Roles of Cell Adhesion Molecules in Tumor Angiogenesis Induced by Cotransplantation of Cancer and Endothelial Cells to Nude Rats

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ABSTRACT

Roles of cell adhesion molecules mediating the interaction of cancer and endothelial cells in tumor angiogenesis were investigated using new in vitro and in vivo model systems with a cultured murine endothelial cell line (F-2) and human cultured epidermoid cancer cells (A431). The A431 cells exhibited typical in vitro cell adhesion to the endothelial F-2 cells. The initial step of adhesion was mediated by sialyl Lewis x (Le x) and sialyl Le a, the carbohydrate determinants expressed on the cancer cells, and E-selectin expressed constitutively on F-2 cells. Prolonged culture led to the implantation of cancer cells into the monolayer of the F-2 cells, which was mediated mainly by αvβ3-integrin. F-2 cells cultured on Matrigel showed evident tube formation, and coculture of F-2 cells with A431 cells led to the formation of A431 nests constantly surrounded by tube-like networks consisting of F-2 cells. This in vitro morphogenesis was inhibited by the addition of anti-sialyl Le x/Le a or anti-β3-integrin antibodies, which led to the formation of cancer cell aggregates that were independent from the F-2 cell networks. This in vitro morphological appearance was exactly reproduced in the in vivo tumors, which were formed when the mixture of A431 and F-2 cells at the ratio of 10:1 were cotransplanted s.c. into the back of nude rats. The tumors of A431 supplemented with F-2 cells were profoundly vascularized throughout by the tubular structures formed by F-2 cells, the lumen of which contained the host rat blood cells. The tumor mass thus formed was an average 5.8-fold as large as control A431 tumors that were grown without F-2 cells. The co-injection of anti-Le x/Le a or anti-β3-integrin antibodies produced a marked reduction in the size of A431 tumors, which were not vascularized and accompanied an independent tiny remnant clump of F-2 cells. The size of these A431 tumors did not differ significantly from those of control A431 tumors raised without F-2 cells. These results indicate that the interaction of tumor cells and endothelial cells in orderly tumor angiomorphogenesis is highly dependent on the action of cell adhesion molecules mediating the adhesion of cancer cells to endothelial cells, inhibition of which remarkably retards tumor growth and angiogenesis.

INTRODUCTION

Adhesion of cancer cells to vascular endothelial cells is initiated by the binding of E-selectin on endothelial cells to the carbohydrate ligands on cancer cells. The carbohydrate determinants, sialyl Le a and sialyl Le x on cancer cells, serve as ligands for E-selectin in the initial adhesion, followed by the secondary adhesion mediated by integrins (1–4). This adhesion has been suggested to be involved in hematogenous metastasis of cancer (1, 5–8). This hypothesis is further supported, although indirectly, by the results of several clinical statistical studies (9–13) indicating that patients with cancer cells that strongly express sialyl Le a and/or sialyl Le x are at high risk of developing hematogenous metastasis and have a significantly poorer postoperative prognosis than other patients.

The direct interaction of cancer cells with endothelial cells may have physiological relevence, however, not only in hematogenous metastasis of cancer, but also in tumor angiogenesis. Cell adhesion mediated by selectins and their carbohydrate ligands has recently been suggested to be involved in the process of angiogenesis by bovine endothelial cells and human umbilical vein endothelial cells (14, 15). These studies were confined to angiogenesis by endothelial cells per se, and the roles of cell adhesion molecules in the interaction of cancer cells and endothelial cells in tumor angiogenesis remained to be studied. Various mechanisms have been proposed for the formation of tumor vasculature, which would involve direct or indirect interaction of cancer cells and endothelial cells, including the interaction of cancer cells with circulating endothelial progenitor cells (16, 17), as well as so-called mosaic blood vessels (18–20). Nevertheless, only a few experimental systems are available to evaluate the interaction of cancer cells and endothelial cells (21, 22).

Considerable study on tumor angiogenesis has emphasized the need for humoral factors, such as vascular endothelial growth factor, basic fibroblast growth factor, and TGFβ, which are secreted from malignant cells and support survival, proliferation, and/or maturation of endothelial cells. The importance of these humoral factors is already well established, but the roles played by the cell adhesion molecules have not been properly evaluated because of a lack of appropriate experimental systems to estimate the adhesive interaction between cancer cells and endothelial cells in the context of tumor angiogenesis, with a few exceptions (21). The presence of actively proliferating and functional endothelial cells is essential for the study of tumor angiogenesis, whereas microvascular endothelial cells in cell culture generally have a limited life span. This could be the reason that the roles of humoral factors that support survival, proliferation, and/or maturation of endothelial cells have dominated the study of tumor angiogenesis, whereas the significance of cell adhesion molecules tends to remain in the background. To date, only a few endothelial cell lines are available that demonstrate enough of the fundamental characteristics of microvascular endothelial cells (21, 23, 24).

In the present study, we use a cultured endothelial cell line, F-2 (23), which does not need particular humoral factors for its survival and proliferation but retains expression of important cell adhesion molecules and an ability to exhibit significant tube formation. We first attempted to characterize in detail the adhesion molecules involved in the interaction of the endothelial cells with human cancer cells and then applied the F-2 cells for in vitro and in vivo studies of tumor angiogenesis. This experimental system was less affected by the humoral factors related to survival or proliferation of endothelial cells.
and served to disclose the inherent roles played by cell adhesion molecules in tumor angiogenesis.

MATERIALS AND METHODS

Cell Culture and Chemical Reagents. A murine endothelial cell line, F-2, was first established from UV-induced tumors in a BALB/c-C57BL/6-F1-nu/nu mouse, as described previously (23), and was maintained in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS. The human cultured epidermoid cancer cell line A431 (American Type Culture Collection, Rockville, MD) was maintained in DMEM supplemented with 10% FCS. Recombinant human IL-1β was kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). Human recombinant TNFα was obtained from Bachem Fine Chemicals Inc. (Torrance, CA), and TGFβ3 was from Biomedical Technologies Inc. (Stoughton, MA).

Antibodies and Flow Cytometric Analysis. Anti-sialyl Leα (SNH-3) was kindly supplied by Dr. Sen-Ichiro Hakomori, Pacific Northwest Research Foundation (Seattle, WA), and anti-sialyl Leα antibody (2D3; both murine IgM) was prepared as described previously (25). An antibody directed to CD29 (β2-subunit; 4B4) was purchased from Coulter Immunology (Hialeah, FL), and antibodies to CD49b (α2-subunit; G8), CD49c (α1-subunit; M-KIID2), CD49d (α2-subunit; HP2/1), and CD49f (α1-subunit; GoH3) were obtained from Immunotech (Marseille, France). Anti-αν-antibody (CD51; MAB1980) was from Chemicon International Inc. (Temecula, CA). Antimurine E-selectin antibody (10E9) was kindly provided by Dr. Dietmar Vestweber, Max-Plank-Institute für Immunobiologie (Freiburg, Germany; Ref. 26).

For coculture experiments, A431 and F-2 cells were mixed at the ratio of 2:5, plated on the Matrigel-coated plates, and cultured for 72 h. In some experiments, F-2 cells were cultured on Matrigel (13.6 mg/ml; Collaborative Biomedical Products, Bedford, MA) to form tube-like structures. For coculture experiments, A431 and F-2 cells were mixed at the ratio of 2:5, plated on the Matrigel-coated plates, and cultured for 72 h. For in vivo tumor formation, A431 cells (5 × 10^6) were injected i.c. into the backs of F344 nude rats with or without 5 × 10^3 cells of the murine endothelial cell line F-2. Rats were sacrificed 21–25 days after transplanation of cells, and the harvested tumors were subjected to histological and immunohistochemical analyses. For inhibition studies, a mixture of anti-sialyl Leα and anti-sialyl Leβ antibodies, or anti-β3-integrin antibody (25 μg/ml), was injected into the backs of nude rats.

RESULTS

Adhesion and Implantation of Human Cancer Cells to the Monolayer of F-2 Cells. Human cultured epidermoid cancer cells (A431) underwent typical cell adhesion to cultured murine endothelial cells (F-2) in vitro as shown in Fig. 1. This adhesion was observed equally well either at 4°C or 37°C and was not affected by rotating the incubation plates at 80 rpm, indicating that the initial adhesion was shear force-resistant and energy-independent. Adhesion reached a plateau 30 min after the addition of the cancer cells to the F-2 cell monolayers (Fig. 1b).

When the plates were incubated longer, the cancer cells began to infiltrate the monolayer of F-2 cells (schematically shown in Fig. 1a). The implantation process was clearly detectable at 3 h after addition of the A431 cells and was almost complete within 8 h (Fig. 1c). This secondary implantation process was observed at 37°C, but was significantly attenuated at or below room temperature. Rotation of the culture plate also significantly inhibited the implantation, indicating that the secondary implantation was shear force-labile and energy-dependent.

Expression of Selectin Ligands and Integrins on Cancer Cells. To characterize the adhesion molecules involved in the initial adhesion and implantation, we analyzed the cell adhesion molecules expressed on A431 cells. A431 cells moderately expressed sialyl Leα and sialyl Leβ, the established ligands for selectins (Fig. 2a). As for integrins, A431 cells strongly expressed β1, αα, αα, and αν and

follows (31). A 24-well plate was coated with 5 μg/ml collagen I (Seikagaku Kogyo, Tokyo, Japan) or 20 μg/ml laminin (Takara Shuzo, Otsu, Japan) at 4°C overnight, and the wells were washed three times with PBS. Unbound surfaces were blocked with 0.5% BSA in PBS for 1 h, and the wells were then washed three times with PBS. A431 cells (5 × 10^5) were added at a volume of 500 μl/well to each substrate-coated well and then incubated for 30 min at 37°C. The wells were then washed three times with PBS to remove unattached cells. The number of attached cells was counted directly under a microscope.

Tube Formation of F-2 Cells and in Vivo Tumor Formation in Nude Rats. In some experiments, F-2 cells were cultured on Matrigel (13.6 mg/ml; Collaborative Biomedical Products, Bedford, MA) to form tube-like structures. For coculture experiments, A431 and F-2 cells were mixed at the ratio of 2:5, plated on the Matrigel-coated plates, and cultured for 72 h. For in vivo tumor formation, A431 cells (5 × 10^6) were injected i.c. into the backs of F344 nude rats with or without 5 × 10^3 cells of the murine endothelial cell line F-2. Rats were sacrificed 21–25 days after transplanation of cells, and the harvested tumors were subjected to histological and immunohistochemical analyses. For inhibition studies, a mixture of anti-sialyl Leα and anti-sialyl Leβ antibodies, or anti-β3-integrin antibody (25 μg/ml), was injected into the backs of nude rats.

Fig. 1. Interaction of A431 cell with the monolayer of cultured murine endothelial cells, F-2. a, schematic illustration of interaction of A431 cells with F-2 cells. b, phase-contrast microscopic appearance 30 min after the addition of A431 cells to the F-2 monolayers, indicating significant adhesion of A431 cells (magnification, ×200). c, 8 h after the addition of A431 cells to the F-2 monolayers indicating implantation (magnification, ×200).
HB-EGF and genistein on the expression of integrins. When A431 cells were cultured in the presence of cytokines, it is acknowledged to share its receptors for EGF and that they are closely involved in the growth of cancers. Because the aim of the present series of experiments was to investigate the interaction of cancer cells and endothelial cells, we studied the effect of cytokine HB-EGF, which is known to be pro-angiogenic. When A431 cells were cultured under typical culture conditions and various stimulations on the expression of E-selectin on F-2 cells. F-2 cells under routine culture conditions significantly expressed murine E-selectin, and its expression was modestly enhanced to 123 ± 18, 116 ± 17, and 110 ± 27% by the addition of IL-1β, TNFα, and LPS, respectively, in terms of fluorescence intensity (Fig. 3b). Addition of a mixture of IL-1β, TNFα, and LPS enhanced the expression of E-selectin up to 147 ± 11%, whereas expression was inhibited to 79 ± 23% by the addition of TGFβ (Fig. 3b). The expression of E-selectin on F-2 cells was also affected by the growth condition of the cells. Proliferating F-2 cells in sparse or subconfluent culture expressed E-selectin more strongly, and its expression in the confluent state decreased to 42.6% of that in the subconfluent state (Fig. 3b). When the F-2 cells were cultured on Matrigel, they formed tube-like structures (see below) and still expressed murine E-selectin at a level similar to that expressed by the cells in the monolayer (Fig. 3b). These results indicated that the expression of E-selectin on F-2 cells was generally stable and that it may well interact with its ligands when the cells form tube-like structures as well as in monolayers. Moreover, cells in the proliferative stage will interact more closely with the cells expressing appropriate selectin ligands.

Analysis of Initial Step of Cancer Cell Adhesion to F-2 Cell Monolayer. The adhesion of A431 cells to the F-2 monolayers was only marginally inhibited by the addition of anti-sialyl Leα or anti-sialyl Le antigen to the incubation medium, but the mixture of both antibodies inhibited adhesion completely (Fig. 4a). This indicated that both sialyl Leα and sialyl Leα were involved in the adhesion of A431 cells to F-2 cells. Anti-integrin antibodies, including anti-β1, α2, α3, α6, and αv, covering the major integrins on the A431 cells did not affect the initial adhesion step of A431 cells to F-2 cells.

Analysis of Secondary Implantation Step of Cancer Cells to F-2 Cell Monolayer. In contrast, the secondary implantation of A431 cells to the F-2 cell monolayer was not affected by the anti-sialyl Leα and anti-sialyl Leα antibodies (Fig. 4a). Whereas it was completely inhibited by anti-β1-integrin antibody (Fig. 4a). The anti-β1-integrin antibody used in this study was directed to human β1-integrin and had no cross-reactivity to murine β1-integrin, indicating that the β1-integrin involved in the implantation process was expressed on the side of human cancer cells. The implantation of A431 cells was also modulated by the addition of cytokines and the density of the F-2 cells.

Fig. 2. Expression of carbohydrate determinants and integrins on A431 cells as analyzed by flow cytometry. a, results of flow cytometric analysis of expression of sialyl Leα, sialyl Leα, and integrins on A431 cells under typical culture conditions. b, effect of HB-EGF and genistein on the expression of α3-integrin on A431 cells. c, effect of HB-EGF and genistein on the expression of other integrins; α2-integrin by HB-EGF on A431 cells. d, time course of induction of α3-integrin by HB-EGF on A431 cells. Mean fluorescence intensity.

Fig. 3. Expression of endogenous selectin and laminin on cultured murine endothelial F-2 cells as analyzed by flow cytometry. a, results of flow cytometric analysis of expression of murine E- and P-selectins and laminin on F-2 cells under typical culture conditions. b, effect of various cytokines, cell density, or tube formation on the expression of murine E-selectin on F-2 cells. Tube formation of F-2 cells was induced by culturing the cells on Matrigel. For experimental details, see “Materials and Methods.”
completely inhibited by murine antihuman α3-integrin antibody (Fig. 4a), suggesting that the molecular species of the integrin involved in the process was mainly α3β1-integrin. The participation of integrin in the implantation step was compatible with the temperature- and energy-dependent characteristics of the reaction. A431 cells exhibited significant binding to laminin and collagen, but not to fibronectin. The binding activity of α3β1-integrin expressed on A431 cells was ascertained by the significant inhibition of binding of A431 cells to laminin by anti-α3-integrin antibody as well as by anti-β1-integrin antibody, as shown in Fig. 4b. In contrast, the binding of A431 cells to collagen was mediated mainly by α2-integrin.

In the analysis of secondary implantation step of cancer cells to the F-2 cell monolayer, the 24-well plate was first centrifuged with F-2 cell monolayers at 300 × g, immediately after the addition of the A431 cancer cells. This protocol was adopted to evaluate only the activities of the molecules involved in the process of implantation, not in the initial step of adhesion. Centrifugation of the plates ensured the close contact of cancer cells with the F-2 cell monolayers from the beginning of the incubation period, excluding any possible effects on the experimental results of the first step of cell adhesion. This may be the reason that the anti-sialyl Lea and anti-sialyl Lea antibodies had no effect on the implantation of the cancer cells in these assays. The overall interactions of cancer cells with the F-2 cells in vivo and in vitro would be expected to require the normal progression of these two sequential steps, i.e., initial adhesion and secondary implantation.

In Vitro Cooperation of Cancer Cells and Endothelial Cells. F-2 cells formed tube-like network structures when cultured on Matrigel, as shown in Fig. 5a. When A431 cells were cocultured with F-2 cells on Matrigel in vitro, tube formation of F-2 cells was invariably
We attempted to reproduce this phenomenon observed, which surrounded small aggregates of A431 cells in an orderly fashion (Fig. 5b). Prolonged culture led to the growth of cancer cell nests formed by A431 cells, which were clearly demarcated by the tubular networks of F-2 cells (Fig. 5c). The morphology, on the whole, closely resembled that of natural cancer tissues, where the cancer nests are surrounded by stroma tissues containing small blood vessels. Inclusion of the mixture of anti-sialyl Le<sup>a</sup>/Le<sup>a</sup> antibodies or anti-β<sub>1</sub>-integrin antibody in the culture medium inhibited the interaction of A431 cells with F-2 cells and led to the accumulation of A431 cell aggregates, which were formed independently from the meshwork of F-2 cells (Fig. 5, d and e).

### Effects of Co-injection of F-2 Cells on Growth of A431 Tumors in Vivo
We attempted to reproduce this phenomenon in vivo and injected nude rats with the mixture of A431 and F-2 cells. Preliminary experiments indicated that the mixture of these cells at a ratio of 10:1 yielded the best results. Injection of F-2 cells at a concentration <2 × 10<sup>6</sup> cells did not induce any tumors in nude rats when injected without A431 cells, whereas at this number they well supported and promoted the tumor formation of A431 cells when co-injected with A431 cells. Therefore, the protocol incorporating 5 × 10<sup>6</sup> A431 cells and 5 × 10<sup>5</sup> F-2 cells was adopted for the experiments described below.

The growth of A431 tumors supplemented with F-2 cells was much more rapid than the growth of tumors formed by the A431 cells only, and the difference was statistically significant (typical results are shown in Table 1). On average, A431 tumors ~5.8-fold larger than control were formed by the co-injection of F-2 cells (Table 1 and Fig. 6). By microscopic observation, the A431 tumors thus formed by co-injection of F-2 cells were characterized by vigorous vascularization, in which blood vessel-like structures developed in almost every cancer cell nest (Fig. 6) to form small, medium-sized, and eventually large blood vessel-like structures (Fig. 7, a–c). This closely resembled the configuration observed in the in vitro coculture of A431 cells with F-2 cells on Matrigel, as indicated in Fig. 5c. The tumors formed by A431 cells alone were very small and contained almost negligible vascularization of host origin (Fig. 6b). These control tumors also exhibited a strong tendency toward parakeratotic degeneration (Fig. 6b). Only cancer cells in the outermost thin layers just beneath the capsule were viable; the rest of the central tumor area contained degenerated eosinophilic materials (Fig. 6b).

When analyzed using specific antirat, antihuman, and antimouse antibodies, the stroma and blood vessel-like structures in the A431 tumor co-injected with F-2 cells were confirmed to be almost

### Table 1. Acceleration of in vivo growth of A431 tumors by cotransplantation with F-2 cells, and the effects of antibodies directed to cell adhesion molecules

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Treatment</th>
<th>Tumor weight (g)</th>
</tr>
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<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A431 only</td>
<td></td>
<td>0.22 ± 0.16 (&lt;i&gt;n&lt;/i&gt; = 5)</td>
</tr>
<tr>
<td>A431 + F-2</td>
<td></td>
<td>1.28 ± 0.77 (&lt;i&gt;n&lt;/i&gt; = 5*)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A431 + F-2</td>
<td>No treatment</td>
<td>0.89 ± 0.26 (&lt;i&gt;n&lt;/i&gt; = 13)</td>
</tr>
<tr>
<td>A431 + F-2</td>
<td>Anti-sialyl Le&lt;sup&gt;a&lt;/sup&gt; and anti-sialyl Le&lt;sup&gt;a&lt;/sup&gt; Ab&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.25 ± 0.16 (&lt;i&gt;n&lt;/i&gt; = 9&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>A431 + F-2</td>
<td>Control IgM</td>
<td>0.65 ± 0.17 (&lt;i&gt;n&lt;/i&gt; = 6)</td>
</tr>
<tr>
<td>A431 + F-2</td>
<td>Anti-β&lt;sub&gt;1&lt;/sub&gt;-integrin Ab</td>
<td>0.25 ± 0.13 (&lt;i&gt;n&lt;/i&gt; = 7&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>A431 + F-2</td>
<td>Control IgG</td>
<td>0.72 ± 0.22 (&lt;i&gt;n&lt;/i&gt; = 4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Compared with controls: <sup>a</sup> <i>P</i> < 0.05; <sup>b</sup> <i>P</i> < 0.0001.

<sup>c</sup> Ab, antibodies.

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Fig. 6. Gross appearance of in vivo tumors formed in nude rats by transplantation of A431 cells with or without F-2 cell supplement. a, a typical tumor formed 21 days after co-injection of A431 cells (5 × 10<sup>6</sup> cells) and F-2 cells (5 × 10<sup>5</sup> cells). A scheme showing the distribution of tissue components is attached. b, typical control tumor formed 21 days after the injection of A431 cells (5 × 10<sup>6</sup> cells) without F-2 cells. Formalin-fixed paraffin sections 7-μm thick were prepared for histological examination from tumor specimens obtained from nude rats and stained with H&E. Areas contained viable cancer cells, keratinoid degeneration, connective tissues, and blood vessel-like structures formed by F-2 cells were identified based on microscopic observation. Bars, 2 mm.
exclusively of murine origin, indicating that they were formed by murine F-2 cells (Fig. 7e). Only the small mesenchymal components in the stroma and outermost capsules were found to be of rat origin, whereas all of the cancer cells were of human origin (Fig. 7f–h). The blood vessel-like structures contained WBCs stained significantly with antirat LFA-1 antibody (Fig. 7d), indicating that the blood vessels were somewhere connected to the systemic circulation of the host nude rats.

These results indicated that the co-injection of the murine endothelial cell line led to the vigorous vascularization of A431 tumors, which encouraged them to grow much faster than control tumors transplanted without F-2 cells.

Significance of Cell Adhesion Molecules in the in Vivo Growth of A431 Tumors. The accelerative effect of F-2 cell co-injection for tumor growth was abrogated by concomitant administration of the mixtures of anti-sialyl Le$^a$/Le$^a$ antibodies or anti-$\beta_1$-integrin antibody (typical experimental results are summarized in Table 1). The sizes of A431 tumors formed in the presence of these antibodies were in the same range as those of tumors raised without F-2 cell co-injection (Table 1). The A431 tumors formed in the presence of these antibod-
DISCUSSION

The murine endothelial cell line F-2 expressed E-selectin and adhered to human cancer cells that expressed sialyl Le\(^\text{a}\) and sialyl Le\(^\text{a}\), the specific carbohydrate ligands for selectin. The adhesion of human cancer cells to F-2 cells was mediated initially by the interaction of E-selectin with sialyl Le\(^\text{a}\) and sialyl Le\(^\text{a}\), and the subsequent implantation was mediated by \(\beta_1\)-integrins. The integrin species mainly involved in the implantation of A431 cells to F-2 cell monolayers was identified as \(\alpha_\text{v}\beta_3\)-integrin, which is known to react with several ligands, including laminin, collagen, and fibronectin (35, 36). The counter receptor on F-2 cells for \(\alpha_\text{v}\beta_3\)-integrin is most probably laminin because it is expressed on F-2 cells and clear inhibition of binding of A431 cells to laminin was obtained by anti-\(\alpha_\text{v}\)-integrin antibody.

Sialyl Le\(^\text{a}\) and sialyl Le\(^\text{a}\) were constitutively expressed on A431 cells, whereas expression of E-selectin on endothelial F-2 cells was affected by cytokines and proliferative states of the cells. The characteristics of the F-2 cells used in this study were in several aspects different from those of cultured human endothelial cells. First, expression of E-selectin on F-2 cells was less affected by treatment with cytokines, such as IL-1\(\beta\) or TNF\(\alpha\), compared with that on cultured human endothelial cells, such as human umbilical vein endothelial cells. Second, the F-2 cells constitutively expressed E-selectin, whereas human endothelial cells usually express virtually no E-selectin under normal culture conditions. F-2 cells in the proliferative stage are thought to more strongly interact with cancer cells expressing selectin ligands. In these aspects, the F-2 cells are very similar to bovine endothelial cells (14). Preferential expression of E-selectin on proliferating endothelial cells in tissues in which growth of microvessels is ongoing has been reported in several human tissues, such as dividing microvascular endothelial cells in placenta and neonatal foreskin (37).

It is noteworthy that HB-EGF, which is produced by endothelial cells (33, 34), augmented the expression of \(\alpha_\text{v}\beta_3\)-integrin on A431 cells. This enhancing effect was abrogated by the inhibitor of the EGF receptor kinase, genistein. Various investigators, including some of the authors of the present report, have shown that growth stimulation by EGF or HB-EGF treatments significantly augments integrin expression on cancer cells (38–40). This suggests preferential enhancement of integrin expression, including \(\alpha_\text{v}\)-chains, on the subpopulation of growth-stimulated cancer cells. Taken together, these findings suggest that the cancer cells and F-2 cells, both in the proliferative stage, preferentially interact and undergo cell adhesion and implantation.

A unique characteristic of F-2 cells is their ability to form three-dimensional tubular network structures when cultured on Matrigel-coated plates (23). Selectin-mediated adhesion and integrin-mediated implantation of cancer cells were consistently observed with cultured endothelial cells either in monolayer or in the form of tubular network structures. Several investigators have suggested the possible involvement of selectin-carbohydrate interactions in the \textit{in vitro} tube formation of cultured endothelial cells (14, 41). The introduction of anti-E-selectin or anti-sialyl Le\(^\text{a}\)/Le\(^\text{a}\) antibodies into F-2 cells failed to inhibit Matrigel-induced tube formation, even after the transfection of E-selectin or glycosyltransferase cDNA to further augment the expression of selectin and its ligands (data not shown). Together with the findings on the apparent normal development of blood vessels in mice with disrupted selectin genes (42, 43), this would indicate the presence of selectin-dependent and -independent pathways in the tube formation of cultured endothelial cells and the highly cell context-dependent role of cell adhesion molecules in angiogenesis. However, angiogenesis sometimes depends on the interaction of endothelial cells with other types of cells, and the roles of cell adhesion molecules in such interaction remain to be studied. It is noteworthy that the \textit{in vitro} angiogenesis of bovine aortic endothelial cells induced by polymorphonuclear leukocytes requires adhesion of leukocytes to endothelial cells through E-selectin and integrin/intercellular adhesion molecule-1 interaction (44, 45). When added to the coculture of F-2 cells with A431 cells, the anti-sialyl Le\(^\text{a}/\text{Le}\(^\text{a}\) antibodies as well as anti-\(\beta_1\)-integrin antibody significantly inhibited the interaction of endothelial cells with cancer cells. The orderly formation of cancer cell nests surrounded by functional vascular networks of F-2 cells was almost completely inhibited by these antibodies both \textit{in vitro} and \textit{in vivo}. Our results indicated that the interaction of cancer cells with endothelial cells through adhesion molecules such as selectins and integrins is critical for generation of functional vascular networks nourishing cancer cell nests and promoting \textit{in vivo} growth of tumors. The novel \textit{in vitro} and \textit{in vivo} model experimental systems described here offer a unique opportunity to study direct or indirect interaction between cancer cells and endothelial cells together with the outcome.

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