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Retroviral transduction of intercellular adhesion molecule-1 enhances endothelial attachment of bladder cancer

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Abstract An intercellular adhesion molecule-1 (ICAM-1)-negative RT4 transitional cell carcinoma (TCC) cell line was transducted with full-length ICAM-1 cDNA via a retroviral vector. Flow cytometry showed that a sense-oriented clone (S20) highly expressed ICAM-1 while an anti-sense clone (AS6) did not. Both S20 and AS6 bound with equal frequency (30 ± 8.7% vs 30 ± 9.4%) to unstimulated human umbilical vein endothelial cells (HUVECs) in cell attachment assays. However, when phorbol myristate acetate (PMA)-activated T lymphocytes, which express lymphocyte function-associated antigen-1 (LFA-1), were cocultured with tumor cells, attachment of S20 increased twofold (60 ± 11.9%) but AS6 showed no change (32 ± 11%). Blocking studies with anti-LFA-1 and anti-ICAM-1 monoclonal antibodies caused an inhibition of the attachment to baseline levels, demonstrating that the enhancement of S20 attachment was dependent upon the LFA-1/ICAM-1 interaction. Enhanced attachment of S20 was not inhibited by the addition of isotypic immunoglobulin G. These results suggest that LFA-1-expressing leukocytes may act as a bridge between the endothelium and tumor cells which express ICAM-1 and, thereby, enhance the potential for hematogenous metastasis.

Key words ICAM-1 · Transitional cell carcinoma · Metastasis · Endothelium

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Introduction

In an analytic study of melanoma, Johnson et al. [4] determined that one of the melanoma-associated antigens, P3.58, an 89-kDa cell surface glycoprotein which is expressed predominantly in metastatic lesions, is identical to intercellular adhesion molecule-1 (ICAM-1). They showed that ICAM-1 was detectable on advanced human melanoma, but not on benign melanocytes or early melanoma, and that the frequency of ICAM-1 expression correlated with the melanoma thickness. They suggested that ICAM-1 expression may contribute to the metastatic or invasive capacity of melanoma cells [4]. Natali et al. [7] confirmed that the expression of ICAM-1 in primary melanoma correlated with the thickness of the lesions and showed that the frequency of expression of ICAM-1 was markedly higher in metastatic lesions than in primary melanomas. These results suggested that expression of ICAM-1 enhances the metastasis of melanoma cells [4, 7].

However, there is no reasonable explanation of the mechanism of ICAM-1-mediated hematogenous metastasis, since very few lymphocyte function-associated antigen-1 (LFA-1) molecules, which are receptors of ICAM-1, are expressed on vascular endothelial cells or tumor cells. These findings mean that tumor cells expressing ICAM-1 can not attach directly to the endothelial cells by the ICAM-1/LFA-1 binding system.

It is well known that activated lymphocytes express LFA-1 molecules and attach to ICAM-1-positive cells, such as vascular endothelial cells and some tumor cells. Tumor cells attached by these activated lymphocytes in blood vessels can bind to endothelial cells via the LFA-1 molecules expressed on the lymphocytes. In this study, we tried to clarify the mechanism of ICAM-1-mediated hematogenous tumor metastasis. Our working hypothesis was that activated lymphocytes expressing LFA-1 could be a bridge between tumor cells and vascular endothelial cells, thereby enhancing hematogenous tumor metastasis.

Materials and methods

Reagent

Murine monoclonal antibodies to ICAM-1 (CD54) and LFA-1 (CD18) were purchased from Becton Dickinson (San Jose, Calif.). Recombinant human cytokines were obtained from the following sources: interferon-gamma (IFN-γ), Biogen (Cambridge, Mass.); tumor necrosis factor-alpha (TNF-α), Cetus (Emoryvill, Calif.); interleukin-one beta (IL-1β), R and D (Minneapolis, Minn.). Tissue culture reagents were purchased from Gibco-BRL (Grand Island, N.Y.) or Whitaker Bioproducts (Walkersville, Md.). Reagents for RNA isolation, gel electrophoresis, and Northern blot analysis were obtained from Boehringer Mannheim (Indianapolis, Ind.) or Sigma (St. Louis, Mo.). Radioisotopes were purchased from New England Nuclear (Boston, Mass.). All other reagents were purchased from Sigma, unless otherwise specified. A plasmid containing a cDNA clone for the ICAM-1 gene was a kind gift from Dr. T. A. Springer, Harvard Medical School.

Cell culture

The amphotropic retroviral packaging cell line, PA317 (American Type Culture Collection, Rockville, MD), was maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS). Human umbilical vein endothelial cells (HUVECs) were isolated by a previously reported method [3] and maintained in MCDB-105 (Sigma), containing 20% FCS, 100 mg/ml epidermal growth factor (EGF; Sigma) and 100 mg/ml heparin (Fujisawa). Human bladder cancer cell line RT4 was maintained in our laboratory. An RT4 transitional cell carcinoma (TCC) cell line was maintained in modified Eagle's medium supplemented with 10% FCS, 0.1 mM nonessential amino acids and 1 mM sodium pyruvate. Cells were recovered for flow analysis by trypsin digestion (0.05% trypsin and 0.53 mM EDTA) for 3 min. Identical results were obtained when EDTA alone was used.

Construction of human ICAM-1 retroviral vectors

A retroviral vector, pBabe [6], was a gift from Dr. Brian Williams (Cancer Biology, The Cleveland Clinic Foundation, Ohio). The full-length human ICAM-1 cDNA was isolated as a *Bam*H-1 restriction fragment from CDM8 containing ICAM-1 cDNA (a kind gift from Dr. Timothy Springer, Boston, Mass.), and ligated into pBabe after the blunt ligation of both ends of the ICAM-1 cDNA.

Production of human ICAM-1 amphotropic virus

High-titer amphotropic virus stocks were prepared as previously described [6]. Briefly, retroviral plasmids were transfected into ecotropic packaging cells by glycerol shock and the virus produced was used to infect the PA317 amphotropic packaging cells. After selection in 500 mg/ml of the aminoglycoside antibiotic, Geneticin (G418), viral supernatants from these cells were sterile-filtered and stored at −80 °C until used. This concentration of G418 killed all cells that did not contain the retroviral plasmid bacterial transposon, which encodes for aminoglycoside 3′-phosphotransferase and provides resistance to G418.

Immunocytometry

Flow immunocytometric analysis was performed using two-color immunofluorescence [5]. Tumor cells were phenotypically identified through the use of fluorescein isothiocyanate (FITC)- and phycoerythrin-conjugated monoclonal antibodies. Isotypic controls were used for each subclass of immunoglobulin. HUVECs treated with IL-1 served as positive controls for ICAM-1 expression. Analyses on the FACScan (Becton Dickinson) were performed using an argon ion laser (Cyosics, San Jose, Calif.) with 15 mW of 488-nm excitation. Optics in the fluorescence path included a focusing objective, beam splitters, dichroic mirrors, a 530-nm bandpass filter, a 585-nm bandpass filter and a 650-nm pass/cut-on filter. Live gating of the forward and orthogonal scatter channels, determined by fluorescence backgating (bright $CD45^+$, $CD14^-$), was employed to selectively acquire events. Multicontour, multiparameter software (Paint-a Bate and LYSIS II, Becton Dickinson) was used to analyze the data. Results were reported as (1) percentage of positive cells and (2) relative intensity, after correction for the nonspecific binding of isotypic controls, determined by measurement of the autofluorescence background.

RNA isolation and Northern blot analysis

Total RNA was extracted with guanidium isothiocyanate and isolated by centrifugation over a cesium chloride cushion, using previously described techniques [9]. The RNA samples were electrophoresed on denaturing 1% formamide-agarose gels and transferred to nylon membranes (MSI, Mass.). Blots were hybridized with ^{32}P-labeled random primed ICAM-1 cDNA. The blots were also stripped and rehybridized with a human β-actin oligonucleotide to normalize for mRNA content.

Phorbol myristate acetate (PMA)-activated T lymphocytes

Peripheral blood was obtained from normal, healthy volunteers for isolation of mononuclear cells. Heparinized blood was layered over Histopaque 1077 (density = 1.077 g/ml) (Sigma) and centrifuged for 30 min at 400 *g*. The buffy coat interface was collected and washed twice with Hank's balanced salt solution, and the cells centrifuged through a layer of FCS to remove the platelets. The resultant mononuclear cell preparation was passed through a nylon wool column to collect T cells, as described previously [1]. The T cells were cultured in the presence of PMA (10 ng/ml) (Sigma) for 24 h before use in the attachment assays.

Adherence assay

The adherence of RT4 TCC cells to HUVECs was examined using a modification of a previously described technique [2]. HUVECs were plated on 48-well flat-bottomed tissue culture plates and allowed to reach confluence. RT4 TCC cells were labeled with ^{51}Cr by incubating for 1.5 h at 37 °C (50 μCi per T25 flask). After trypsin digestion for 3 min, the labeled RT4 TCC cells were washed three times. PMA-activated or non-activated resting T lymphocytes were added to the HUVEC monolayers in the 48-well flat-bottomed tissue culture plates (1×10^6 T lymphocytes per 0.15 ml medium per well) and incubated at 37 °C for 10 min. The ^{51}Cr-labeled RT4 TCC cells were then added to the HUVEC monolayers (3.5×10^4 RT4 cells per 0.15 ml medium per well) and incubated at 37 °C for a further 30 min (lymphocyte : RT4 TCC cells = 30 : 1). Nonadherent cells were gently removed, and each well was washed three times with RPMI 1640 with 0.6% bovine serum albumin (BSA). The remaining adherent cells were then lysed with 1% Triton X-100, and the supernatants were harvested and counted. Each treatment was done in duplicate. The spontaneous release of ^{51}Cr was also determined, and remained below 10% of the total count in each experiment. The percentage of adherent RT4 TCC cells was calculated as follows: percent bound = [adherent c.p.m./(maximum c.p.m. − spontaneously released c.p.m.)] × 100%. In some wells, monoclonal antibody to ICAM-1 (10 μg/ml), LFA-1 (10 μg/ml), or control IgG (10 μg/ml) was added to the wells when the T lymphocytes were added to the HUVEC monolayers. All adherence and Cr-release assays were repeated at least five times, using a different source of lymphocytes each time. Data are expressed as the average of five experiments.

Results

Transduction of RT4 with human ICAM-1

High-titer amphotropic retroviruses were prepared and used to infect RT4 TCC cells. More than 70% of PA317 cells were stably infected as determined by the number of cells sensitive to G418 selection and by flow cytometric analysis (data not shown).

To determine whether ICAM-1 was successfully transducted, total RNA was prepared from G418-resistant populations of transducted cells and analyzed for ICAM-1. Northern blot analysis revealed that parental RT4 (non-transducted) did not show any ICAM-1 message. Also, cytokine (IFN-γ, IFN-α, 1L-1β)-stimulated RT4 TCC cells did not express any ICAM-1 messages in Northern blot analysis. Contrary to these findings, transducted RT4 TCC cells with sense- and anti-sense-oriented ICAM-1 showed an ICAM-1 message (Fig. 1).

To determine whether ICAM-1 was appropriately expressed on the cell surface, transducted RT4 TCC cells were evaluated by flow cytometry. The majority (over 90%) of transducted RT4 TCC cells with sense-oriented ICAM-1 (S20) expressed ICAM-1 compared with control cells, which were transducted with anti-sense-oriented ICAM-1 (AS6) and parental RT4 TCC cells (Fig. 2). ICAM-1 was detected with monoclonal anti-ICAM-1 antibody, indicating that the molecule was appropriately folded on the cell surface.

Cell attachment assay

Transducted RT4 TCC cells with sense-oriented ICAM-1 (S20) showed 30 ± 8.7% attachment to HUVECs. Also, RT4 TCC cells transducted with anti-sense-oriented ICAM-1 (AS6) showed 30 ± 9.4% attachment to HUVECs. These findings indicated that ICAM-1 expression on RT4 TCC cells did not affect the cell attachment to endothelial cells (HUVECs). In contrast, a greater number of RT4 TCC cells (60 ± 11.9%) attached to HUVECs when PMA-activated lymphocytes were cocultured with S20 which express ICAM-1 on RT4. However, AS6 did not show any increase in attachment to HUVECs (32 ± 11%), even though PMA-activated lymphocytes were cocultured. Also, non-stimulated lymphocytes which do not express the activated LFA-1 molecules did not enhance the attachment of S20. These findings suggest that the ICAM-1/LFA-1 system, in combination with activated T lymphocytes, plays an important role in the attachment of RT4 TCC cells to endothelial cells. To confirm this possibility, blocking studies were performed. Both anti-LFA-1β and anti-ICAM-1 monoclonal antibodies completely blocked the increase in attachment of S20 cocultured with activated T lymphocytes, whereas control IgG did not (Fig. 3).

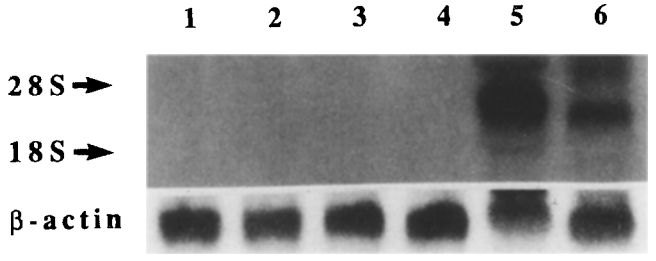

Fig. 1 Expression of intercellular adhesion molecule-1 (ICAM-1) messages in RT4 transitional cell carcinoma (TCC) cells. To determine whether ICAM-1 was successfully transducted, total RNA was prepared from G418-resistant populations of transducted cells and analyzed for ICAM-1. Northern blot analysis revealed that parental RT4 (non-transducted) did not show any ICAM-1 message (*lane 1*). Also, cytokine (IFN-γ, IFN-α, 1L-1β)-stimulated RT4 TCC cells did not express any ICAM-1 messages in Northern blot analysis (*lanes 2, 3, 4*, respectively). Contrary to these findings, transducted RT4 TCC cells with sense-oriented (*lane 5*) and anti-sense-oriented (*lane 6*) ICAM-1 showed an ICAM-1 message

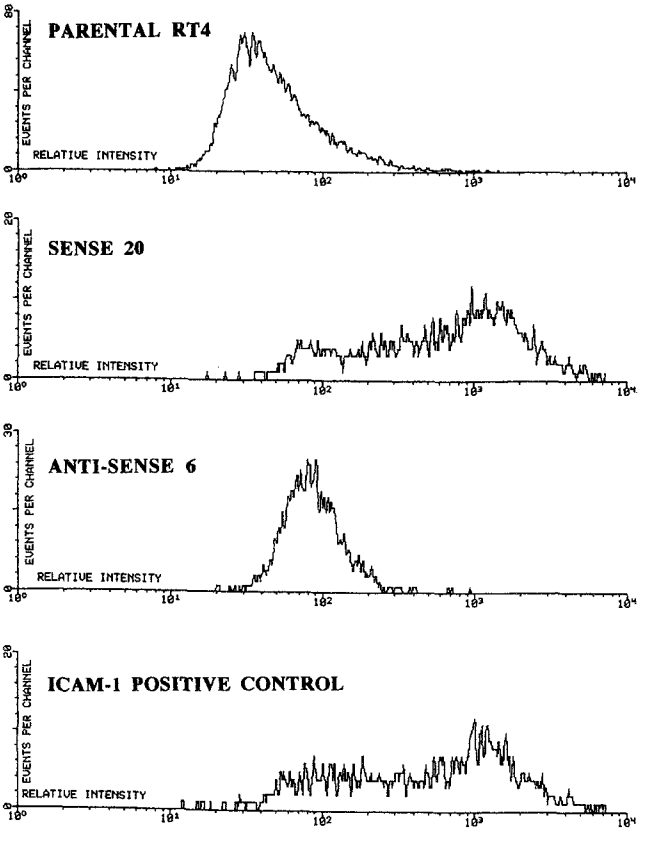

Fig. 2 Flow cytometric analysis of ICAM-1 expression on RT4 TCC cells. To determine whether ICAM-1 was appropriately expressed on the cell surface, transducted RT4 TCC cells were evaluated by flow cytometry. The majority (over 90%) of transducted RT4 TCC cells with sense-oriented ICAM-1 (*Sense 20*, S20) expressed ICAM-1 when compared with control cells which were transducted with anti-sense-oriented ICAM-1 (*Anti-Sense 6*, AS6) and parental RT4 TCC cells

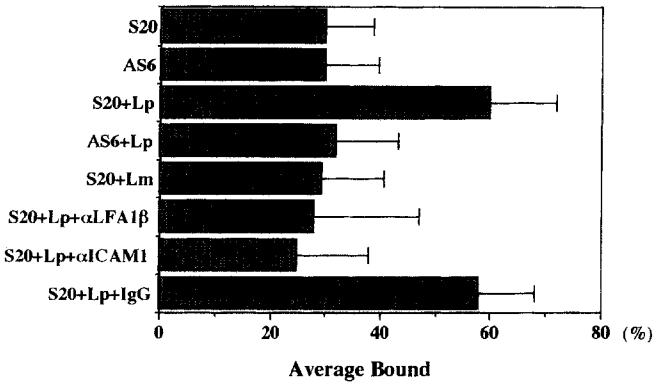

Fig. 3 Adherence of RT4 TCC cells to human umbilical vein endothelial cells (HUVECs): effect of cocultured activated T lymphocytes and monoclonal antibody to ICAM-1 or lymphocyte function associated antigen (LFA-1). Details of the experiment can be found in Materials and methods. Data are expressed as the average of five experiments. *S20* RT4 transducted with sense-oriented ICAM-1, *AS6* RT4 transducted with anti-sense-oriented ICAM-1, *Lp* phorbol myristate acetate (PMA)-stimulated T lymphocytes, *Lm* non-stimulated T lymphocytes cultured with medium alone, *αLFA1β* anti-LFA1β monoclonal antibody; *αICAM1* anti-ICAM-1 monoclonal antibody. S20 and AS6 showed 30 ± 8.7% and 30 ± 9.4% attachment to HUVECs, respectively. Contrary to these findings, a greater number of RT4 TCC cells (60 ± 11.9%) attached to HUVECs when PMA-activated lymphocytes were cocultured with S20. However, AS6 did not show any increase in attachment to HUVECs (32 ± 11%) even though PMA-activated lymphocytes were cocultured. Also, nonstimulated lymphocytes which did not express the activated LFA-1 molecules did not enhance the attachment of S20. Blocking studies were performed and both anti-LFA-1β and anti-ICAM-1 monoclonal antibodies completely blocked the increase in attachment of S20 cocultured with activated T lymphocytes, whereas control IgG did not

Discussion

The results of the present study suggest that LFA-1-expressing leukocytes may act as a bridge between endothelium and tumor cells which express ICAM-1 and, thereby, enhance the potential for hematogenous metastasis.

During an analytical study of melanoma, Johnson et al. [4] determined that one of the melanoma-associated antigens is expressed predominantly in metastatic lesions and is identical to ICAM-1. They showed that ICAM-1 was detectable on advanced human melanoma, but not on benign melanocytes or early melanoma, and suggested that ICAM-1 expression may contribute to the metastatic or invasive capacity of melanoma cells [4]. They proposed several mechanisms to explain the correlation between ICAM-1 molecule expression and tumor metastasis. They speculated that melanoma cells can establish heterotypic cell contact with leukocytes present in the tumor infiltrate. This might lead to a reduction in homotypic adhesion between melanoma cells while enhancing tumor cell adhesion to migratory and invasive leukocytes, enabling individual cells to dissociate from the primary tumor [4]. The de novo expression of ICAM-1 by melanoma leads to heterotypic adhesion between melanoma cells and LFA-1-bearing leukocytes and, thereby, contributes to dissemination of the cells from the primary tumor [4]. Natali et al. [7] confirmed that expression of ICAM-1 in primary melanoma correlated with the thickness of the lesions and showed that the frequency of expression of ICAM-1 was markedly higher in metastatic lesions than in primary melanomas. This may reflect the stage of differentiation of melanocytes from which melanoma cells have originated, since expression of ICAM-1 on cells of certain hematopoietic lineages is a differentiation-related phenomenon. They also speculated that an alternative, but not exclusive, mechanism may be that ICAM-1 mediates the interaction with LFA-1-bearing cells of melanoma cells, thus facilitating their dissociation from the lesion and their metastatic spread [7].

Recently, Opdenakker [8] hypothesized that chemotactic factors give vectorial power to invading and metastasizing tumor cells, not only actively but also by a passive countercurrent mechanism. The chemoattracted leukocytes pave the "shortest" way for the tumor cells to move towards the blood vessels or lymphatics. This is made possible through the chemokine-mediated expression of integrins which facilitate margination, adherence and effective diapedesis where the chemokine is most abundant, namely at the site of the chemokine-producing tumor [8]. However, these hypotheses cannot adequately explain the role of the ICAM-1/LFA-1 system in tumor metastasis, since they failed to show any direct evidence that ICAM-1 enhances the dissociation of tumor cells. Furthermore, the authors did not describe any mechanisms of tumor attachment to the vascular or lymphatic endothelial wall, but only suggested those of tumor dissociation from the primary site [4, 7, 8].

There is no reasonable explanation for the mechanism of ICAM-1/mediated hematogenous metastasis, since the LFA-1 molecule, which is a receptor of ICAM-1, was not expressed on tumor cells or vascular endothelial cells. These findings mean that tumor cells expressing ICAM-1 can not attach to the endothelial cells directly by the ICAM-1/LFA-1 binding system. Our working hypothesis is that leukocytes which attack the tumor cells might be a bridge between tumor cells and blood vessel endothelium and enhance lodging of the tumor cells at a site distant from the primary tumor. In immune surveillance, many tumor cells in the blood vessel or stroma will be destroyed by immunocompetent cells. However, once the tumor cells survived, LFA-1-expressing leukocytes (activated T lymphocytes) attached on tumor cells seemed to enhance the tumor cell adhesion on endothelial cells through the ICAM-1/LFA-1 system (Fig. 3). The present study showed that activated T lymphocytes may increase the attachment of ICAM-1-expressing tumor cells to vascular endothelial cells and, thereby, may enhance the potential for tumor cell metastasis.

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