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Intercellular Adhesion Molecule-3 on Endothelial Cells

Expression in Tumors but Not in Inflammatory Responses

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Intercellular adhesion molecule-3 (ICAM-3) was identified as the third counter-receptor for lymphocyte function-associated antigen-1. ICAM-3 is absent on endothelial cells in normal tissues but found on endothelial cells in lymphomas. Here, we examined ICAM-3 expression on vascular endothelial cells in lymphomas, nonlymphoid malignancies, benign tumors, and inflammatory diseases. We compared the expression of ICAM-3 on endothelial cells with the severity of inflammatory infiltrates and with the presence of E-selectin and VCAM-1. We found that ICAM-3 expression on endothelial cells was high on both benign and malignant tumors whereas it was low in inflammatory diseases. In contrast to E-selectin, ICAM-3 expression on endothelial cells was not correlated to the severity of inflammatory infiltrates. In hemangiomas, we showed by Northern blot analysis and immunocytochemistry that ICAM-3 expression was induced and that it was localized in immature areas that sustain the early stages of angiogenesis. Therefore, expression of ICAM-3 on blood vessels does not seem to play a role in the recruitment of leukocytes during inflammation but rather is correlated with angiogenesis and tumor development. (Am J Pathol 1996, 148:465–472)

Cell adhesion molecules (CAMs) such as intercellular CAM (ICAM)-1, ICAM-2, vascular CAM (VCAM)-1, and E-selectin play crucial roles in leukocyte migration to sites of inflammation, extravasation, lymphocyte activation, and proliferation.[1–9] These molecules are expressed on endothelial cells, bind to counter-receptors present on leukocytes, and mediate leukocyte adhesion to the endothelium. The expression of several of these adhesion molecules is modulated by inflammatory cytokines. For example, interleukin (IL)-1α and tumor necrosis factor (TNF)-α induce up-regulation of E-selectin, VCAM-1, and ICAM-1 *in vitro*, whereas interferon (IFN)-γ induces up-regulation of ICAM-1, and IL-4 induces up-regulation of VCAM-1.[2]

Recently, ICAM-3, a third ICAM family member, was identified.[10,11] Although it shares with the other CAMs the capacity to bind the integrin lymphocyte function-associated antigen (LFA)-1, it is distinguished from them by its constitutive expression at high levels on resting lymphocytes and on antigen-presenting cells (Langerhans cells). The high levels of expression of ICAM-3 on resting leukocytes, its expression on antigen-presenting cells, and its capacity to activate lymphocytes suggest a crucial role of ICAM-3 in the genesis of the immune response.[10–15]

In contrast to the other ICAMs, ICAM-3 is not expressed *in vitro* on primary cultures of endothelial cells even after cytokine stimulation.[10] However, *in vivo*, ICAM-3 is expressed on small vessels in lymphoid malignancies.[13,14] To understand the mechanism that triggers expression of ICAM-3 on blood vessels *in vivo*, we analyzed ICAM-3 expression on malignant lymphoid and nonlymphoid tumors, on benign tumors, on tissues in inflammatory diseases, and on normal tissues. We compared the expression of ICAM-3 on endothelial cells with the severity of inflammatory infiltrates and with the presence of E-

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Table 1. *Expression of Adhesion Molecules in Nonlymphoid Malignancies*

Diagnosis	Endothelial cells			Cases with infiltrating cells
	ICAM-3	E-selectin	VCAM-1	
Inflammatory disease				
Granulomatosis	3/7	5/5	3/4	7/7
Graft-versus-host disease (skin)	0/4	ND	ND	4/4
Colitis	0/19	19/19	0/1	19/19
Heart transplant (acute rejection)	0/2	2/2	1/2	2/2
Vasculitis	0/2	ND	ND	2/2
Total	**3/34** (1 %)	**26/26** (100%)	**4/7** (57 %)	
Benign tumors				
Angioma	25/30	27/27	11/12	8/30
Oligodendroglioma	3/5	1/2	0/2	4/5
Low grade astrocytoma	2/4	1/3	1/1	2/4
Ependymoma	0/1	0/1	ND	0/1
Total	**30/40** (75%)	**29/33** (88 %)	**12/15** (80%)	
Malignant tumors				
Neuroblastoma	7/7	4/5	1/3	6/7
Medulloblastoma	3/3	1/2	2/2	1/3
Tubulopapillary renal cell carcinoma	1/4	0/2	0/2	3/4
High grade astrocytoma	2/2	0/2	0/1	0/2
Total	**13/16** (81%)	**5/11** (45 %)	**3/8** (37 %)	

Results are shown as number of positive cases/cases studied.
ND, not determined.

selectin and VCAM-1. In addition, we studied the expression of ICAM-3 on tumor cells in 80 Hodgkin's and non-Hodgkin's lymphomas of various types.

We show here that ICAM-3 is absent on endothelial cells in normal tissues but is frequently induced on endothelial cells in benign or neoplastic proliferations. We also show that ICAM-3 is poorly expressed on vessels in inflammatory diseases and that there is no correlation between the intensity of inflammatory infiltrates and the expression of ICAM-3 on endothelial cells ($P > 0.05$). In contrast, E-selectin expression on endothelial cells is statistically related to the inflammatory reaction ($P = 0.001$). Therefore, it is unlikely that ICAM-3 expression on endothelial cells induces the recruitment of leukocytes during inflammation, but rather, we suggest that ICAM-3 may be associated with angiogenesis during tumor progression.

Materials and Methods

Tissues, Reagents, and Staining Procedure

Samples of human tissues were collected at surgery and divided to be either snap-frozen in isopentane/liquid nitrogen and stored at −80°C or fixed in formalin for standard histology. Lymphomas were classified according to the revised classification of the American-European classification from the International Lymphoma Study Group.[16] We also examined at least three different tissue blocks of normal lymph node, tonsil, thymus, spleen, heart, lung, liver, gut, muscle, skin, and kidney. Each tissue section was checked for the presence of the following three adhesion molecules: ICAM-3 (ICR1.1, ICR3.1, ICOS Corp.), VCAM-1 (E 1/7; Pharmigen, France) and E-selectin (H 4/18; Pharmigen). Monoclonal primary antibodies were labeled with a three-stage indirect immunoperoxidase technique.[17]

Scoring and Statistical Analyses

We performed a semiquantitative evaluation of ICAM-3, E-selectin, and VCAM-1 on endothelial cells and of interstitial leukocyte infiltrates on each tissue block at magnification ×25 on the whole specimen. Adhesion molecule expression was scored 0 when endothelial staining was absent, 1 when less than 50% of the endothelial sections were positive, and 2 when more than 50% of the endothelial sections were stained. The intensity of inflammatory infiltrates was scored 0 when few or no leukocytes were scattered on the parenchyma, 1 when infiltrate was focal, and 2 when infiltrating cells were diffusely distributed. Statistical analysis was performed using this grading system. In Tables 1 and 3, positive cases correspond to cases scored 1 and 2, and negative cases correspond to cases scored 0. The relationship between the expression of the different mole-

cule and between the intensity of infiltrating cells was made with the χ^2 test.

Isolation of RNA and Northern Blot Analysis

We extracted polyA$^+$ mRNA from frozen human skin angiomas, inflammatory small intestine, heart, and tonsil and from the human epithelial and monocytic cell lines A549 and U937. Total mRNA was extracted using RNA STAT60 mRNA isolation reagents (Tel-test "B", Friendswood, TX); polyA$^+$ mRNA was then purified by chromatography on oligo-dT cellulose columns. PolyA$^+$ mRNA samples (5 mg each) were fractionated on a 1% agarose gel containing 0.66 mol/L formaldehyde and then transferred to a nylon membrane. Anti-sense ICAM-3 RNA probes were labeled by *in vitro* transcription using ^{32}P-labeled UTP. Membranes were hybridized overnight at 65°C in 50% formamide, 5X standard saline citrate (SSC), 50 mmol/L Tris-Hcl, pH 7.6, 0.1% sodium pyrophosphate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mmol/L EDTA, 1% sodium dodecyl sulfate (SDS), and 150 mg/ml denatured salmon sperm and then washed at 65°C twice in 2X SSC, 0.1% SDS and twice in 0.1X SSC, 0.1% SDS for 15 minutes each. The same membranes were hybridized with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

Results

Expression of ICAM-3 on Endothelial Cells in Lymphomas, Tumors, and Inflammation

We studied the expression of ICAM-3 on blood vessels in 40 benign tumors (30 angiomas and 10 other cases), 16 malignant nonlymphoid tumors, 80 lymphomas, and 34 cases of various types of inflammatory diseases. We first examined the expression of ICAM-3 on the endothelial cells in malignant, benign, and nonlymphoid tumors. ICAM-3 on endothelial cells was detected in 81% (13 of 16 cases) of malignant tumors (Figure 1A) and in 75% (30 of 40 cases) of benign tumors (Figure 1D and Table 1). In addition, the level of ICAM-3 was high in malignant tumors (in 10 of 16 cases, more than 50% of the vascular sections were stained) whereas it was moderate or high in angiomas and other benign tumors (Table 2). In hemangiomas, ICAM-3 expression was inversely correlated with the vessel maturity. That is, ICAM-3 was strongly expressed within the immature area (90% of cases) and expressed weakly (40% of cases) or not at all (30% of cases) within the well canalized portion of the tumor (Figure 1D).

On the other hand, ICAM-3 expression on endothelial cells in lymphomas was lower. ICAM-3 was expressed on blood vessels in 76% of Hodgkin's disease, in 50% of non-Hodgkin's lymphomas, and in most cases in less than 50% of the vascular sections (Table 3). There was no correlation between the subtypes of lymphomas, their grade of malignancy, and the expression of ICAM-3 on blood vessels.

Surprisingly, however, ICAM-3 was poorly expressed in inflammatory diseases (3 of 34 cases; Figure 1B) with a low level of expression (Table 1). ICAM-3 expression on endothelial cells was not statistically correlated to the intensity of infiltrating cells either in tumors or in inflammatory diseases ($P > 0.05$). Moreover, the endothelial cells that expressed ICAM-3 were not always in close proximity to infiltrating cells (Table 1). Therefore, although the ICAM-3 expression on vessels seemed to increase in relation to the degree of proliferation and malignancy of the nonlymphoid tumors, it was not correlated with the degree or the location of the inflammatory infiltrates. Both large and small vessels, except high endothelial venules (Figure 1C), were positive for ICAM-3 in all stained sections. In all cases, lymphatic vessels did not express ICAM-3 (data not shown).

Comparison between Expression of ICAM-3, E-Selectin, and VCAM-1 on Endothelial Cells in Tumors and Inflammation

We next compared the expression of E-selectin (Figure 1E) and VCAM-1 (Figure 1F) with the expression of ICAM-3 on endothelial cells. We also correlated the expression of these three molecules on endothelial cells with the intensity of the inflammatory infiltrates on serial sections.

We found ICAM-3, E-selectin, and VCAM-1 expression on endothelial cells only in pathological lesions and not in normal tissues. E-selectin was expressed in 100% of inflammatory diseases, 45% of malignant tumors, 88% of benign tumors (Table 1), 96% of non-Hodgkin's lymphomas, and 100% of Hodgkin's lymphomas (Table 3). VCAM-1 was expressed in 57% of inflammatory diseases, 37% of malignant tumors, 80% of benign tumors (Table 1), 58% of non-Hodgkin's lymphomas, and 90% of Hodgkin's lymphomas. E-selectin expression on endothelial cells was both co-localized and correlated with the intensity of the inflammatory infiltrates ($P = 0.0001$). When we compared the expression of

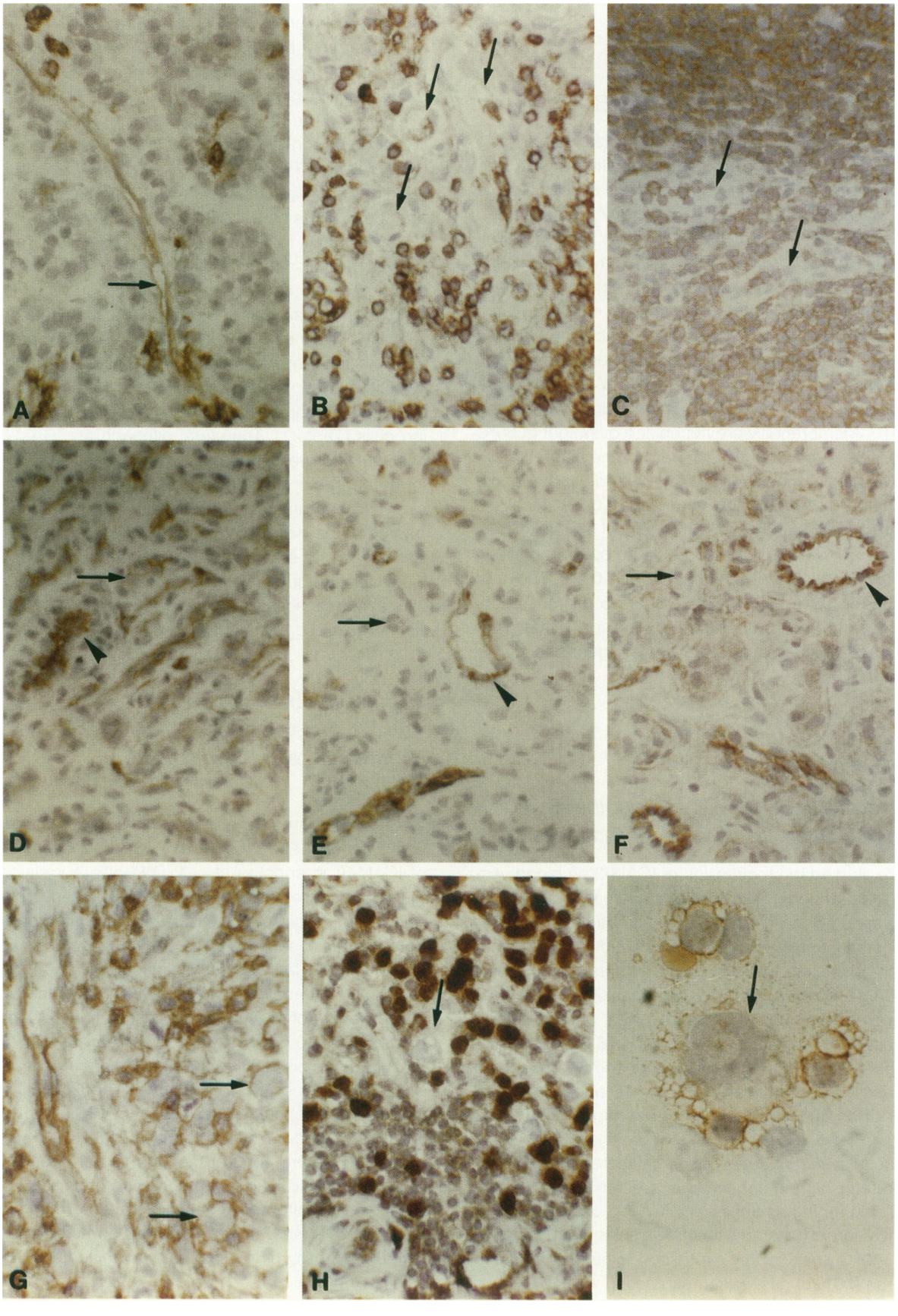
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Table 2. *Semiquantitative Grading of ICAM-3 Expression on Endothelial Cells in Pathological Tissues*

Diagnosis	*n*	ICAM-3* 0	1	2
Non-Hodgkin's lymphomas				
Follicular center cell	8	4	4	0
Lymphoplasmocytoid	2	2	0	0
Mantle cell	5	4	1	0
Diffuse large B cell	17	7	6	4
Burkitt's	6	0	5	1
Peripheral T cell	7	5	2	0
Anaplastic large cell null cell type	13	7	4	2
T cell type	5	2	2	1
Total	63	31	24	8
Hodgkin's disease				
Mixed cellularity	3	1	0	2
Nodular sclerosis	14	3	9	2
Total	17	4	9	4
Malignant tumors	16	3	3	10
Benign tumors	40	10	13	17
Inflammatory diseases	34	31	2	1

*0, absent; 1, less than 50% of endothelial sections; 2, more than 50% of endothelial sections.

ICAM-3 with the expression of VCAM-1 and E-selectin, the expression of these three molecules varied by the number and by the type of vessels stained. In some cases, the same vessels were stained by ICAM-3, E-selectin, and VCAM-1, and the difference was essentially represented by the number of labeled vessels. In other cases, the same vessels expressed E-selectin and VCAM-1, but ICAM-3 stained distinct vessels.

In hemangiomas, the expression of E-selectin and VCAM-1 was limited to a few scattered endothelial cells and was not correlated with vessel maturity (Figure 1, E and F). The expression of ICAM-3 on endothelial cells did not correlate with either the number of infiltrating leukocytes ($P > 0.05$) or with the expression of E-selectin on vessels ($P > 0.05$; Table 1). Therefore, in contrast to ICAM-3, the expression of E-selectin and VCAM-1 on endothelial cells was higher in inflammatory diseases than in tumors.

Expression of ICAM-3 on Tumor Cells in Hodgkin's and Non-Hodgkin's Lymphomas

We also examined expression of ICAM-3 in the tumors themselves. ICAM-3 was expressed on tumor cells in 61 of 63 non-Hodgkin's lymphomas (Figure 1G) but only 2 of 17 Hodgkin's cases ($P < 0.05$; Table 3). The 2 lymphomas that did not express ICAM-3 were 1 case of Burkitt's lymphoma and 1 case of anaplastic large T cell lymphoma. Reed-Sternberg cells in Hodgkin's lymphomas did not express ICAM-3 (Figure 1H) except in 2 cases classified as nodular sclerosing Hodgkin's diseases. The absence of expression of ICAM-3 on Reed-Sternberg cells was confirmed on cells isolated by imprints that were performed in 8 cases (Figure 1I).

Northern Blot Analysis of the Angiomas

Angiomas that showed no signs of inflammation expressed ICAM-3 on endothelial cells as determined by immunocytochemistry (Figure 1C). To determine whether ICAM-3 was synthesized by the angiomas or whether soluble ICAM-3 was synthesized elsewhere and adsorbed by endothelial cells, mRNA expression of ICAM-3 was analyzed by Northern blotting (Figure 2). Indeed, a transcript migrating at approximately 2.2 kb corresponding to ICAM-3 was detected in the angioma mRNA. The same transcript was found in polyA$^+$ mRNA purified from inflammatory intestinal lesions, from tonsil, and from U937 cell lines that were used as positive controls. This transcript was not detected in polyA$^+$ mRNA purified from heart tissue and from the lung epithelial cell line A549, which were used as negative controls. Therefore, ICAM-3 mRNA is inducible and synthesized by endothelial cells.

Expression of ICAM-3 in Normal Tissues

The general pattern of expression of ICAM-3 has been reported previously.[10–14] To facilitate comparisons with the results of the other laboratories, we undertook a series of control analyses on normal tissues to establish that the monoclonal antibodies used here to identify a pattern of ICAM-3 expression on normal cells was equivalent to that reported with other reagents. In normal lymph node, spleen, thymus, and tonsil, ICAM-3 was strongly expressed on B and T lymphocytes of the paracortical and follicular mantle areas. In contrast, activated B lymphocytes of the germinal center did not express ICAM-3.

Figure 1. A *to* C: *Immunoperoxidase staining with ICAM-3 antibodies. ICAM-3 was expressed on inflammatory cells and on endothelial cells in a small vessel* (arrow) *in tubulopapillary renal cell carcinoma* (A) *but was not expressed in small vessels* (arrows) *in a wound healing area in severe colitis* (B) *and was not expressed in high endothelial venules of follicular lymphoma* (arrows; C) *Magnification, ×250.* D *to* F: *Serial sections of the immature area of a hemangioma. Immature vessels* (arrows) *were labeled with ICAM-3 antibodies* (D) *but were not labeled with E-selectin* (E) *and VCAM-1 antibodies* (F). *Larger, mature vessels* (arrowheads) *were labeled with all three antibodies. Magnification, ×250.* G *and* H: *ICAM-3 was expressed both by tumor cells* (arrows) *and endothelial cells in anaplastic lymphoma* (G), *whereas it was not expressed on Reed-Sternberg cells* (arrow) *in Hodgkin's disease* (H). *Magnification, ×250.* I: *Imprint of Hodgkin's disease. The Reed-Sternberg cell* (arrow) *surrounded by lymphocytes did not express ICAM-3. Magnification, ×630. All tissue sections were counterstained with hematoxylin.*

Table 3. *Expression of Adhesion Molecules in Lymphoid Malignancies*

Diagnosis	Endothelial cells			Tumor cells
	ICAM-3	E-selectin	VCAM-1	ICAM-3
Non-Hodgkin's lymphomas				
Follicular center cell	4/8	5/5	0/3	8/8
Lymphoplasmocytoid	0/2	ND	ND	2/2
Mantle cell	1/5	2/2	1/1	5/5
Diffuse large B cell	10/17	11/11	2/5	17/17
Burkitt's	6/6	2/3	3/3	5/6
Peripheral T cell	2/7	4/4	1/2	7/7
Anaplastic large cell null cell type	6/13	4/4	3/4	13/13
T cell type	3/5	1/1	1/1	4/5
Total	32/63 (50%)	27/28 (96%)	11/19 (58%)	61/63 (98%)
Hodgkin's disease				
Mixed cellularity	2/3	3/3	2/3	0/3
Nodular sclerosis	11/14	11/11	8/8	2/14
Total	13/17 (76%)	14/14 (100%)	10/11 (90%)	2/17 (12%)

Results are shown as number of positive cases/cases studied. ND, not determined.

Langerhans cells in the skin strongly expressed ICAM-3, whereas follicular dendritic cells and most tissue macrophages did not. Only some alveolar but not mesenchymal macrophages of the lung expressed ICAM-3. Kupffer cells in the liver and lamina propria macrophages in the bowel did not express ICAM-3. In liver, gut, kidney, lung, heart, skin, and muscle only small mononuclear cells located in the lumen of blood vessels and interstitium were stained. In all tissues, we failed to detect ICAM-3 in normal epithelial cells, endothelial cells, mesenchymal cells, and extracellular matrix. These results are essentially equivalent to that reported in the literature for normal tissues with other ICAM-3-specific reagents.

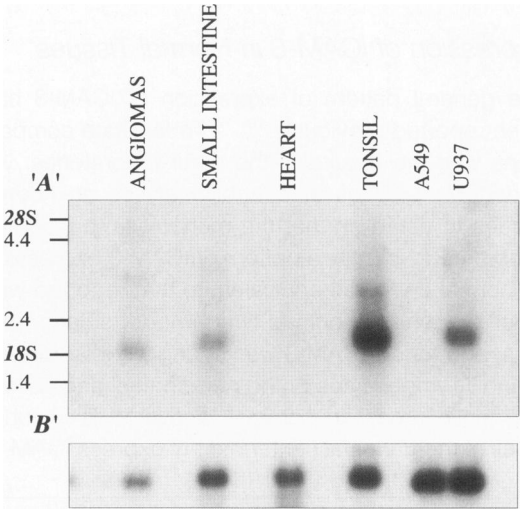

Figure 2. *ICAM-3 mRNA expression in angiomas.* A: *PolyA⁺ mRNA isolated from angiomas and from control tissues and cell lines were analyzed with an ICAM-3 probe by Northern blotting. The position of the 18S and 28S ribosomal RNA is indicated as well as the size markers (kilobases).* B: *To assess variability in sample loading, the same blot was hybridized with a glyceraldehyde-3-phosphate dehydrogenase probe.*

Discussion

We found that ICAM-3 was poorly expressed on vessels in inflammatory diseases and that its expression was not correlated with the intensity and the location of inflammatory infiltrates in contrast to the expression of E-selectin. Expression of adhesion molecules like E-selectin, VCAM-1, and ICAM-1 is triggered by specific cytokines and induces the recruitment of leukocytes to the sites of inflammation.[2,7,8] Therefore, ICAM-3 on endothelial cells, in contrast to its expression on hematopoietic cells,[18,19] does not seem likely to play a role in the recruitment of leukocytes to the inflammation sites.

ICAM-3 was expressed by endothelial cells in malignant nonlymphoid tumors, in lymphomas, and in benign tumors. It was found not only on small vessels but also on large vessels in contrast to previously published results.[14] The level of ICAM-3 expression was high in immature areas of hemangiomas, whereas it was low in mature areas. In addition, there was no ICAM-3 expression on endothelial cells in wound healing areas found in severe colitis. Normal and pathological angiogenesis are regulated by different mechanisms, and in conditions such as wound healing, newly induced capillaries mature by apposition of pericytes and smooth muscle cells. In contrast, tumor cells tend to induce new capillaries continuously without complete differentiation.[20,21] Therefore, our results suggest that ICAM-3 may play a role during pathological angiogenesis. For example, ICAM-3 could participate in angiogenesis either directly or indirectly by inducing the recruitment of leukocytes and especially of monocytes/macrophages that are necessary to sustain angiogenesis and tumor growth.[22]

Interestingly, VCAM-1 and E-selectin expression was lower in malignant tumors than in benign tumors. Tumor cells can suppress VCAM-1 expression on endothelial cells, which was suggested as a mechanism to escape host defenses.[23]

In addition to the analysis of ICAM-3 expression on endothelial cells, we analyzed expression of ICAM-3 on tumor cells. We found that ICAM-3 was not expressed by Reed-Sternberg cells in 15 of 17 cases of Hodgkin's disease, whereas in contrast, it was expressed on tumor cells in 17 of 18 cases of anaplastic large cell lymphomas ($P < 0.05$). The differential diagnosis between Hodgkin's disease and anaplastic large cell lymphoma is sometimes difficult because they look morphologically similar and both express CD30.[24] They cannot always be distinguished by the expression of CD15, epithelial membrane antigen, and Epstein-Barr virus latent membrane protein antigens.[25] Therefore, the differential expression of ICAM-3 could be helpful to distinguish between these tumors that have a different prognosis and treatment.

In conclusion, additional experiments *in vitro* and on animal models are necessary to investigate the role of ICAM-3 on endothelial cells in tumorigenesis and during the early stages of angiogenesis. ICAM-3 might be a useful molecule to target to reduce tumor growth.

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