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Expression of E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in non-small-cell lung carcinoma

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Abstract Vascular cell adhesion molecules (VCAM) play an important part in the regulation of inflammation and are considered to be important in the process of malignant tumour growth. The present study describes the immunohistochemical staining patterns of E-selectin, intercellular adhesion molecule (ICAM)-1 and VCAM-1 on endothelial cells of the vessels in tumour stroma and other cell types in non-small-cell lung carcinoma (NSCLC; n=43) in association with inflammatory cells. Expression of E-selectin was dominant on endothelial cells in the stromal areas of the tumour, especially at the borders, and was confined to endothelial cells. Moderate to strong staining for ICAM-1 was demonstrated on endothelial cells irrespective of size or localization of the vessels. Compared with ICAM-1, fewer vessels were positive for VCAM-1, and stained with lesser intensity. ICAM-1 expression was demonstrated on NSCLC cells, the basal cells of bronchial epithelium, type II pneumocytes, lymphocytes and fibroblasts. VCAM-1 was clearly expressed on NSCLC cells in 4 of the 43 cases and on lymphocytes and fibroblasts. The staining patterns observed on endothelial cells support the idea of an active status of NSCLC vessels. This phenotypic pattern looks similar to the vascular component of inflammation. The presence of ICAM-1 and VCAM-1 on NSCLC cells suggests a functional role in the process of chemotaxis for tumour cells.

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Introduction

Vascular cell adhesion molecules play an important role in the regulation of inflammatory processes. Adhesive interactions between cells and extracellular matrix are necessary for the adhesion and extravasation of leucocytes. The upregulation of adhesion molecules on endothelial cells is a characteristic feature of inflammatory status [4, 36]. Adhesion and transmigration of leucocytes is a multiple step process consisting of rolling of leucocytes, triggering by chemokines and strong adhesion of the leucocytes to the endothelial cells, followed by transendothelial migration of the leucocytes (for reviews see, e.g. [21, 24, 34]). One of the family of selectins, E-selectin, is involved in the process of rolling of the mononuclear cells. It is expressed on vascular endothelial cells, but only when the latter are activated by inflammatory processes in vivo or by bacterial endotoxin and/or cytokines in vitro [3, 20, 31]. Intercellular adhesion molecule (ICAM)-1 (CD54) and vascular cell adhesion molecule (VCAM)-1 belong to the immunoglobulin supergene family and are both involved in the process of strong adhesion of mononuclear cells to the endothelial cells [4, 21, 24, 34, 36]. ICAM-1 is constitutively expressed on endothelial cells and is up-regulated in response to endotoxin, TNF-α, IL-1 or IFN-γ [30, 31, 36]. It is also present on the surface of mononuclear phagocytes and other non-immune cells such as fibroblasts and epithelial cells [30, 31, 36]. VCAM-1 is not constitutively expressed on endothelial cells, but can be induced in the presence of IL-1, TNF-α or IL-4 [23, 28, 39]. VCAM-1 is expressed on several non-vascular cell types, including populations of dendritic cells found in lymph node and skin, bone marrow, stromal cells and synovial cells in inflamed joints [36].

Adhesive interactions between tumour cells and adjacent cells or extracelluar matrix are important for the process of malignant tumour growth. The activation of endothelial cells by inflammatory cytokines causes an increase in adhesion of tumour cells in vitro [2, 7, 27]. Alterations in the expression of VCAM have been reported in a variety of malignant tissues in vivo [9, 13, 15, 16, 32]. E-selectin is absent from normal skin, but is strongly expressed by dermal endothelium in both squamous cell carcinomas and basal cell carcinomas [9, 13]. Increased ICAM-1 expression is correlated with melanoma progression and an increased risk of metastasis [15], while VCAM-1 expression has been demonstrated in nasopharyngeal carcinomas with a lymphoepitheliomatous aspect and in melanomas [16, 32].

In an earlier study we investigated the systemic inflammatory response in non-small-cell lung carcinoma (NSCLC) patients [38]. Enhanced levels of soluble (s) tumour necrosis factor (TNF) receptors and acute phase proteins in plasma were demonstrated in NSCLC patients. In addition, we found elevated levels of sICAM-1 in peripheral blood of NSCLC patients, while sE-selectin levels were not different from those in healthy controls.

The aim of the present study was to investigate the expression of adhesion molecules, as markers of immune activation, on endothelial cells in tumour stroma and on NSCLC cells and other cell types in the environment of the tumour. To this end, the expression of E-selectin, ICAM-1 and VCAM-1 in association with inflammatory cells was investigated.

Materials and methods

Tissues were collected from consecutive resection specimens taken from 43 patients undergoing thoracotomy for non-small-cell lung carcinoma in the University Hospital of Maastricht. The TNM classification was used for staging [22]. Tissue samples were selected from each specimen and frozen in isopentane. Samples were immersed in liquid nitrogen and stored at –70°C.

Paraffin sections were dewaxed with xylene and rehydrated through graded concentrations of ethanol. To prevent endogenous peroxidase activity, sections were immersed in 0.6% hydrogen peroxide in methanol for 5 min. Cryostat sections were cut at 5 μm and taken up on poly-L-lysine-coated slides. Slides were air-dried under a fan for 15 min and fixed in acetone at –20°C. Sections were not immersed in hydrogen peroxide. Consecutive sections were immunostained with monoclonal mouse antibodies directed against the following adhesion molecules: E-selectin (ENA-1), 1:25 dilution [20], ICAM-1 (RR-1, a kind gift from Dr. R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, Conn.), 1:800 dilution [30] and VCAM-1 (1G11, kindly provided by Dr. D.O. Haskard, Rheumatology Unit, Hammersmith Hospital, London, UK), 1:25 dilution [40]. Polyclonal rabbit-anti-factor VIII (A082, Dako, Glostrup, Denmark) at 1:2000 dilution was used to detect vessels in the sections. The antibodies were diluted in Tris-buffered saline (TBS) with 1% BSA and 0.1% Tween. Sections were incubated with the primary antibody for 45 min. After rinsing with TBS, rabbit-anti-mouse biotin (E413, Dako) incubation was performed for 30 min. Subsequently slides were incubated with streptavidin-biotin-horseradish peroxidase complex (Dako) for 30 min. Peroxidase activity was visualized in a substrate solution containing diaminobenzidine (DAB). Immidazole (0.1 M in Tris/HCl) was added to enhance staining intensity. After optimal colour development, sections were counterstained with Mayer's haematoxylin. For negative controls, primary antibodies were omitted and replaced by normal serum of mice as control. Negative controls showed only distinct isolated staining in some macrophages (see Fig. 1D).

The following antibodies were used for cell identification: DAKO-MAC (Dako), 1:100 dilution, for CD68; Pan-B (Dako), 1:1000 dilution, for CD20; OKT-3 (Dako), 1:200 dilution, for CD3; and Dako-HLA-DR (Dako), 1:50 dilution. Subsequent paraffin slides were cut and investigated for the specific markers of macrophages/monocytes and T- and B-lymphocytes.

The distribution and intensity of immunohistochemical staining with the various antibodies were assessed semi-quantitatively in two ways: (a) percentage of each cell type showing immunoreactivity (absent, <25%, 25–75%, >75%) and (b) amount of staining product, recorded as absent, slight, moderate or strong immunoreactivity. The number of vessels staining positive for the adhesion molecules was subjectively compared with the number of vessels detected with factor VIII in consecutive sections.

The relation between immunohistochemical staining patterns of the different cell types and tumour stage or histological differentiation was tested using the Friedman test for non-parametric data. The statistical analyses were performed using the SPSS/PC+ 4.0 package [37].

Results

Forty-three NSCLC specimens were examined for immunoreactivity with E-selectin, ICAM-1 and VCAM-1. The clinical characteristics and the distribution of the histological classification are summarized in Table 1.

To differentiate the inflammatory infiltrate around the tumour cells, specific markers for T- and B-lymphocytes and macrophages/monocytes were used on paraffin sections. In general, lymphoid aggregates were present to a variable extent at the edge of the NSCLC fields or in between the outer fields of carcinoma cells. The aggregates consisted mainly of B-lymphocytes with scattered T-lymphocytes. Lymphocytes were found in tumour stroma of NSCLC. These lymphocytes usually consisted of T-lymphocytes, except for areas with a very dense lymphocytic infiltrate where occasionally B-lymphocytes were demonstrated. In some sections, T-lymphocytes were focally present between carcinoma cells.

CD68 reactive monocytes were present in great numbers in tumour stroma. CD68 was also detected on small cells in between carcinoma cells and on macrophages in alveolar compartments. HLA-DR positivity was found in tumour stroma and also between carcinoma cells. Pneumocytes (type II) were frequently positive for HLA-DR.

Table 1 Clinical and histological characteristics (*n* number of patients)

Clinical characteristics	Sex; male/female	34/9
	Age (years) (mean ± SD)	65±10
Histological type	Differentiation grade	*N*
Squamous cell carcinoma	Well	3
	Moderately	9
	Poorly	5
Adenocarcinoma	Well	6
	Moderately	2
	Poorly	10
Adenosquamous carcinoma	Moderately	1
Large-cell carcinoma	Poorly	7

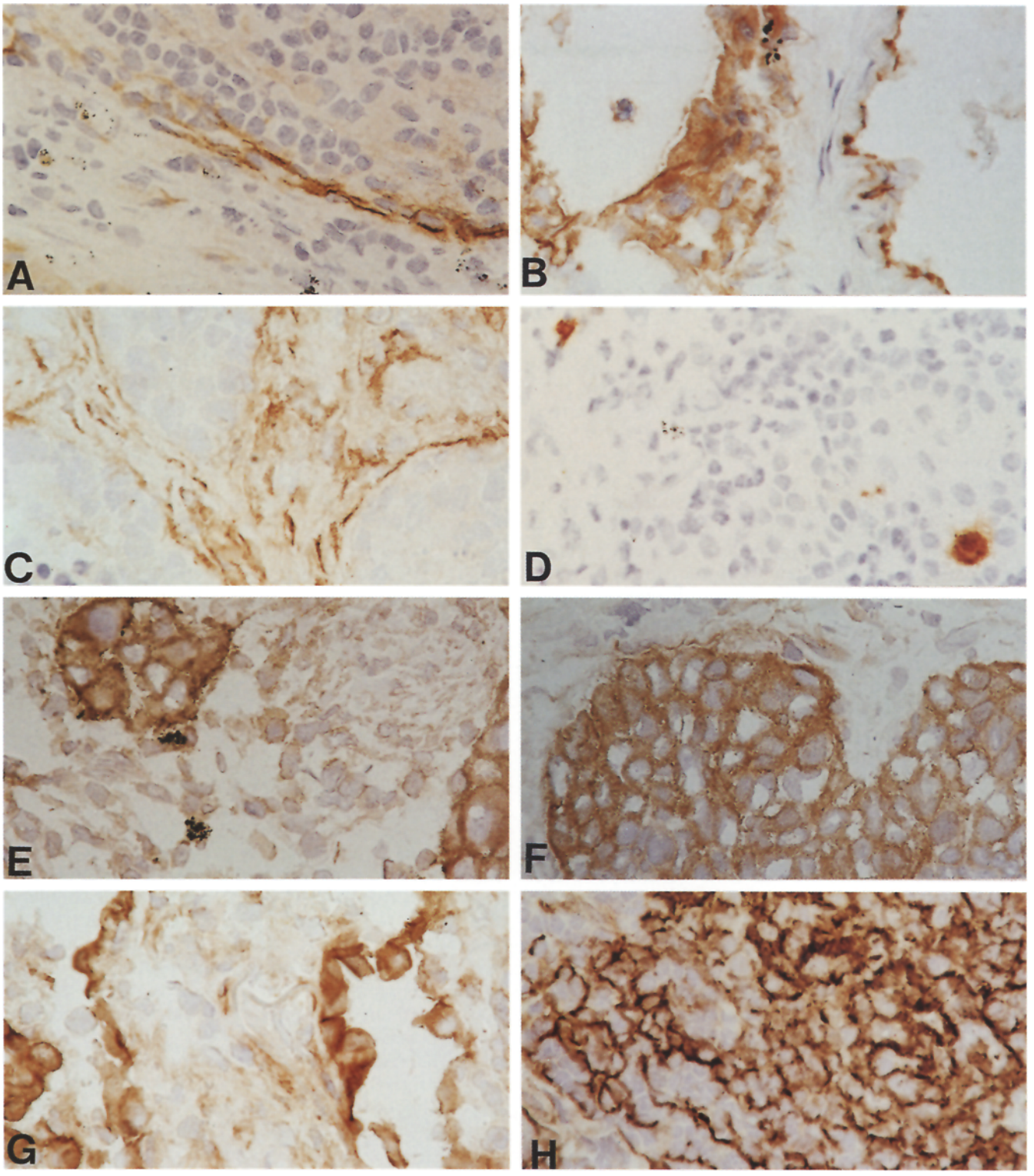

Fig. 1A–H Immunohistochemical staining is shown for different antibodies. Immunoreactivity on endothelial cells is shown for **A** E-selectin, **B** intercellular adhesion molecule (ICAM)-1 and **C** vascular cell adhesion molecule (VCAM)-1. Distinct staining of solitary macrophages is shown in the negative control (**D**). NSCLC cells are clearly immunoreactive for **E** ICAM-1 and **F** VCAM-1. **G** ICAM-1 expression is demonstrated on type II pneumocytes. **H** A typical membranous staining pattern of lymphocytes was observed for VCAM-1. ×450 throughout

Follicular aggregates were heterogeneously stained for HLA-DR. Some aggregates with germinal centres were strongly positive, while the majority of aggregates, showing no germinal centres, were negative. Occasionally, HLA-DR positivity was found on tumour cells.

Immunoreactivity for E-selectin in the different cell types is summarized in Table 2. E-selectin expression was confined to endothelial cells (see Fig. 1A). No other

Table 2 Description of the immunohistochemical results (– absent, + slight, ++ moderate, +++ strong, *N* percentage of positive cases, *NSCLC* non-small-cell lung carcinoma, *ICAM* intercellular adhesion molecule, *VCAM* vascular cell adhesion molecule)

	E-selectin		ICAM-1		VCAM-1	
	Intensity	*N*	Intensity	*N*	Intensity	*N*
NSCLC						
Squamous cell ca.	–		+/++	76%	+	12%
Adenocarcinoma	–		+/++	72%	+	11%
Adenosquamous ca.	–		–	–	–	–
Large cell ca.	–		+/++	57%	–	–
Bronchial epithelium	–		+/++	71%	–	–
Pneumocytes (type II)	–		+/++	53%	–	–
Monocytes and macrophages	–		–	–	–	–
Lymphocytes	–		+++	100%	+++	93%
Endothelial cells venules	++/+++	79%	++/+++	97%	+/+++	97%
Endothelial cells capillaries	++/+++	79%	++/+++	100%	+/++	100%

cell types expressed E-selectin. Between 25% and 75% of the vessels showed strong immunostaining for E-selectin in 34 of 43 sections. In all sections there were more vessels positive for factor VIII than for E-selectin. Differences in size were made arbitrarily between capillaries on the one hand and larger vessels containing at least one layer of smooth muscle cells in the wall on the other. There was no difference between the smaller and larger vessels in staining intensity for E-selectin. E-selectin expression was dominant in the stromal areas of the tumour, especially at the borders. E-selectin expression on endothelial cells was independent of tumour type or tumour stage.

ICAM-1 was demonstrated on endothelial cells, NSCLC cells, basal cells of bronchial epithelium, pneumocytes type II, lymphocytes and fibroblasts (Table 2). More than 75% of the endothelial cells of vessels stained moderately to strongly for ICAM-1 (see Fig. 1B), irrespective of the central or peripheral localization of the vessels in the tumour. Moderate to strong immunohistochemical staining for ICAM-1 was found in >75% of the tumour cells in 30 out of 43 sections (see Fig. 1E). ICAM-1 expression on NSCLC cells was independent of tumour type (P=0.44) or stage (P=0.81). When bronchial epithelium was present (n=7), ICAM-1 expression (moderate to strong) was demonstrated in the basal layer of bronchial epithelium. Type II pneumocytes were present in eight sections and showed slight to moderate staining (see Fig. 1G). Fibroblasts in tumour stroma were positive for ICAM-1 in all sections. From 25% to 75% of the lymphocytes were positive for ICAM-1 and had a typical membranous staining pattern with a strong intensity. These staining patterns were also demonstrated in the germinal centres when present. The other cells of the mononuclear infiltrate did not express immunostaining for ICAM-1.

Next to the endothelial cells, immunohistochemical staining for VCAM-1 was demonstrated on NSCLC cells, lymphocytes and fibroblasts (Table 2). Endothelial cells of the capillaries were slightly to moderately stained for VCAM-1, while larger vessels showed immunoreactivity for VCAM-1 in 25–75% of the vessels with a slight to strong intensity (see Fig. 1C). Compared with the staining of endothelium for ICAM-1, fewer vessels stained positive for VCAM-1, and the staining intensity for VCAM-1 was also less marked than for ICAM-1. No correlation between VCAM-1 expression on endothelial cells and tumour stage or histological type could be demonstrated. The majority of tumour cells did not show immunoreactivity for VCAM-1. However, distinct immunostaining for VCAM-1 was demonstrated in 4 NSCLC cases (see Fig. 1F). Bronchial epithelium and type II pneumocytes did not show any immunoreactivitiy for VCAM-1. Fibroblasts in the tumour stroma were positive for VCAM-1 in all sections. From 25% to 75% of the lymphocytes were positive for VCAM-1 and had a typical membranous staining pattern with a strong intensity (see Fig. 1H). These staining patterns were also demonstrated in the germinal centers when present. The monocytes and alveolar macrophages did not express immunostaining for VCAM-1.

Discussion

The present study describes the immunohistochemical staining patterns of E-selectin, ICAM-1 and VCAM-1 on endothelial cells of the vessels in the tumour stroma of NSCLC. In addition, the expression of ICAM-1 and VCAM-1 was demonstrated on NSCLC cells and other cell types in the vicinity of the tumour cells.

The contribution of tumour blood vessels to the process of immune cell infiltration and host immune response is essential for the process of growth and metastasis. In the absence of local capillary proliferation and delivery of oxygen and nutrients, neoplasms cannot grow. The density of vessels in the tumour is of importance for its rapidity of growth [5, 11, 12]. Tumour vessels, in contrast to normal blood vessels, have been reported to be fragile, thin walled and devoid of normal supportive nervous and muscular components essential for normal vasomotor responses [14]. In addition to being irregular in size, shape and distribution, they are lined with highly proliferative immature endothelial cells [14].

E-selectin and VCAM-1 are normally not expressed on endothelial cells in the lung, but are up-regulated during inflammatory conditions [4, 23, 28, 39]. The active involvement of blood vessels in the local inflammatory

process around tumour cells is supported by the present finding of expression of E-selectin and VCAM-1 on endothelial cells. VCAM-1 and ICAM-1 expression on the vessels do not differ with regard to the localization (central or peripheral) of the vessels in the tumour. However, several sections show more immunostaining for E-selectin on vessels at the borders than in the centre. E-selectin is more involved in the acute phase of inflammation, and VCAM-1 and ICAM-1 are more prominent in the chronic phase of the inflammatory process, as has been demonstrated in other studies [4, 21, 24, 34, 36]. We hypothesize that the vascular outgrowth in the periphery of NSCLC is related to the vascular process of acute inflammation, and similarly, the activation status of endothelial cells in the centre of the tumour may resemble the status present in chronic inflammation.

In an earlier study, we investigated the immunohistochemical staining pattern of cytokines and cytokine receptors on both NSCLC cells and immune cells of the host. Both NSCLC cells and monocytes/alveolar macrophages showed immunoreactivity for the cytokines TNF-α, IL-6 and IL-8, but only a minority of the mononuclear cells in tumour stroma were in an activated status (A.J. Staal, unpublished). In general, mononuclear cells are responsible for the up-regulation of adhesion molecules on endothelial cells and for the attraction of other immune cells to the site of inflammation [4, 21, 24, 34, 36]. However, tumour cells themselves may also be responsible for the up-regulation of the adhesion molecules E-selectin and VCAM-1 on endothelial cells and attraction of immune cells, by secretion of chemotactic cytokines [1, 18, 29, 44]. In addition, the tumour cells may promote angiogenesis by the production of IL-8, which is known to be an angiogenic factor and thus to favour the growth of the tumour [35].

The expression of adhesion molecules on NSCLC cells and other cell types in the environment of the NSCLC cells was also investigated. E-selectin expression was confined to endothelium, while ICAM-1 and VCAM-1 were also expressed on NSCLC cells and other cell types. ICAM-1 positivity was demonstrated in more than 75% of the NSCLC cells, irrespective of tumour stage or histological type. Other studies, however, demonstrated differences in ICAM-1 positivity according to tumour histology. Ruco et al. [32] and Vitolo et al. [42] found more ICAM-1 expression on squamous cell carcinoma cells than on adenocarcinoma cells, while Passlick et al. [25] found increased ICAM-1 expression in adenocarcinoma cells compared with squamous cell carcinoma cells of the lung. One explanation for these variations in outcome might be the use of different antibodies. Although controversy exists concerning the relationship between ICAM-1 staining and histological type, what is certain is that ICAM-1 has been demonstrated in several cases of NSCLC of all histological types. Moreover, ICAM-1 expression has also been found on other types of tumour cells, such as lymphomas, melanomas, and pancreas and colon carcinomas [8, 9, 33, 39, 41], suggesting a similar pattern of activation in the different tumours. The inability to detect immunoreactivity for ICAM-1 in some cases of NSCLC could be explained by reduced ICAM-1 expression on these NSCLC cells. Further studies are needed to explore the significance of reduction or absence of ICAM-1 expression for tumour behaviour in NSCLC patients.

VCAM-1 was expressed distinctly by NSCLC cells in 4 different cases. So far, Ruco et al. [32] have shown VCAM-1 expression in nasopharyngeal carcinomas, while other authors have not been able to demonstrate VCAM-1 expression in epithelial tumours. Recently Cunningham et al. demonstrated VCAM-1 expression on normal bronchial epithelium and isolated type II pneumocytes [6]. The role of VCAM-1 and ICAM-1 expression on NSCLC cells might be explained by the active involvement of NSCLC cells in inflammatory processes, including the function of chemotaxis of mononuclear cells of the host.

The third group of cells investigated was that of the immune cells. The inflammatory infiltrate around the NSCLC consists predominantly of monocytes and lymphocytes. The latter are mainly T lymphocytes, while B-cells are a minor proportion as demonstrated in the present study and by other groups [25, 43]. A typical membranous staining pattern for ICAM-1 and VCAM-1 expression was found on lymphocytes in tumour stroma. The staining patterns of ICAM-1 and VCAM-1 on lymphocytes and in germinal centres were similar, with staining patterns seen at sites of chronic inflammation in tonsils and reactive lymph nodes [17, 26]. These data together support the idea of an activated status of lymphocytes and germinal centres in the environment of the NSCLC cells. Tumour-infiltrating monocytes did not express any immunohistochemical staining for the adhesion molecules and reflect a reduced state of activation, as supported by other studies [30, 31, 36].

Fibroblasts were immunostained for ICAM-1 and VCAM-1. Human fibroblasts do not constitutively express ICAM-1 in the lung, but ICAM-1 expression is induced by stimulation with IL-1, TNF-α or IFN-γ [31]. The observed VCAM-1 expression on fibroblasts was also shown by Vitolo et al. in NSCLC [42]. The increased expression of ICAM-1 and VCAM-1 on fibroblasts surrounding the tumour nests might promote the adhesion of different inflammatory cells to fibroblasts and could thus direct the immune cells to the tumour cells [10, 19].

In conclusion, the presence of E-selectin, ICAM-1 and VCAM-1 on endothelial cells in tumour stroma supports the idea of an active status of NSCLC vessels. This phenotypic pattern looks similar to the vascular component of inflammation. The presence of ICAM-1 and VCAM-1 on NSCLC cells suggests a functional role in the process of chemotaxis for tumour cells.

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