Evaluation of the Balance between Angiogenic and Antiangiogenic Circulating Factors in Patients with Breast and Gastrointestinal Cancers

Daniele Morelli, Debora Lazzerini, Sara Cazzaniga, Paolo Squicciarini, Paola Bignami, Jeanette A. M. Maier, Lucia Sfondrini, Sylvie Ménard, Maria I. Colnaghi, and Andrea Balsari

Divisions of Experimental Oncology E [D. M., D. L., S. C., L. S., S. M., M. I. C.], General Surgical C [P. S.], and General Surgical A [P. B.], Istituto Nazionale Tumori; Ospedale San Raffaele [J. A. M. M.]; and Institute of Pathology, University of Milan [A. B.], 20133 Milan, Italy

ABSTRACT

Angiogenesis is a critical determinant of tumor growth. Tumor cells produce or induce angiogenic molecules that act specifically on endothelial cells (ECs) but also release angiostatic molecules. Thus, tumor angiogenesis represents a net balance between positive and negative regulators of neovascularization.

Sera from patients with breast or gastrointestinal cancers were evaluated for their capacity to selectively modulate the proliferation of human umbilical vein ECs; sera from 15 of 78 (19%) breast cancer patients and 8 of 53 (15%) gastrointestinal cancer patients induced human umbilical vein EC growth, whereas sera from 4 of 78 (5%) breast cancer patients and 1 of 53 (2%) gastrointestinal cancer patients inhibited EC proliferation. Growth-stimulatory sera were significantly more frequent among postmenopausal (14 of 53) than premenopausal (1 of 25) breast cancer patients; inhibitory activity was observed in 3 of 25 premenopausal patients versus 1 of 53 postmenopausal individuals. The half-life of serum-stimulating and -inhibiting factors seemed to differ, because stimulatory activity but not inhibitory activity was decreased at 5 days after surgery. The levels of vascular endothelial growth factor were elevated in about 45% of patients with growth-stimulatory sera, whereas the serum inhibition of EC growth was found to be due, at least in part, to high levels of soluble thrombospondin.

INTRODUCTION

Angiogenesis is an essential step in tumor growth and metastasis (1, 2). Recent studies in breast cancer and other tumor types have shown that quantitation of angiogenesis can serve as an independent prognostic factor (3–5). Angiogenesis quantified based on staining ECs with antibodies to factor VIII-associated antigens (6) or to CD31 (7) showed that increased vascular density in the tumor was associated with poor prognosis. To stimulate angiogenesis, tumors up-regulate the production of a variety of angiogenic factors, including FGF-1, FGF-2, and VEGF (8). However, many malignant tumors also generate inhibitors of angiogenesis, including TSP (9), angioostatin (10), and endostatin (11). It is becoming clear that angiogenesis is the result of a net balance between these positive and negative regulators of neovascularization.

These factors act in a paracrine or autocrine fashion in the interaction between endothelial cells and tumor cells in situ, and there is increasing evidence for an endocrine function of these regulators based on the detection of elevated levels of angiogenic factors such as FGF-2, transforming growth factor α, hepatocyte growth factor, and VEGF in the sera of patients with gastric, renal, lung, or breast cancer (12, 13). FGF-2 has also been detected in urine from cancer patients (14) and children with acute lymphoblastic leukemia (2). Circulating angiogenesis inhibitors have also been documented (15), which, if produced by the primary tumor and secreted in the blood, can suppress angiogenesis and the formation of distant metastases. In that case, removal of the primary tumor releases the angiogenesis blockade, allowing the formation of new capillaries and rapid metastatic spread (14). Together, the evidence points to the potential value of assessing angiogenic activity in the sera of cancer patients in prognosis and in follow-up of response to anticancer therapy.

To evaluate the balance between angiogenic and antiangiogenic factors, we tested the in vitro effect of sera from patients with breast or gastrointestinal cancers on the proliferation of HUVECs. Several sera stimulated the growth of these cells, and, in some cases, this stimulation correlated with elevated concentrations of soluble VEGF. Sera from a few patients exhibited an inhibitory effect on HUVEC proliferation that was due, at least in part, to high levels of soluble TSP.

MATERIALS AND METHODS

Patients. Ninety-five individuals (32 males and 63 females) with no evidence of diseases, 131 malignant cancer patients from 15 of 78 (19%) breast cancer patients and 8 of 53 (5%) gastrointestinal cancer patients, were evaluated for their capacity to selectively modulate the proliferation of human umbilical vein ECs; sera

Received 7/21/97; revised 1/21/98; accepted 2/3/98.

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1 Partially supported by an Associazione Italiana Ricerca sul Cancro/Federazione Italiano Ricerse sul Cancro grant.

2 To whom requests for reprints should be addressed, c/o Division of Experimental Oncology E, Istituto Nazionale Tumori; Ospedale San Raffaele, 20133 Milan, Italy. Phone: 39-2-2390566; Fax: 39-2-2362692.

3 The abbreviations used are: EC, endothelial cell; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; TSP, thrombospondin.
patients, and 14 patients with nonmalignant cancer were enrolled in this study. In the malignant cancer group, 78 patients had breast carcinoma (38% stage 1, 51% stage 2, and 11% stage 3), 10 patients had gastric cancer, and 43 patients had intestinal carcinoma (18% Dukes’ A, 36% Dukes’ B, and 46% Dukes’ C). The 14 patients with nonmalignant cancer had a fibroadenoma or a phyllole tumor.

Serum Samples. Venous blood samples were drawn into tubes and centrifuged at 2000 rpm for 20 min; serum was stored in aliquots at -20°C. The study was approved by a medical ethics committee, and informed consent to take the blood samples was obtained from all of the patients. Healthy volunteers gave an oral statement of permission.

Cell Culture. HUVECs were derived from the umbilical cords of neonates at delivery and used up to passage 4. HUVECs were grown in Medium 199 with 10% FCS, 10 units/ml heparin, 10 μg/ml EC growth factor (Boehringer, Moheza, Italy), and antibiotics (Sigma, St. Louis, MO) on gelatin (Sigma)-coated plastic. A431, MeWo, and SKBr3 cell lines were obtained from the American Type Culture Collection and maintained in RPMI 1640 with L-glutamine supplemented with 10% FCS and antibiotics. Peripheral blood lymphocytes were obtained from the heparinized blood of normal donors by fractionation on Ficoll-Hypaque (Pharmacia). Lymphocytes were then stimulated for 2 days with phytohemagglutinin (Life Technologies, Inc.) and subsequently grown in RPMI 1640 supplemented with 10% human serum and interleukin 2 (30 units/ml).

Proliferation Assay. Cells were seeded at a density of 5 x 10⁴ cells/well in medium supplemented with 1% FCS on gelatin-coated 96-well culture plates (Iwaki; Bibby Sterilin, Staffordshire, United Kingdom) and incubated overnight at 37°C. Sera from healthy donors or patients were added to cells at a 6% final concentration. After 3 days, the cells were stained with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) for 4 h and evaluated spectrophotometrically at 550 nm. Experiments were performed in triplicate, and the variation was less than 10%. To evaluate the test reproducibility, HUVEC cultures were repeated 4 times in the presence of 10 selected patient sera (3 with stimulatory activity, 4 with proliferative activity in the range of healthy donor sera, and 3 with inhibitory activity). In all of the repeats, the selected sera maintained their original activity. To inhibit the activity of selected sera, they were preincubated with 5 μg/ml murine antihuman VEGF monoclonal antibody (Sigma) or 300 μg/ml rabbit antihuman TSP antibody (ammonium-precipitated immunoglobulin fraction) kindly provided by Dr. G. Taraboletti (Mario Negri, Bergamo, Italy).

VEGF ELISA. Wells of 96-well microtiter plates (Iwaki; Bibby Sterling) were coated with 10 μg/ml rabbit antihuman VEGF polyclonal antibody (Cabru, Peregallo di Lesmo, Italy) in 0.1 m NaCl and 0.025 m carbonate buffer (pH 9.0), incubated overnight at 4°C, and further coated with 1% BSA, 0.2 m carbonate buffer (pH 9.0), and 0.1 m NaCl for 3 h at 25°C.
cancer patients induced HUVEC growth, and sera from 4 of breast cancer patients and 8 of 53 (15%) gastrointestinal differences in the distribution were observed when normal eratim activity determined by the sera of healthy donors proliferation of HUVECs using a colonimetric assay. Prolif-

bated overnight with $100^{-\circ}$ boiling in sample buffer with reducing agents, separated by twice with PBS containing 0.5 reaction was developed with K-Blue substrate (Oxford Biomed-

ical Research, Inc., Oxford, MI) and blocked with 1 0.1% BSA) were added to each well and incubated for 1 h at 25°C. Wells were washed again and incubated with 100 μl of antimonous IgG (Fc-specific) peroxidase conjugate (Sigma). The reaction was developed with K-Blue substrate (Oxford Biomedical Research, Inc., Oxford, MI) and blocked with 1 m H$_2$SO$_4$.

**Western Blot Analysis.** Human sera (1 ml) were incubated overnight with 100 μl of heparin-Sepharose (Pharmacia). Heparin-Sepharose was recovered by centrifugation and washed twice with PBS containing 0.5 m NaCl. Protein was eluted by boiling in sample buffer with reducing agents, separated by 7.5% PAGE, transferred to Hybond-C nitrocellulose membranes (Amersham, Little Chalfont, United Kingdom), and incubated with 40 μg/ml rabbit antihuman TSP antibody, followed by antirabbit immunoglobulin horseradish peroxidase-linked antibodies (1:10000; Amersham). Visualization was by the enhanced chemiluminescence detection system (Amersham) according to the supplier’s instructions.

**RESULTS**

**The Effect of Sera on HUVEC Proliferation in Vitro.** Fig. 1 shows the mitogenic activity of sera from healthy donors, from patients with benign tumors, and from breast and gastrointestinal cancer patients as detected by in vitro proliferation of HUVECs using a colorimetric assay. Proliferation activity determined by the sera of healthy donors ranged from +17% to -15% of the mean. No significant differences in the distribution were observed when normal sera were divided according to age, menopausal status, or gender (data not shown). The activity of sera from patients with benign tumors on HUVECs was similar to that of healthy donor sera. In contrast, sera from 15 of 78 (19%) breast cancer patients and 8 of 53 (15%) gastrointestinal cancer patients induced HUVEC growth, and sera from 4 of 78 (5%) breast cancer patients and 1 of 53 (2%) gastrointestinal cancer patients inhibited EC proliferation. The frequency of growth-stimulatory sera from breast carcinoma patients was significantly higher ($P = 0.04$) in postmenopausal (14 of 53) than in premenopausal (1 of 25) women, whereas inhibitory activity was observed in 3 of 25 premenopausal women versus 1 of 53 postmenopausal individuals (Fig. 2). The stimulatory or inhibitory activity in the sera of breast cancer patients did not correlate with tumor stage or grade or with estrogen and progesterone receptor expression (data not shown).

Among gastrointestinal cancer patients, the sera from 1 of 10 gastric cancer patients inhibited HUVEC growth, whereas the sera from 8 of 43 patients with intestinal cancer showed a stimulatory activity.

These stimulatory and inhibitory activities were specific for ECs, because no significant differences were observed in the sera from cancer patients versus controls on the proliferation of peripheral blood lymphocytes and of three non-EC lines (data not shown).

Two additional blood samples were obtained after surgery from each of 13 patients with stimulatory sera and from 3 patients with inhibitory sera. Five days after the operation, all of the sera from patients with inhibitory serum activity maintained their effect on HUVECs, whereas only 23% of sera from stimulatory patients still induced endothelial proliferation (Fig. 3). Within 90 days from surgery, the tested sera induced a proliferative activity in the range of that of healthy donors; note that most of these patients were undergoing chemotherapy or radiotherapy. The activity of sera from patients with benign tumors before and after surgery showed no deviation with respect to that of the controls.

An analysis of VEGF concentrations with respect to the mitogenic activity of selected sera from healthy donors and cancer patients revealed VEGF levels below 0.23 ng/ml in the healthy donor sera, whereas levels of VEGF higher than 0.23 ng/ml were observed in 8 of 18 sera with high proliferative activity on HUVECs (Fig. 4). The preincubation of high-VEGF sera with antibodies against VEGF resulted in a marked decrease (>50%) of HUVEC proliferative activity, whereas only

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**Fig. 3** Duration of serum modulatory activity on HUVEC growth. Sera from 13 patients with stimulatory sera (A) and 3 patients with inhibitory sera (B) were collected again at 5 days and about 90 days after surgery and tested for activity on HUVEC proliferation.
Fig. 4  Correlation between HUVEC proliferative activity and VEGF levels. The cutoff levels (vertical lines) and the modulation of HUVEC growth were calculated as described in the Fig. 1 legend. The cutoff level of VEGF (horizontal line) includes all normal sera. Cancer patients, □; healthy donors, ●.

A slight inhibition (<15%) or no inhibition of HUVEC proliferation was observed when stimulatory sera with low VEGF levels were preincubated with anti-VEGF antibodies.

TSP-1 is one of the inhibitors of angiogenesis produced by malignant tumors. This multifunctional extracellular glycoprotein has been shown to suppress the angiogenic response in vivo and in vitro (9). Western blot analysis of two inhibitory sera from cancer patients revealed elevated levels of TSP-1 in both cases (Fig. 5A, Lanes 1 and 3). The preincubation of these inhibitory sera with antibodies against TSP-1 released the block of endothelial proliferation (Fig. 5B).

DISCUSSION

Tumors can express several angiogenic factors, some of which may synergize and lead to a highly metastatic phenotype (16). However, factors that negatively regulate the angiogenic process have also been identified, including a negative angiogenesis regulator produced by Lewis lung carcinoma cells in mice (14); on the other hand, Lewis lung carcinomas also produce the angiogenic factor VEGF (17), suggesting the importance of the balance between positive and negative regulators for tumor angiogenesis.

We evaluated the sera from cancer patients and healthy donors for their ability to up- or down-regulate EC proliferation as a simple and reproducible in vitro approach, gaining information about the angiogenesis/antiangiogenesis balance. Indeed, not all serum samples with high levels of VEGF induced endothelial growth, probably because of the simultaneous presence of antiangiogenic molecules. Conversely, not all of the stimulatory sera showed high levels of VEGF, indicating that factors other than VEGF are involved. It is well known, for instance, that high levels of FGF-2, transforming growth factor α, and hepatocyte growth factor can be detected in cancer patients (12, 13). We found that serum-stimulatory activity was reduced at 5 days after surgery in about 75% of the cases, whereas the inhibiting activity was still present at the same time point. This finding raises the possibility that stimulating and inhibiting factors have different half-lives. The accumulation of serum proteins with a very short half-life is intriguing; it is conceivable that tumors produce molecules that stabilize these polypeptides.

A higher frequency of sera with angiogenic activity was found among postmenopausal versus premenopausal patients, suggesting the role of hormones in altering the levels of angiogenic modulators. However, the available in vitro and in vivo studies do not help to explain this result. Indeed, estrogens augment experimental angiogenesis (18), partly through the
induction of FGF-2, whereas progesterone up-regulates TSP-1, an inhibitor of angiogenesis (19). Insight into the interactions between hormones and angiogenesis modulators in vivo and in vitro awaits further study.

No significant correlation was found between the serum-stimulating activity on HUVECs and the stage of the disease, although three of four inhibitory sera observed in breast carcinoma patients were obtained from patients with small-sized tumors who were node-negative for tumor infiltration.

Whereas tests to identify positive and negative regulators of angiogenesis are cumbersome, our in vitro assay to measure the activity of cancer patient sera on ECs provides a simple avenue to functional information on the net balance between angiogenic and antiangiogenic factors. Such information may be of value in the prognosis of cancer and in monitoring the tumor response to cancer therapy.

ACKNOWLEDGMENTS

We thank L. Mameli for manuscript preparation and M. Azzini for photograph reproductions.

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