 1 μm



Figs. 7a–d. Higher magnification of immunoelectron microscopy for ELAM-1 in endothelial cells of venules. **a** Ulcerative colitis. Immunoreactivity is present along the outer surface of luminal cell membrane (*arrows*) as well as on the cell membrane, in well-developed rough endoplasmic reticulum and in perinuclear space (*arrowheads*). ×30000. *Bar* = 0.5 μm. **b** Ulcerative colitis. *Arrows* indicate immunoreactive round granules. *L*, Lumen. ×27000. *Bar* = 0.5 μm. **c** Ulcerative colitis. Exocytotic process of ELAM-1 is observed (*arrow*). Immunoreactivity is also present in rough endoplasmic reticulum and perinuclear space (*arrowheads*). *L*, Lumen. ×27000. *Bar* = 0.5 μm. **d** Crohn's disease. *Large arrows* indicate exocytosis of ELAM-1. *Small arrow* indicates a round, immunoreactive granule. ×27000. *Bar* = 0.5 μm

in the skin (Groves et al. 1991). In active inflammation in IBD, small capillaries and capillary buds (immature capillaries with slit-like lumen) were abundant, as a result of angiogenesis. However, they may not be involved in the permeation of neutrophils, since most of them lack ELAM-1 expression. ELAM-1 expression was mainly in venules and partly in capillaries. Since venules are sparse in normal mucosa, its occurrence was mainly related to microvascular changes associated with angiogenesis in IBD. We have already found that nearly all capillaries and venules in IBD are positive for intercellular adhesion molecule-1 (ICAM-1, CD54) (Nakamura et al. 1991), suggesting that permeation of lymphocytes positive for lymphocyte function-associated antigen-1 (LFA-1) may occur in a wider range of microvessels. A recent study revealed that inducible cell adhesion molecule 110 (INCAM-110), a receptor for lymphocytes and monocytes, was also expressed in venules in acute and chronic inflammation (Rice et al. 1991). These data suggest that venules in IBD share common features with HEV in leucocyte permeation. In other words, the venules in IBD, increased by remodelling or angiogenetic stimuli, actively permit the permeation of inflammatory cells by induction of adhesion molecules, thereby sustaining the inflammatory response.

By immunoelectron microscopy we have elucidated the expression of ELAM-1 antigen along the luminal plasma membrane of endothelial cells. This is consistent with its character of a transmembrane receptor. Its localization in rough endoplasmic reticulum and perinuclear cisternae shows a probable synthetic process in endothelial cells, suggesting that ELAM-1 is actively produced in activated endothelial cells in severely inflamed sites. Exocytosis of ELAM-1 was an unexpected finding. We have already observed similar exocytosis concerning von Willebrand factor in the stroma of gastrointestinal carcinoma and in the oral mucosa (Ohtani and Sasano 1987; Ohtani and Nagura 1990; Takeuchi et al. 1988). The present study suggests to us that ELAM-1 also has a secretory function. We have no definite explanation for this phenomenon, but the following facts concerning GMP140 (CD62) may be suggestive. GMP140, which is part of the LECAM family with ELAM-1 and Mel-14, is expressed on the cell surface of endothelial cells and platelets via the fusion of granules which contain GMP140 (Isenberg et al. 1986). A deletion form of GMP140 is known, which lacks the transmembrane domain (soluble form of GMP140) (Johnston et al. 1989), and these findings lead us to speculate that GMP140 may also function as a secretory substance. Since our data are limited to morphological evidence, we need functional information from further studies.

We have shown that HEV in normal Peyer's patches and lymph nodes also express ELAM-1. Ruco et al. (1990) has already reported that HEV in lymph nodes were positive for ELAM-1 in reactive lymphadenitis and Hodgkin's disease. They ascribed this change to issue expression of IL-1 and TNF-α. Koch et al. (1991) reported expression of ELAM-1 in venules and capillaries in rheumatoid arthritis, a chronic inflammatory lesion of the synovial membrane. The lesions mentioned above are not usually infiltrated with neutrophils. Therefore, it is difficult to explain the ELAM-1 expression in capillaries and venules in these lesions from the viewpoint of adhesion to neutrophils. Recently, memory T cells have been shown to bind to ELAM-1 (Picker et al. 1991; Shimizu et al. 1991). Therefore, it is conceivable that ELAM-1-positive HEV in lymphoid tissue and venules in chronic inflammation may be involved in the adhesion to certain subsets of lymphocytes. Further studies are in progress.

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