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Endothelial Intercellular Adhesion Molecule-1 Expression Is Suppressed in Human Malignancies: The Role of Angiogenic Factors[1]

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ABSTRACT

Intercellular adhesion molecule 1 (ICAM-1) is involved in the recirculation of blood leukocytes and, presumably, in the infiltration of cytolytic effector leukocytes into tumors. The present report describes a down-regulated expression of vascular ICAM-1 on tumor-infiltrating endothelial cells (EC) in renal cell carcinoma. This finding was obtained by flow cytometric analysis of tumor EC compared to EC obtained from healthy tissue. Since growth of solid tumors is dependent on the formation of new blood vessels (angiogenesis), we hypothesized that angiogenic factors are responsible for the down-regulation of ICAM-1. This hypothesis was investigated *in vitro* using human umbilical vein- and dermis-derived EC. Using flow cytometry, we found a biphasic regulation of ICAM-1 during stimulation of cultured EC with the angiogenic agent basic fibroblast growth factor (bFGF). Although 16–24 h after activation a marked up-regulation of ICAM-1 was observed, expression was significantly decreased after 48 h. The longevity of this down-regulation was at least 7 days. Northern blot analysis revealed down-regulation of the steady-state mRNA level of the gene. ICAM-2 showed similar results of initial up- and later down-regulation. Functional relevance for the changes in ICAM-1 expression was demonstrated by a corresponding biphasic regulation of EC-leukocyte adhesion after EC activation by bFGF. The described effects are specific for bFGF since other angiogenic factors (such as vascular endothelial growth factor, transforming growth factor β, and interleukin 8) did not affect adhesion molecule expression. Subsequent experiments showed that angiogenic factors decrease the sensitivity of EC to activation with tumor necrosis factor-α in regard to adhesion molecule expression. The present results reveal a tumor-derived escape mechanism from cytolytic effector leukocytes by down-regulation of vascular adhesion molecules *in vivo* and *in vitro* and decreased responsiveness to proinflammatory cytokines.

INTRODUCTION

Treatment of cancer patients with adoptive immunotherapy (1, 2) is dependent on the selective accumulation of leukocytes in the tumor. Cellular immunity against tumor cells requires adhesion molecules on vascular endothelium that mediate arrest and extravasation of leukocytes (3–5). Expression of EAM[3] is controlled by cytokines such as TNF-α, IL-1, and IFN-γ. These cytokines facilitate leukocyte adhesion to EC and extravasation into extravascular tissues by inducing an enhanced expression of ICAM-1, VCAM-1, and E-selectin (3, 6–8). TGF-β, in contrast, reduces adhesion of resting and activated lymphocytes to EC, although without affecting expression of ICAM-1 and VCAM-1 (9, 10).

Angiogenesis, the formation of new blood vessels, is a process occurring in tumors and wound healing (11, 12). This process, regulated by angiogenic factors, expands the endothelial surface and thus the possible interaction between leukocytes and vessel wall. In tumors, however, these interactions might be detrimental to the tumor itself. Cytotoxic effector cells form a major threat to the tumor; therefore, escape mechanisms avoiding infiltration of leukocytes into the tumor may have evolved. Recent reports suggest that protective mechanisms, controlled by the tumor cells, are operative to prevent infiltration of leukocytes (13–15). Although altered expression of certain tumor endothelium-associated antigens has been described (16–19), insight into the regulation of adhesion molecule expression and endothelial functions by angiogenic factors is largely lacking. We asked, therefore, whether angiogenic factors are involved in tumor escape from host defense by interfering with the mechanism of effector cell extravasation. bFGF (20) and VEGF are the most carefully investigated angiogenic factors that play a role in tumor angiogenesis. In the present paper, we quantitatively studied EAM expression in renal cell cancer. Regulatory mechanisms controlled by angiogenic factors were studied *in vitro* using umbilical vein and dermal EC. Evidence is provided that tumors have protective mechanisms against increased exposure to leukocytes by actively down-regulating EAM expression and thereby diminishing leukocyte adhesion to vascular endothelium via angiogenic factors.

MATERIALS AND METHODS

Monoclonal Antibodies and Cytokines. Anti-adhesion molecule antibodies MEM111 anti-ICAM-1, 1G11B1 anti-VCAM-1, ENA1 anti-ELAM-1, CLB/CD31 anti-PECAM/CD31, QBEND10 CD34, EN4 EC-marker (Sanbio, Uden, the Netherlands), F10.2 anti-ICAM-1 (a gift of Dr. Bloem, Dept. of Immunology, University Hospital Utrecht, Utrecht, the Netherlands), BT1 anti-ICAM-2 (a gift of Dr. Gahmberg, Dept. of Biochemistry, University of Helsinki, Helsinki, Finland), and TP1/24 and HP2/19 anti-ICAM-3 (a gift of Dr. Sanchez-Madrid, Dept. of Immunology, Hospital de la Princesa, Madrid, Spain) were of monoclonal origin. Polyclonal antibodies against HLA class I and II were from Dako (Glostrup, Denmark). The cytokines TNF-α, IL-8, bFGF, VEGF, and TGF-β were all purchased from R&D Systems (Abington, United Kingdom).

Cells and Cultures. HUVEC were harvested from normal human umbilical cords by perfusion with 0.125% trypsin, as described previously (21). DMECs, isolated from foreskins, were supplied by Dr. Koolwijk (Dept. of Vascular and Connective Tissue Research, Gaubius Laboratory TNO, Leiden, the Netherlands; Refs. 22 and 23). Cells were cultured in fibronectin-coated tissue culture flasks in RPMI 1640 with 20% HS, supplemented with glutamine and antibiotics. Confluent HUVEC cultures were passed 1:3. After reaching confluence in the second passage, EC were seeded 1:3 in flat-bottomed tissue culture plates and used for adhesion experiments and cytokine incubations when the cells reached 80–90% confluence. In some experiments the HUVEC-derived SV40 large-T transformed cell line ECL4n was used as a model.[4] Peripheral blood mononuclear cells were prepared by density gradient centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) of heparinized peripheral blood from healthy adult donors. PHA-blasts were prepared by culturing 2 × 10^6 peripheral blood mononuclear cells/well (24-well plates)

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[3] The abbreviations used are: EAM, endothelial adhesion molecule; TNF, tumor necrosis factor; EC, endothelial cells; ICAM, intercellular adhesion molecule; VCAM-1, vascular endothelial cell adhesion molecule-1; TGF, transforming growth factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; IL, interleukin; HUVEC human umbilical vein EC; DMEC, dermal microvascular EC; HS, human serum; PHA, phytohemagglutinin; RCC, renal cell carcinoma; DiI-LDL, DiI-labeled low density lipoprotein.

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with 10 μg/ml PHA. After 3 days, cells were used for adhesion assays. The (promyelocytic) cell line HL60 was also used in the adhesion assays.

Flow Cytometric Analysis of Tumor-derived Endothelial Cells. Tumor and normal tissues, obtained from surgical procedures as first treatment of RCC, were processed immediately after resection under sterile conditions. An ethical review committee-approved informed consent procedure was used to obtain the relevant materials. Normal and tumor tissue was minced and treated for 1 h with 1 mg/ml collagenase and 1 mg/ml dispase (both from Sigma Chemical Co, St. Louis, MO) in PBS. Tissue was subsequently sieved, and single cells were washed and resuspended in RPMI 1640/20% HS. Enzyme treatment and sieving was repeated two times, and cells were collected and cultured overnight in a fibronectin-coated culture flask in RPMI 1640/20%HS. Then, nonadhering cells and debris were discarded, and adhering cells were stained with anti-adhesion molecule antibodies and counterstained for EC by culturing for 2 h with DiI-acetylated-LDL (1:50; Biomedical Biotechnology; Ref. 24) or by indirect staining with EC-specific antibody EN4-biotin and streptavidin-PE (Dako). DiI-LDL labeling and staining with EN4 antibodies identified the same cells, demonstrating the validity of both methods.

Immunofluorescence. Immunofluorescence using indirect PE- or FITC-conjugated reagents required three separate incubations. EC cells (0.1×10^6; either trypsinized or harvested with a cell scraper) were washed in cold PBS, resuspended in 20 μl appropriately diluted monoclonal antibody, and incubated for 1 h on ice. Subsequently, cells were washed three times in PBS/0.5% BSA and incubated for another 30 min with biotinylated goat-antimouse immunoglobulin (Dako). After another three washings, cells were incubated with streptavidin-phycoerythrin conjugate. Stained cells were analyzed on a FACScan flow cytometer. Of each sample, foreward scatter, side scatter, green fluorescence (525/20 nm) and red fluorescence (640/20 nm) signals of 5000 cells were recorded. Electronic settings for scatter signals were adjusted for these experiments, since settings used for leukocyte flow cytometry did not permit proper analysis of EC. Data analysis was performed using PClysys software (Becton Dickinson). Statistical significance of observed differences was determined using the Student's *t* test.

Northern Blot. RNA from subconfluent EC cultures (75-cm^2 Petri dishes) incubated with bFGF for different time points was isolated using an RNA-zol kit (Campro Scientific, Houston, TX). Total RNA (10 μg) for each sample was separated in a 0.8% formaldehyde-denaturing gel, transferred to nitrocellulose (Hybond N^+; Amersham International, Amersham, United Kingdom) and hybridized to a ^{32}P-labeled 1.9-kb cDNA probe, containing the functional sequence of the human *ICAM-1* gene (a gift from Dr. B. Seed, Massachusetts General Hospital, Boston, MA). Membranes were washed at a high stringency in 0.2× SSC-0.1% SDS at 50°C for 1 h. Filters were exposed to X-ray films (Kodak X-Omat; Eastman Kodak Company, Rochester, NY) using an intensifying screen at −80°C for not less than 12 h. Autoradiograms were subjected to scanning using a laser densitometer (Model GS670; Bio-Rad, Hercules, CA), and data were analyzed with the Molecular Analyst PC software. The intensity of the major ICAM-1 mRNA transcript was normalized with respect to actin mRNA expression used as a control (25).

Adhesion Assay. Confluent cultures of EC in 96-well plates were used for adhesion assays. HL-60 or PHA-blasts were cultured for 24 h in the presence of 5 μCi [^3H]thymidine/ml (87 Ci/mmol; Amersham International). Cells were washed twice and subsequently added (in quadruplo; 5×10^4–2×10^5 cells/well) to the growth factor-pretreated EC cultures. After an adhesion step of 2 h at 37°C, unadhered cells were removed by washing the wells gently with prewarmed culture medium using a multichannel pipette. Adhered cells were first enumerated using an inverted microscope; subsequently, [^3H]thymidine activity was measured using liquid scintillation.

Cell Proliferation. EC proliferation was measured using a [^3H]thymidine incorporation assay. EC were seeded 1:10 in flat-bottomed tissue culture plates and grown for 3 days, in the absence or presence of growth factors, in RPMI/20% HS and antibiotics. During the last 16 h of the assay, the culture was pulsed with 0.5 μCi [*methyl*-^3H]thymidine/well. Results are expressed as the arithmetic mean cpm of quadruplicate cultures.

RESULTS

Single-cell suspensions of tumor and normal tissue can be analyzed by flow cytometry. EC were characterized by either staining with EC-specific EN4 antibody or by incubation with DiI-LDL (24). Additional criteria for EC determination were CD31 and EC-specific scatter signals. These criteria excluded the measurement of leukocytes. Adhesion molecule expression on EC can be studied by double staining with FITC-conjugated antibodies. This method generates

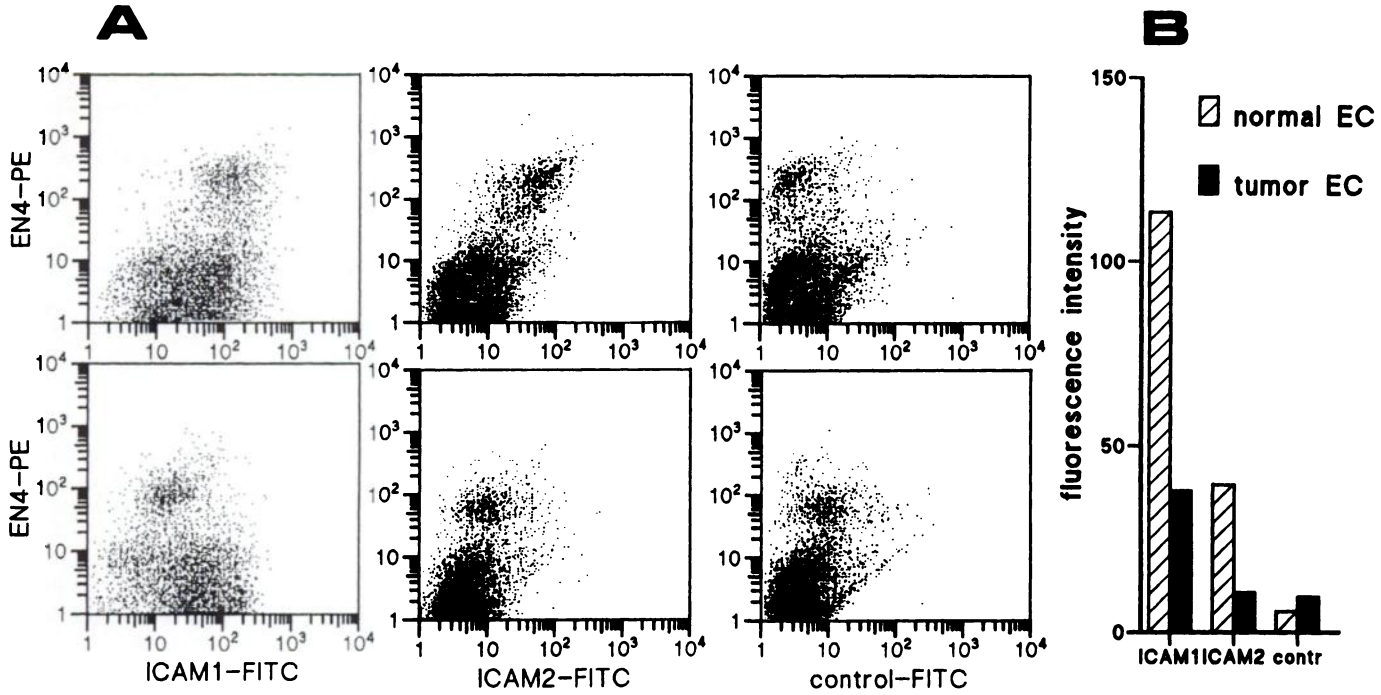

Fig. 1. ICAM-1 is suppressed on tumor-derived endothelial cells. *A*, FACS analysis of normal renal tissue (*upper panels*) and RCC-derived (*lower panels*) adherent cells, stained for EC with EN4-biotin/streptavidin-PE (FL2) and gated on EC foreward/sideward scatter characteristics. *B*, ICAM-1 and ICAM-2 are down-regulated on RCC-derived EC (■) as compared to EC of normal renal tissue from the same patient (▨). Mean FITC-fluorescence of EN4-positive, foreward/sideward scatter gated cells is shown of one representative experiment of seven.

Table 1 *Suppressed endothelial ICAM-1 and ICAM-2 expression in renal cell carcinoma*[a]

	ICAM-1		ICAM-2	
Tumor[b]	Normal	Tumor	Normal	
Expt 1	209[c]	136	137	95
Expt 2	596	288	128	27
Expt 3	450	76	120	25
Expt 4	1650	446	780	20
Expt 5	445	358	291	116
Expt 6	311	26	231	10

[a] Data represent mean fluorescence intensities of EN4-PE-positive cells (see Fig. 1) gated on foreward and sideward scatter characteristics of EC.
[b] Expt, experiment.
[c] Intensity values not corrected for background (<20) fluorescence.

quantitative data on adhesion molecule expression, evading one of the major drawbacks of immunohistochemistry, and allows phenotypic comparison of EC originating from different origins (Fig. 1*A*). It was found that EC from RCCs display a significantly lowered expression of ICAM-1 and ICAM-2 as compared to EC originating from normal renal tissue of the same patient (Fig. 1). Similar results were obtained for EC derived from seven different RCCs and normal kidney tissues. Table 1 shows ICAM-1 and ICAM-2 values from six different donors. Mean expression of ICAM-1 on tumor EC was 44% of the expression seen on EC from normal tissue ($P < 0.001$, $n = 7$). For ICAM-2, the mean expression was 30% ($P < 0.001$, $n = 7$) from normal tissue. In these experiments, differences were not found in expression of PECAM/CD31 and HLA class I molecules.

Since tumors provide their own supply of new blood vessels by production of angiogenic factors, we hypothesized that the inhibited ICAM expression in tumors is due to the exposure of EC to these factors. bFGF is one of the most potent angiogenic factors involved in many tumor types. We found that incubation of HUVEC for 3 days in 10 ng/ml bFGF resulted in a marked decrease of ICAM-1 expression. The longevity of this down-regulation was at least 7 days. Measurements of this effect at different time points revealed that during early stages of activation, ICAM-1 expression is elevated (Fig. 2; Table 2). Similar results were obtained with different anti-ICAM-1 antibodies. The same biphasic effect was found for ICAM-2, another LFA-1 ligand that is mainly expressed by EC. ICAM-3, which is absent on normal EC, is not induced by bFGF under these conditions. In experiments with DMEC, the same results were obtained (data not shown). In dose-response experiments, it was found that the described effects of bFGF on the expression of ICAM-1 (both the stimulatory effect after 16–24 h and the inhibitory effect after 3–7 days) were clearly dose dependent and maximal at 10 ng/ml bFGF, while a concentration of 1 ng/ml already revealed significant responses (Fig. 3). The early transient up-regulation of ICAM preceded proliferation, as measured by [^{3}H]thymidine incorporation (Fig. 4).

To see whether bFGF may act on modulating *ICAM-1* gene expression, we treated HUVEC with 10 ng/ml bFGF for different intervals of time. bFGF was able to transiently up-regulate the expression of the gene as early as 4 h, with a very rapid decrease of the ICAM-1 mRNA transcript reaching undetectable levels within 24 h (Fig. 5*A*). In the endothelial cell line ECL4n, bFGF was able to decrease the mRNA transcript 4 h after the treatment, switching off the gene expression in the presence of bFGF (Fig. 5*B*). The rapid decrease of mRNA content in ECL4n is in accordance with the lack of increased protein expression (Table 2) in these cells at the studied time points.

To identify the functional impact of these findings, adhesion assays were performed. We found that the observed regulation of ICAM-1 expression on EC after treatment with bFGF correlated well with the adhesion of the leukocyte cell line HL-60 and PHA-activated peripheral blood T lymphocytes. Both cell types express the ligand for ICAM-1. Pretreatment of EC for 16–24 h resulted in an augmented adhesion of HL-60 and peripheral blood T-cell blasts. Incubation of EC for 4 days with 10 ng/ml bFGF decreased the adhesion of these cells to background levels (Fig. 6).

To study the phenotype of EC that have received angiogenic signals, a panel of antibodies was tested on untreated and bFGF-stimulated EC. Next to the regulation of ICAM, we found that the hemopoietic progenitor cell antigen CD34, which is also expressed by a subset of EC, is down-regulated during the culture with bFGF. Three days after activation of EC, CD34 is almost completely modulated from the plasma membrane. Both the fluorescence intensity and the number of positive cells is decreased by culture in the presence of bFGF (Table 2).

Another molecule that exhibits an altered expression after activation of EC by bFGF is the leukocyte homing receptor CD44. CD44 is present on EC, and in contrast to ICAM-1, ICAM-2, and CD34, expression is increased 24 h after exposure to bFGF. This 2–3-fold up-regulation is transient, and at day 7, CD44 expression is comparable with expression on cells that were not exposed to bFGF (Table 2).

Expression of PECAM/CD31, the endothelial cell marker EN4, and HLA-class I molecules was not affected by the activation of cultured EC with bFGF. In addition, adhesion molecules such as VCAM-1 and E-selectin, which are described to be up-regulated upon stimulation with the inflammatory cytokines IL-1, TNF-α, and IFN-γ, were not induced by bFGF (Table 2). Similar results were obtained with HUVEC and DMEC.

The potency of bFGF to act as a stimulator of EC proliferation prompted us to test the possibility that regulation of ICAM-1 expression is the result of the strong mitogenic response induced by bFGF. Therefore, a number of angiogenic factors were tested for their capacity to influence expression of ICAM-1. The culture of HUVEC with mitogenic [bFGF (10 ng/ml) and VEGF (100 ng/ml)] and non-mitogenic [TGF-β (10 ng/ml) and IL-8 (4 ng/ml)] angiogenic factors

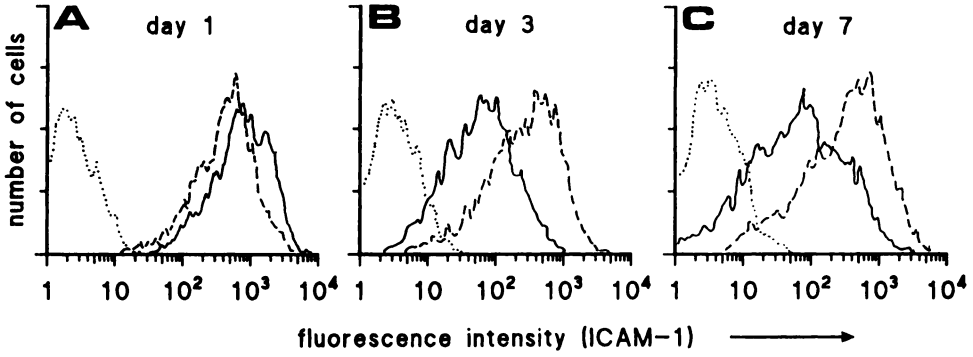

Fig. 2. Biphasic regulation of ICAM-1 expression on bFGF-treated HUVEC. Measurements were performed 1 day (*A*), 3 days (*B*), and 7 days (*C*) after activation. Cells treated with 10 ng/ml bFGF (———) were compared with untreated cells (----). ·····, conjugate control. One representative experiment of six is shown.

Table 2 *EAM expression after bFGF activation*[a]

	Day 0	Day 1	Day 3	Day 7
HUVEC				
ICAM-1	297 ± 14	425 ± 64[b]	87 ± 6[c]	99 ± 2[c]
ICAM-2	736 ± 147	1201 ± 92[b]	512 ± 23[d]	348 ± 1047[b]
ICAM-3	8 ± 9	4 ± 2	4 ± 1	4 ± 1
VCAM-1	3 ± 3	3 ± 3	4 ± 2	ND[e]
E-selectin	1 ± 0	1 ± 1	1 ± 0	ND
CD34	29 ± 4	25 ± 3	10 ± 3[b]	ND
CD44	58 ± 17	110 ± 7[b]	143 ± 9[c]	72 ± 2
PECAM	211 ± 117	223 ± 103	277 ± 213	ND
HLA class I	217 ± 47	231 ± 26	133 ± 60	232 ± 153
EN4	201 ± 48	185 ± 95	147 ± 61	ND
Cell line ECL4n				
ICAM-1	137 ± 21	162 ± 12	74 ± 17[b]	ND[e]

[a] Mean fluorescence intensity from four independent experiments (± SD). Intensities are corrected for background (in all cases <5) fluorescence (subtraction). At all time points, EC samples without bFGF treatment were processed and used for correction of inter-assay variation (see Fig. 1). Values identified with asterisks are significantly different from day 0 values.
[b] Student's *t* test, $P < 0.02$.
[c] Student's *t* test, $P < 0.001$.
[d] Student's *t* test, $P < 0.05$.
[e] ND, not done.

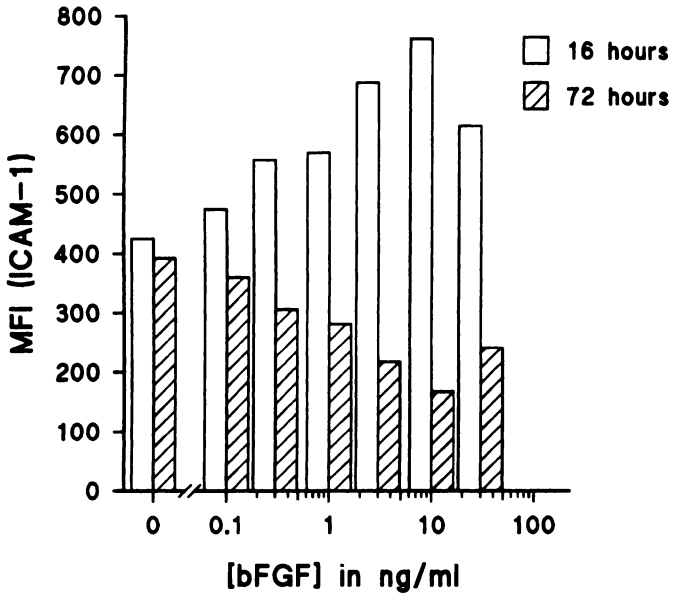

Fig. 3. Dose-response study of ICAM-1 expression after bFGF activation. HUVEC were grown for 16 h (□) and 72 h (▨) with various concentrations of bFGF. Mean fluorescence intensity (*MFI*) is shown of one representative experiment of three.

and subsequent measurement of cell proliferation and adhesion molecule expression was performed. In one representative experiment of three, ICAM-1 and ICAM-2 fluorescence intensities after 4 days culture with bFGF, VEGF, TGF-β and IL-8 were (respectively): ICAM-1, 166, 421, 426, and 418 (control 388); and ICAM-2, 830, 968, 1208, and 1024 (control 933). Independent of their mitogenic capacity, VEGF, TGF-β and IL-8 did not alter ICAM-1 and ICAM-2 expression on EC, in contrast to the bFGF effect.

Preventing the development of an effective inflammatory infiltrate by production of angiogenic factors was suggested to serve as an escape from immunity. In a subsequent set of experiments, the hypothesis was tested whether angiogenic factors decrease the sensitivity of EC for proinflammatory cytokines. Therefore, the influence of prior exposure to bFGF on the up-regulation of ICAM-1, VCAM-1, and E-selectin by TNF-α (4 ng/ml for 16 h) was measured. As shown in Fig. 7, ICAM-1 levels induced by TNF-α in the presence of bFGF are lower than that in the absence of bFGF. The magnitude of the response to TNF-α is similar under both conditions (3–4-fold up-regulation with TNF-α, both with or without exposure of EC to bFGF). Up-regulation of VCAM-1 and E-selectin expression by TNF-α is also inhibited by bFGF.

DISCUSSION

The present study describes the suppressed expression of ICAM-1 and ICAM-2 on EC from RCC compared to healthy renal tissue. The regulatory mechanisms involved in this phenomenon were addressed *in vitro* using cultured umbilical vein- and dermis-derived EC. The suppressed expression of adhesion molecules on EC from RCC was found using a novel method to quantitatively analyze the phenotype of tumor-derived EC by flow cytometry. Absence of changes in other molecules expressed by these isolated EC makes unlikely that the differences in EAM are an artifact. The *in vitro* experiments strongly support the *in vivo* observation. Lack of adhesion molecules abrogates leukocyte extravasation. Inhibition of leukocyte infiltration into tumors may be a strategy to escape immunological attack. Recent data have demonstrated that tumor-derived activities can inhibit the expression of adhesion molecules instrumental for the accumulation of blood-borne leukocytes in tumors. On metastasized melanoma and carcinoma, cytokine-induced VCAM-1 expression is down-regulated by tumor factors (14). Also, endothelial E-selectin has been observed to be repressed in melanoma (26). This down-regulation was suggested to be mediated by tumor-derived TGF-β (9). The ability of tumors to down-regulate leukocytic adhesiveness seems, therefore, not an exclusive property for certain tumors. The underlying mechanisms remained to be clarified. We here describe that one of the mechanisms involves angiogenic factors produced by the tumor. bFGF, one of the commonest angiogenic factors active in a variety of different tumors, down-regulates endothelial ICAM-1 and ICAM-2 expression at the protein and mRNA level. Although a transcriptional basis of ICAM-1 down-regulation has also been found in the IL-4-induced inhibition of the IFNγ-driven ICAM-1 augmentation (27) and

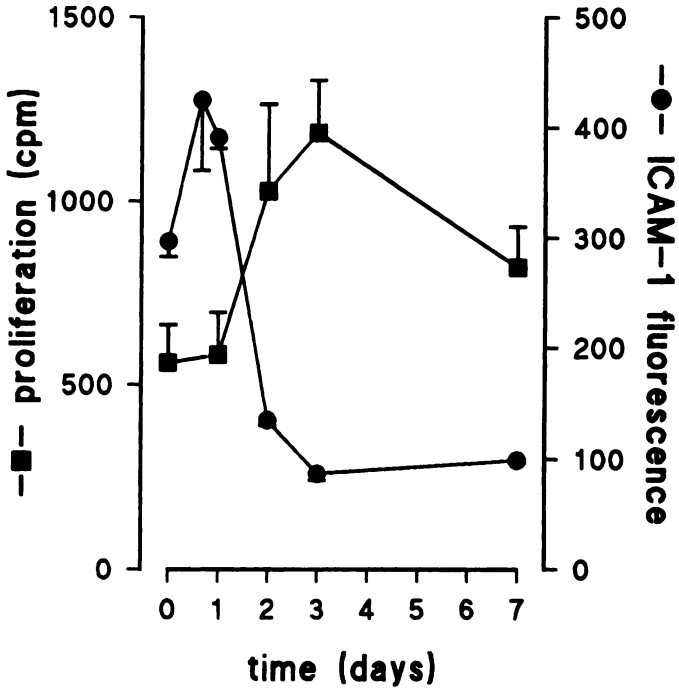

Fig. 4. Time kinetics of ICAM-1 expression on HUVEC induced by bFGF (10 ng/ml) in relation to [^{3}H]thymidine incorporation.

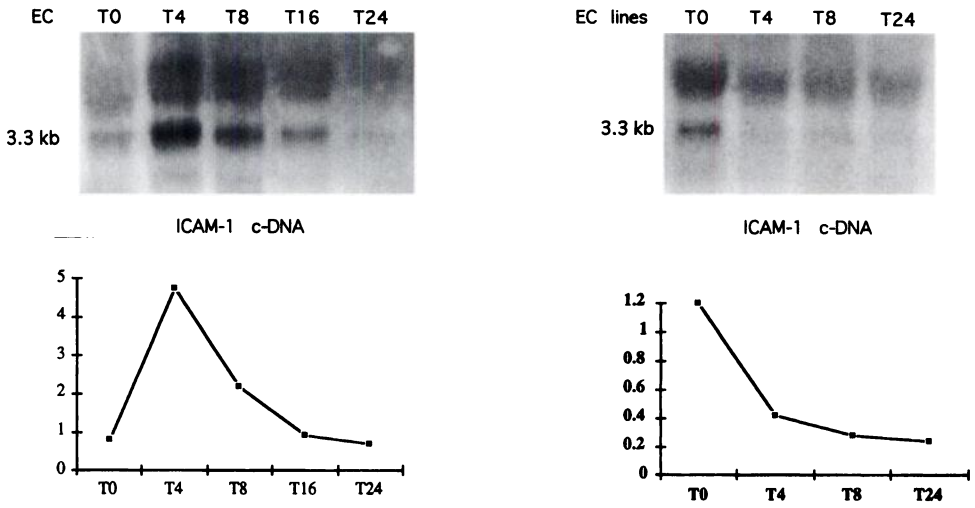

Fig. 5. Analysis of ICAM-1 mRNA expression in HUVEC (*left panels*) and the endothelial cell line ECL4n (*right panels*) various time points after treatment with bFGF. EC were cultured in 75-cm^2 Petri dishes with 10 ng/ml bFGF. After various time points, mRNA was prepared, and Northern blot analysis was performed (*upper panels*). Densitometric analysis of autoradiographed bands was performed normalizing *versus* β-actin mRNA (data not shown) as a control.

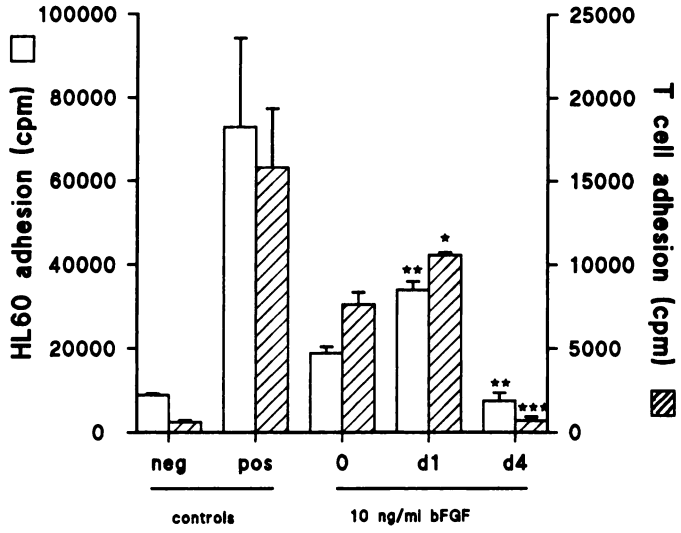

Fig. 6. Adhesion of leukocytes is regulated by bFGF. HL-60 promyelocytic cell line (□) and PHA-stimulated peripheral blood T-cell blasts (▨) were adhered for 2 h on bFGF (10 ng/ml) prestimulated HUVEC. Adhesion is expressed as mean [^{3}H]activity of quadruplicate measurements; *bars*, SD. Positive control is represented by incubation with TNF-α for 16 h. Negative control is performed by paraformaldehyde fixation prior to adhesion. One representative experiment of four is shown. *Bars* identified with *asterisks*, significantly different from day 0 values (*, $P < 0.005$; **, $P < 0.001$; ***; $P < 0.0001$).

in the dexamethasone inhibition of TNF-α-induced up-regulation of ICAM-1 (28), a decreased stability of ICAM-1 mRNA might also explain our data. However, initial increased mRNA suggests regulation at the gene level. Future studies will address this issue. In a variety of systems, ICAM-1 has been described as an inducible molecule. ICAM-2, however, has never been observed to exhibit a regulated expression *in vitro*. Our report is the first observation that expression of ICAM-2 can be regulated on EC *in vitro* and *in vivo*. Production of angiogenic factors is a essential for growth of tumors. Therefore, tumor escape from leukocyte infiltration by down-regulation of ICAM molecules is most likely not a unique property of RCC. Although we were not able to collect relevant control (ovarian) tissue, we also found a very low expression of ICAM-1 and ICAM-2 on ovarian carcinoma-associated endothelium (data not shown).

Functional relevance for the observed findings is presented by the adhesion assays. A corresponding biphasic regulation of leukocyte adhesion is demonstrated after bFGF activation. Although bFGF induces a strong mitogenic effect, adhesion values measured at time points during EC proliferation were not corrected for the number of EC in the assay. For time points 16 and 24 h, it is demonstrated that the number of cells is not increased (Fig. 4), indicating that not the cell number but the level of ICAM-1 expression is responsible for up-regulation of adhesion. Correction for the number of EC at 3–7 days will not change the conclusions from the experiments since this would have resulted in even lower adhesion values. It is assumed that the strong adhesion of leukocytes to EC necessary for trans-EC migration is mainly mediated by the LFA-1/ICAM-1 adhesion receptor ligand pair. This seems valid for the present experiments since adhesion via VLA-4 and carbohydrate ligands requires expression of VCAM-1 and E-selectin on the endothelium. Expression of these adhesion receptors is low or even absent on resting EC and is not affected by bFGF treatment (Table 2). The latter finding suggests that the adhesion measured at day 3 is mainly mediated by LFA-1/ICAM-1. In a recent study, down-regulation of ICAM-1 on bFGF-stimulated HUVEC has been suggested to cause a suppressed emigration of CD4$^+$ T lymphocytes,

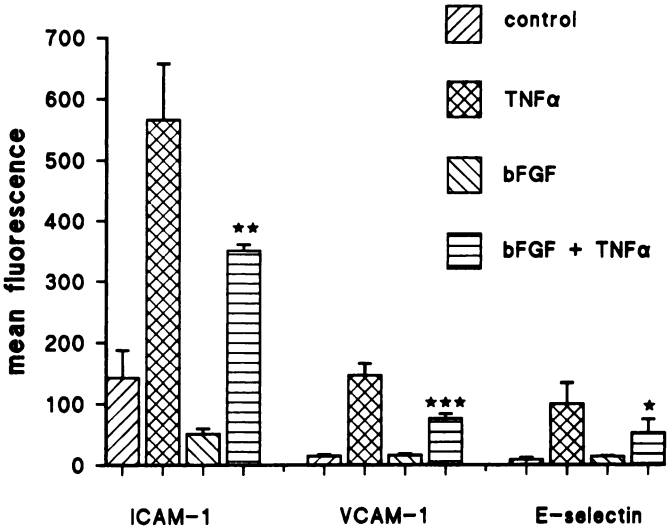

Fig. 7. Inhibition of TNF-α-induced up-regulation of ICAM-1, VCAM-1, and E-selectin by bFGF. Cultured HUVEC were preincubated with bFGF (10 ng/ml) for 3 days, during the last 16 h of which, TNF-α (4 ng/ml) was added. One representative experiment of four is shown. Mean fluorescence intensity values are shown of two independent experiments of unrelated donors; *bars*, SEM. *Bars* identified with *asterisks* (*bFGF + TNFα*), significantly different from values of TNF-α alone (*, $P < 0.05$; **, $P < 0.002$; ***, $P < 0.001$).

leading to insufficient helper function in local immune responses (15). Our data support these findings.

In addition to the down-regulated expression of ICAM-1, a marked decrease of CD34 expression is observed after treatment with bFGF. CD34, an antigen that is expressed on hematopoietic stem cells, is described in immunohistochemical studies to be expressed by a subset of EC (29) that is present at the tip of vascular sprouts and on cells with proliferating morphology. Expression of CD34 as a proliferation marker has been suggested (30). The observed loss of CD34 expression during stimulation with bFGF seems, therefore, controversial. Also, the inverse correlation of CD34 and VCAM-1/E-selectin (31, 32) is not confirmed in the present study since CD34 disappears without induction of VCAM-1 and E-selectin. Our findings suggest that CD34 is not required during the proliferative phase of angiogenesis, which may correlate with the loss of polar distribution of CD34 on growing vascular sprouts (30). The regulated expression of CD34, a ligand for L-selectin, may also contribute to the prevention of initial attachment of leukocytes under shearing conditions (33) and thereby the escape from the immune system. The precise role of CD34 on EC, however, is not known, and the function of changes in expression of CD34 upon EC activation needs further investigation.

It is an interesting observation that early upon EC activation, a transient up-regulation of certain adhesion receptors (ICAM-1, ICAM-2, and CD44) occurs. An attractive hypothesis concerning this issue would be that the initial events during angiogenesis, like sprouting of new vessels, is dependent on adhesion events to extracellular matrix components. The recent finding that ICAM-1 can serve as a receptor for hyaluronan (34) supports this hypothesis. The transient up-regulation of CD44 (receptor for the ligand molecules hyaluronic acid, laminin, and fibronectin) would also fit well in this hypothesis. In tumors, the effect on leukocyte adhesion is probably insignificant since tumor EC are exposed to tumor-derived factors for longer periods of time.

In recent reports by other investigators (15), it is described that bFGF influences the sensitivity of EC to inflammatory cytokines. We show here that TNF-α-induced ICAM-1 expression reaches lower absolute levels in the presence than in the absence of bFGF. Based on our observation that the magnitude of elevation is similar in both cases, it is suggested that bFGF does not interfere in the signaling pathway of TNF-α that augments ICAM-1 expression. Indeed, it has been described that whereas the inducible expression of ICAM-1 is regulated by nuclear factor-κB, other transcription factors regulate the constitutive expression (25, 28, 35, 36). In our view, bFGF interferes only with the constitutive ICAM-1 expression. For VCAM-1 and E-selectin, transcriptional regulation has not been elucidated; hence, bFGF signaling might directly inhibit TNF-α induced up-regulation. These experiments show that immunological attack by cytotoxic effector cells may also be inhibited by modulation of TNF-α-induced inflammatory responses.

The present study demonstrates that angiogenic factors are involved in the intricately regulated expression of various adhesion molecule systems, which in the *in vivo* situation of tumor growth may contribute to the prevention of the infiltration of relevant leukocytes into cancer tissue. For improvement of results of immunotherapies against tumors, it might be useful to develop strategies to abolish the reduced EAM expression caused by bFGF. It remains to be established whether antiangiogenic therapies, presently starting in clinical experimental settings, are in part dependent on the effect on endothelial adhesion molecule expression.

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