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Cancer Cell Binding to E-Selectin Transfected Human Endothelia

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Summary: Human endothelial cells were transiently transfected with E-Selectin which enabled us to study tumor cell/endothelial interactions following engagement of E-Selectin without the added complications of metabolic stimulation, morphological changes, and/or up regulation of other adhesion molecules due to cytokine induction. Similar results were received from *in vitro* binding studies and FACS analyses on both Tumor Necrosis Factor-alpha activated and E-Selectin transfected endothelial cells. These data suggest that this methodology is appropriate for dissecting the individual activities of E-selectin while minimizing the participation of other adhesion molecules, thereby allowing us to develop a better understanding of the role of E-Selectin and endothelia in metastatic disease.

Introduction: E-Selectin, a 115 kD endothelial cell surface glycoprotein (1), has an extremely low basal expression on resting endothelial cells and maximal expression following four hours of stimulation by cytokines (2). E-Selectin is a member of the selectin (LEC-CAM) family bearing multiple extracellular domains including the N-terminal lectin recognition domain, EGF-like domain and six complement-like repeats (3). It has been shown that the ligand for E-Selectin is composed of the carbohydrate structures $Lewis^x$ and $Lewis^a$ antigens (4,5,6). E-Selectin plays a critical role in recruitment of neutrophils (7), however, it is also involved in the adhesion of tumor cells to activated endothelia (8,9,10,11). $Lewis^x$ and $Lewis^a$ are tumor antigens found in the serum of cancer patients (12,13,14). Since E-Selectin has been shown to bind both these moieties, the concept of it's importance in metastatic disease has gained strength. Therefore, E-Selectin may be instrumental in the diapedesis of metastatic cancer cells which have escaped from the primary location and are in the process of colonizing distant sites.

To investigate the contribution of E-Selectin, we studied the binding of a variety of colon carcinoma cell lines to this adhesion molecule transfected into Huvecs . The rationale for these experiments stems from the fact that, thus far, research involving E-

Abbreviations: Huvecs, human umbilical vein endothelial cells; TNF-α, tumor necrosis factor-alpha.

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Selectin expression on endothelial cells has used inflammatory mediators to elicit the appearance of E-Selectin (10,15,16,17). Stimulation of the endothelia by pleotrophic growth factors causes modulation of a variety of metabolic activities including increased synthesis of colony stimulating factor, interleukins, Class II MHC, platelet activating factor, von Willebrand factor, and platelet-derived growth factor, as well as up regulating multiple adhesion molecules, and altering endothelial proliferation and migration (18). Elucidation of the specific effects resulting from E-Selectin expression or binding, rather than cytokine stimulation, is difficult. Hence, we have transiently transfected Huvecs with an E-Selectin cDNA and examined the endothelial ability to bind tumor cells *in vitro*. The methodology used herein will allow us the opportunity to further understand the contribution of E-Selectin during tumor cell extravasation.

Materials and Methods:

Tissue Culture: Huvecs were purchased from Cell Systems Corporation (Kirkland, WA) and grown according to manufacturer's instructions. Four colon carcinoma cell lines (DLD-1, Colo-201, Caco-2, and HT-29) were purchased from American Type Culture Collection (Rockville, MD). HCT-116 and RKO colon carcinoma cell lines were the generous gift of Dr. M. Brattain, Baylor University, Houston, TX.

Reagents and Antibodies: TNF-α was made in house by Dr. P.F. Lin, Virology Department, Bristol-Myers Squibb, Wallingford, CT. Factor VIII and E-Selectin monoclonal antibodies were purchased from AMAC, Inc. (Westbrook, ME).

Transfections: E-Selectin cloned into the pCDM8 vector (the generous gift of Dr. A. Aruffo, Bristol Myers-Squibb, Seattle, WA; 6) was transfected into Huvecs using calcium phosphate-DNA transfections (19). Control cultures were mock transfected using the same protocol minus the E-Selectin cDNA. Transfection efficiencies were established using duplicate cultures transfected with either pRSV-Luciferase (20) or pCH110 containing the β-galactosidase gene driven by the SV-40 promoter (Pharmacia LKB Biotechnology, Piscataway, NJ).

FACS: Following transfections, endothelial monolayers were trypsinized, centrifuged and allowed to recover 4 h in a 37°C shaker. Cells were then incubated on ice for 1 h with primary antibodies, rinsed, centrifuged, and resuspended with FITC-conjugated secondary antibodies for 30 min on ice. FACS analysis was performed using the FACStar instrument (Becton Dickinson, Mountainview, CA)

Adhesion Assays: A variety of colon carcinoma cell lines were added to either TNF-α activated (100 U/ml, 4 h, 37°C) or E-Selectin transfected endothelial monolayers and allowed to incubate for 1 h, 37°C. Background binding was established using negative controls including quiescent, non-TNF-α treated endothelia or mock transfected endothelial cultures. Competition studies included preincubation of cancer cells with conditioned medium from either E-Selectin-IgG or mock transfected COS7 cells (the generous gift of Dr. A. Aruffo, Bristol Myers-Squibb, Seattle, WA; 21) for 1 h in a 37°C shaker prior to adding the tumor cells to endothelial monolayers for an additional hour. The cultures were washed, fixed in 10% formalin/2% glutaraldehyde for 1 h, RT, rinsed, and hematoxylin stained for 20 min. Percent inhibition of binding was determined by counting six random 200 X microscopic fields, obtaining the average, dividing this number by the value of the positive controls, and multiplying by 100 to express the value as a percentage.

Results and Discussion: The major threat of cancer is generally not the primary lesion; rather the danger lies in metastatic spread. Endothelial binding by cancerous

cells is an essential step during extravasation from the blood vessel and the establishment of malignant foci. E-Selectin has been implicated as the primary mediator of colon carcinoma cell adhesion to activated endothelia (17). With the majority of cancers classified as carcinomas, it is important to begin to decipher the role E-Selectin plays in tumor cell diapedesis during the metastatic process.

Confluent Huvec monolayers were incubated with a variety of colon carcinoma cell lines including Colo-201, DLD-2, HT29 and HCT-116 with similar results. Colo-201 cells used as a representative cell line bound to Huvecs stimulated 4 h with 100 U/ml TNF-α (Figure 1a), but did not bind to resting endothelia (Figure 1b). The E-Selectin binding specificity was established by preincubating the cancer cells in undiluted conditioned medium from COS7 cells transfected with an E-Selectin-IgG chimera (Figure 1c) which blocked tumor cell adherence to TNF-α stimulated endothelia. Conditioned medium from mock transfections (Figure 1d) did not inhibit tumor cell binding. Caco-2 cells were used as a negative control since, in our hands, they did not bind stimulated endothelial cells (Merwin and Basson, unpublished

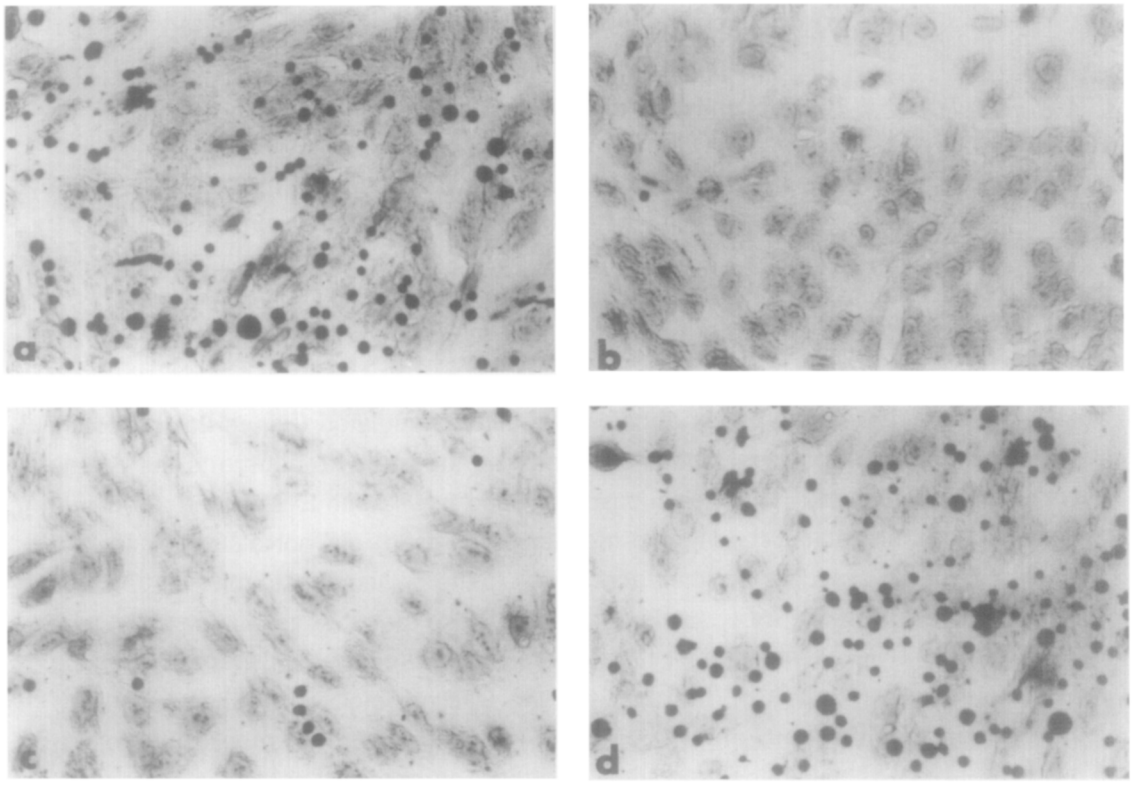

Figure 1: E-Selectin binding studies using Colo-201/Huvec co-cultures. Endothelia were co-cultured with Colo-201 cells under the following conditions. (a) TNF-α activated Huvecs. (b) Quiescent Huvecs. (c) TNF-α activated Huvecs co-cultured with Colo-201 cells preincubated with conditioned medium from E-Selectin-IgG transfected COS7 cells containing soluble E-Selectin-IgG antigen or (d) with conditioned medium from mock transfected cells. Magnification = 190 X.

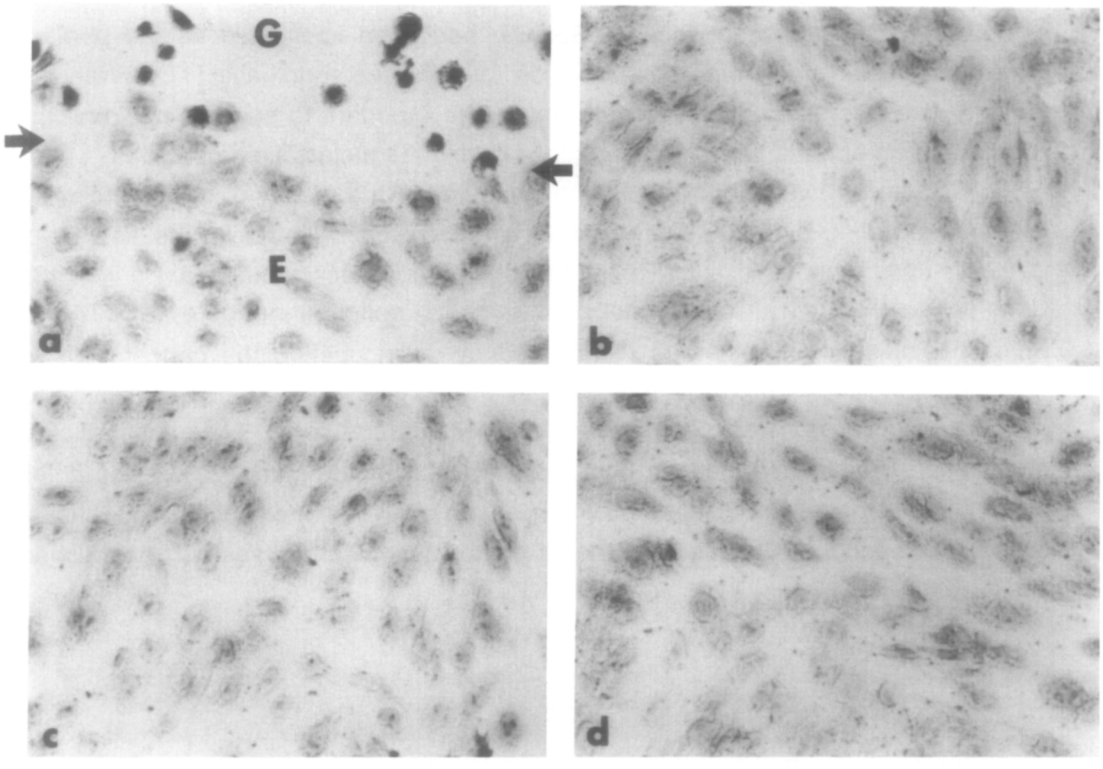

Figure 2: E-Selectin binding studies using Caco-2/Huvec co-cultures. Endothelia were co-cultured with Caco-2 cells under the following conditions. (a) TNF-α activated Huvecs. (b) Quiescent Huvecs. (c) TNF-α activated Huvecs co-cultured with Caco-2 cells preincubated with conditioned medium from E-Selectin-IgG transfected COS7 cells containing soluble E-Selectin-IgG antigen or (d) with conditioned medium from mock transfected cells. Caco-2 cells were used as a negative control as they did not bind endothelia; however, they did adhere to the substratum (a). G = gelatin; E = endothelial cells; Arrows = edge of cell monolayer. Magnification = 190 X.

observation). There was a lack of binding to either stimulated (Figure 2a) or resting (Figure 2b) Huvecs; however, these cells retained the ability to adhere to the gelatin substratum (Figure 2a). The preincubation of Caco-2 cells with E-Selectin-IgG (Figure 2c) or mock transfected conditioned medium (Figure 2d) did not alter their lack of binding to endothelial cells nor their attachment to the gelatin substratum. RKO colon carcinoma cells were an exception in our studies. Like the other carcinoma cells, they did adhere to TNF-α stimulated endothelial monolayers (Figure 3a) and not to resting endothelial cells (Figure 3b), but their binding was not blocked by incubation with conditioned medium from either E-Selectin-IgG (Figure 3c) or mock (Figure 3d) transfected COS7 cells. These data suggest, perhaps, a unique repertoire of adhesion molecules expressed on RKO cell surfaces.

The actual cell numbers bound to the endothelial monolayer varied from ~1000 HCT-116/cm^2 to ~400 Colo-201/cm^2 (Figure 4A) with no apparent correlation between cell number bound and differentiation state of the tumor cell lines (data not

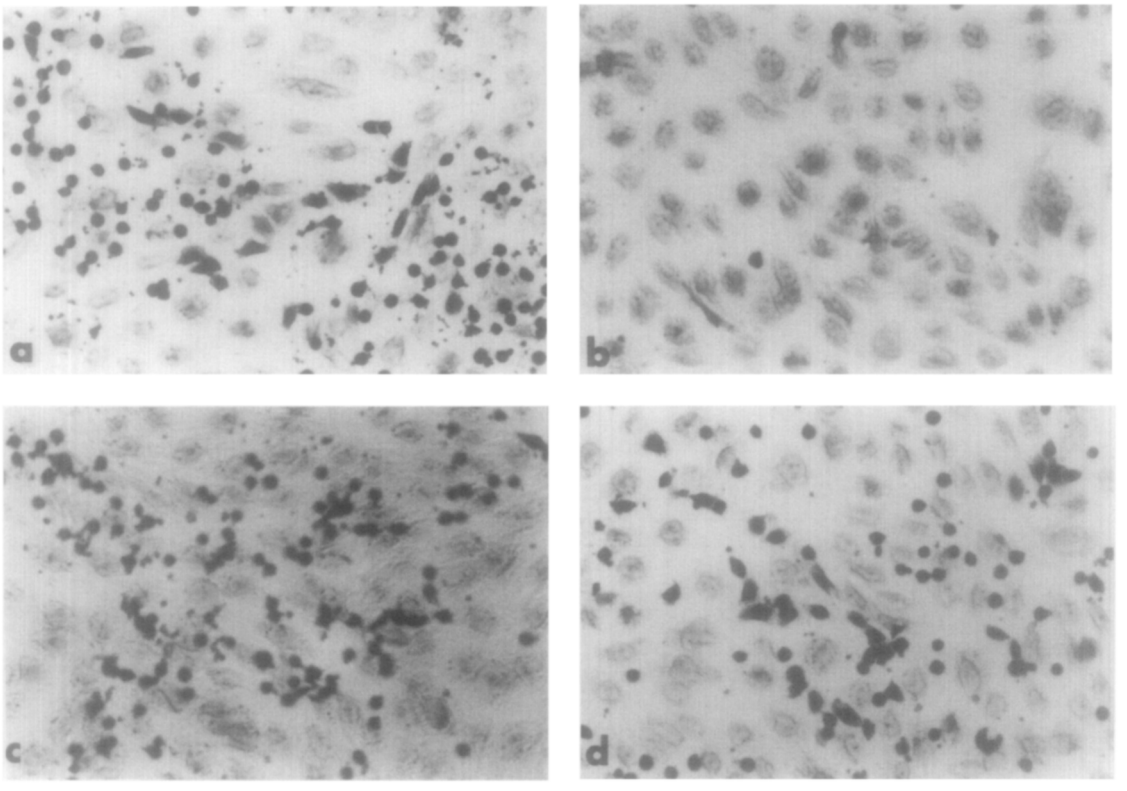

Figure 3: RKO colon carcinoma cell binding to endothelia. Endothelia were co-cultured with RKO cells under the following conditions. (a) TNF-α activated Huvecs. (b) Quiescent Huvecs. (c) TNF-α activated Huvecs co-cultured with RKO cells preincubated with conditioned medium from E-Selectin-IgG transfected COS7 cells containing soluble E-Selectin-IgG antigen or (d) with conditioned medium from mock transfected cells. Magnification = 190 X.

shown). Competition assays suggest colon carcinoma cell binding to activated Huvecs is E-Selectin dependent since soluble E-Selectin-IgG chimeric antigen inhibited binding 71 to 84% (Figure 4B). Previous reports of E-Selectin binding competition studies (22,23), have shown inhibition ranging from 30 to 50% in contrast to our findings of 70 to 80%. This may be due to the use of different cell types (leukocytes versus colon carcinoma cells) or the possibility that E-Selectin is the major mediator of colon carcinoma cell binding to activated endothelial cells (17) while leukocyte-endothelial cell adhesion may be mediated by several ligand pairs. The differences in binding profiles between colon carcinoma cell lines (our inability to compete off RKO binding with soluble E-Selectin-IgG antigen and Caco-2 cells which bound substratum but not endothelia) is consistent with the concept of tumor cell heterogeneity.

In order for cancer cells to bind to endothelial cells, the endothelia must first be stimulated to express specific adhesion molecules on their surface. The role of E-Selectin in cell adhesion has been studied *in vitro* by activating endothelial cells with

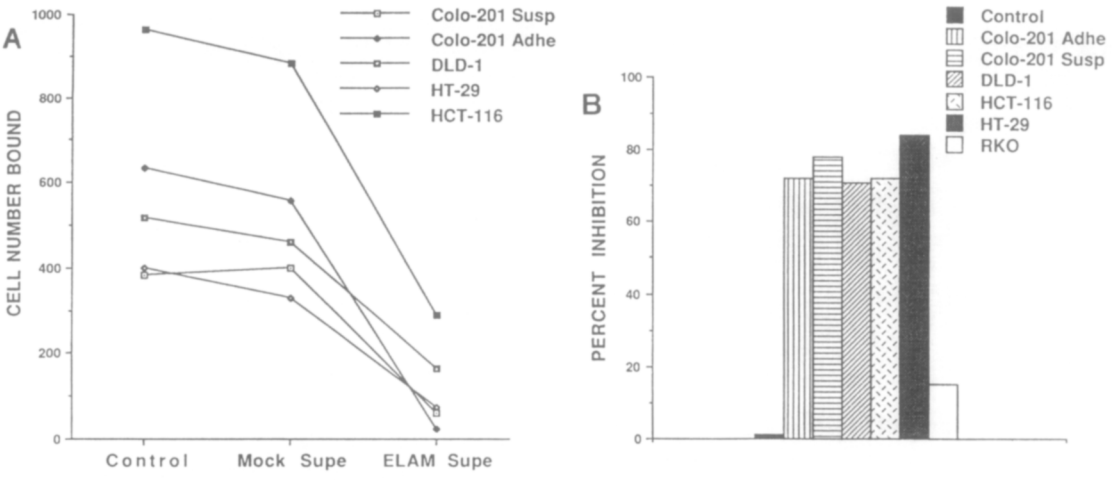

Figure 4: Binding profiles of colon carcinoma cells to Huvecs. (A) The actual number of cancer cells which bound to the Huvecs varied from ~400 Colo-201 cells/cm^2 to ~1000 HCT 116 cells/cm^2. Condition medium from E-Selectin-IgG transfected COS7 cells competed off binding, while conditioned medium from mock transfected cell did not. (B) Percent inhibition of cancer cell binding to activated endothelia is a result of preincubation with conditioned medium from E-Selectin-IgG transfected COS7 cells. Control cells had minimal initial binding and therefore, showed no inhibition. Statistical analyses showed a significant difference between endothelial cells binding untreated versus E-Selectin-IgG treated tumor cells ($p = < 0.05$). However, there was no significant difference between endothelia binding untreated tumor cells versus tumor cells treated with conditioned medium from mock transfected COS7 cells ($p = > 0.5$).

cytokines; in particular TNF-α. While TNF-α does, in fact, up regulate E-Selectin expression, it's presence also triggers the induction or modulation of a large number of proteins including a combination of several adhesion molecules (18). In order to elucidate the role that E-Selectin plays in the egression of tumor cells from the blood vessel, it is necessary to decrease the variables within the culture system. We have accomplished this by performing calcium phosphate-DNA mediated transient transfections using pCDM8-E-Selectin on endothelial cells. In this manner we were

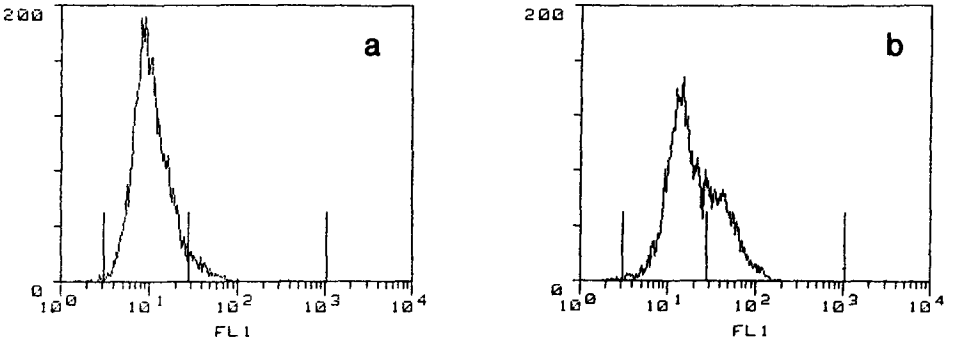

Figure 5: FACS analyses of E-Selectin transfected endothelial cells. Mock transfected Huvecs (a) gave a negative profile, while E-Selectin transfected cells (b) gave ~30% positive staining.

able to more readily distinguish the direct effects elicited by E-Selectin expression on endothelial cell surfaces and binding by tumor cells without the complication of cytokine activation. To determine and optimize the transfection efficiency, we used pCH110, a vector expressing β-galactosidase under the control of a SV-40 promoter, to obtain reproducible transfection efficiencies of between 30 to 50% (data not shown). To quantitate E-Selectin expression, FACS analyses were performed on transfected cultures. E-Selectin staining on mock transfected Huvec cultures was virtually nil (Figure 5a), while the transfection profile was ~30% positive (Figure 5b). As a positive

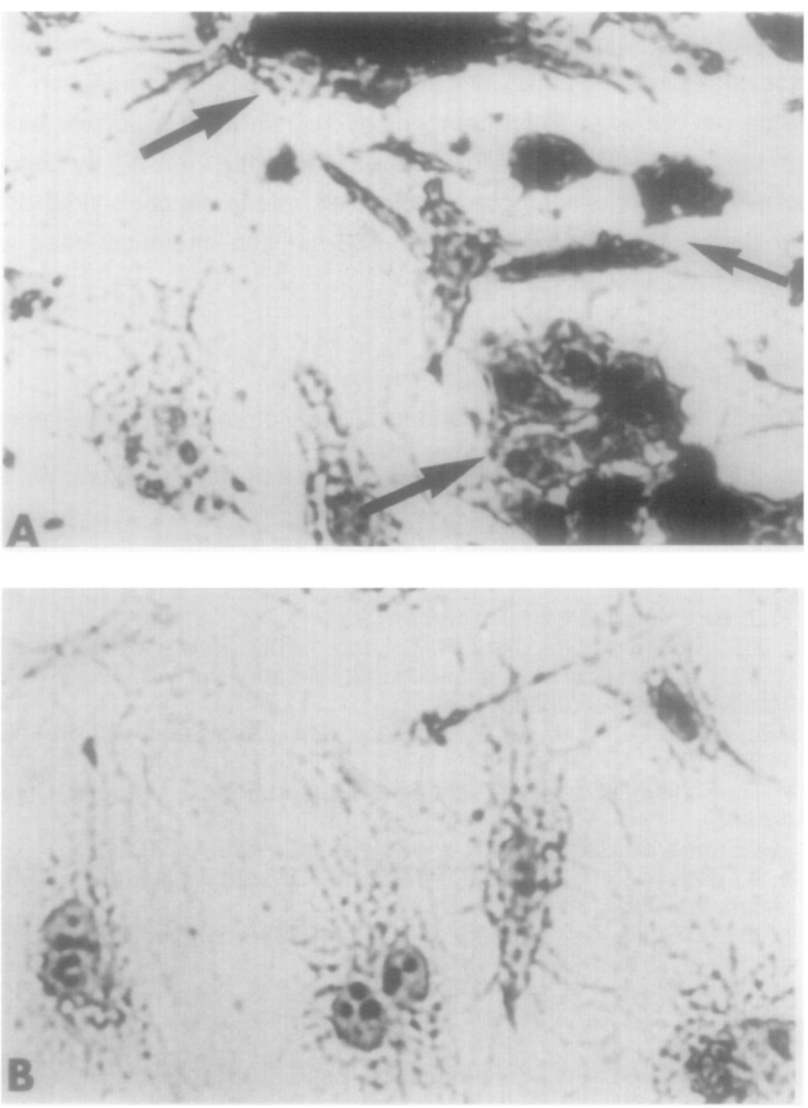

Figure 6: Colon carcinoma HCT-116 cells binding to E-Selectin transfected endothelia. Huvecs were transfected with either pCDM8-E-Selectin or mock cDNA. Following recovery, they were incubated for 1 hour with HCT-116 which bound to positive transfectents (A) but were unable to bind mock transfected endothelial cells (B). Arrows = tumor cells bound. Magnification = 200 X.

control, both mock and E-Selectin transfected endothelia revealed similar positive staining with anti-Factor VIII. Similar binding profiles were observed using E-Selectin transfected Huvecs (Figure 6) as seen with TNF-α activated endothelial cells (Figure 1). We also observed equivalent competition using the conditioned medium containing E-Selectin-IgG (data not shown).

In conclusion, we have shown that a variety of colon carcinoma cells adhere to the E-Selectin molecule residing on TNF-α stimulated endothelial surfaces. Employing transient transfection protocols to constitutively express E-Selectin, we were able to duplicate the binding studies while eliminating the significant changes in metabolic activity and up regulation of other adhesion molecules caused by cytokine activation. Parallel experiments using rat epididymal fat pad microvessel endothelial cells in place of Huvecs revealed similar results in binding, competition and FACS analyses (data not shown). This methodology will allow us to further examine the alterations in the endothelial cells following the binding of E-Selectin by tumor cells. It will also assist us in understanding this molecule's role in cancer cell egression from the vascular lumen, through the endothelial barrier into interstitial sites where the establishment of metastatic foci occur.

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