Association of Serum Endoglin with Metastasis in Patients with Colorectal, Breast, and Other Solid Tumors, and Suppressive Effect of Chemotherapy on the Serum Endoglin

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ABSTRACT

In this report, we present data indicating that the increased serum endoglin (EDG; CD105) quantitated by a double-antibody sandwich assay is associated with metastasis in patients with solid tumors including colorectal and breast carcinomas. In addition, we show that chemotherapy exerts a suppressive effect on the serum EDG. EDG is a proliferation-associated cell membrane antigen of human vascular endothelial cells. Furthermore, EDG is essential for angiogenesis. We generated two anti-EDG monoclonal antibodies (mAbs), termed SN6a and SN6h, defining different epitopes of EDG and developed a double-antibody sandwich assay to quantitate serum EDG in patients with solid tumors. SN6h possesses an exceedingly high antigen-binding avidity (K, 1.38 x 10^1 liters/mol), whereas SN6a possesses an ordinary avidity for a mAb directed to a cell surface antigen (K, 2.85 x 10^8 liters/mol). We measured serum samples from 101 patients with solid tumors (34 colorectal cancers, 16 breast cancers, and 51 other cancers), 8 patients with benign diseases, and 31 healthy volunteers. The serum level of EDG was significantly elevated in the patients with metastatic cancers. The mean serum EDG in the 42 metastasis-positive patients was 34.0 ± 26.8 ng/ml (median value, 37.2 ng/ml), whereas the value in the 59 metastasis-negative patients was 27.9 ± 26.8 ng/ml (median value, 37.2 ng/ml). The difference in EDG levels between the two groups is statistically significant (P < 0.005). In additional studies, we found that chemotherapy suppressed serum EDG levels in cancer patients. Of the 54 metastasis-positive patients with solid tumors, the mean serum EDG in the 32 chemotherapy-receiving (chemotherapy(+) ) patients was 44.7 ± 41.9 ng/ml (median value, 36.1 ng/ml), whereas the value in the 22 chemotherapy(-) patients was 102.4 ± 99.5 ng/ml (median value, 64.8 ng/ml). The difference in serum EDG between the two groups is statistically significant (P < 0.005). In the majority of metastasis-positive patients who were not receiving chemotherapy, serum EDG was elevated. The results suggest that serum EDG may be a useful marker for monitoring early signs of metastasis and cancer relapse in a long-term follow-up of solid tumor patients.

INTRODUCTION

The major cause of death from cancer is metastasis that is resistant to conventional therapies (1–3). Therefore, early detection of tumor metastasis is extremely important, and there is a critical need for reliable MTS markers (4). Recently, several investigators reported that genetic, micrometastatic, and other markers may serve as predictors or indicators of metastatic potential of solid tumors (4–13). Despite these interesting findings, a serum marker for MTS and a simple assay for such a marker would be highly valuable for diagnosis and/or monitoring MTS in cancer patients whose primary tumors had been resected. For the past several years, we have been studying immunological assays to quantitate serum EDG in cancer patients.

EDG is a homodimer cell-membrane glycoprotein that was initially reported by us as a human leukemia-associated homodimer cell-membrane antigen (14). Later it was also found on endothelial cells as well as on leukemia cells (15). The existence of two forms of EDG was later reported, i.e., the smaller form

The difference in EDG levels between the two groups was statistically significant (P = 0.012). Of the colorectal cancer patients, the difference in EDG levels between the 19 metastasis-negative patients and the 15 metastasis-positive patients was statistically significant (P = 0.02). In addition, the difference between the normal control (n = 31) and the 15 metastasis-positive colorectal cancer patients was statistically significant (P = 0.04). Of the breast cancer patients, the difference in EDG levels between the 11 metastasis-positive patients and the normal control was statistically significant (P < 0.005). In additional studies, we found that chemotherapy suppressed serum EDG levels in cancer patients. Of the 54 metastasis-positive patients with solid tumors, the mean serum EDG in the 32 chemotherapy-receiving (chemotherapy(+) ) patients was 44.7 ± 41.9 ng/ml (median value, 36.1 ng/ml), whereas the value in the 22 chemotherapy(-) patients was 102.4 ± 99.5 ng/ml (median value, 64.8 ng/ml). The difference in serum EDG between the two groups is statistically significant (P < 0.005). In the majority of metastasis-positive patients who were not receiving chemotherapy, serum EDG was elevated. The results suggest that serum EDG may be a useful marker for monitoring early signs of metastasis and cancer relapse in a long-term follow-up of solid tumor patients.

The abbreviations used are: MTS, metastasis/metastases; EDG, endoglin; Ab, antibody; mAb, monoclonal Ab; VEGF, vascular endothelial growth factor; CEA, carcinoembryonic antigen; TGF, transforming growth factor; RPCI, Roswell Park Cancer Institute.

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1 Supported by United States Army Medical Research Grant DAMD17-97-1-7197 and American Cancer Society Grant IM-741/RPG-91-005-05-IM. A report on SN6h was previously submitted to the 6th International Workshop and Conference on Human Leukocyte Differentiation Antigens. In the Workshop/Conference, we briefly reported reactivity of SN6h with vascular endothelium of paraffin-embedded malignant breast tissues (21). However, we have not reported other properties of SN6h nor the procedures by which SN6h was generated. SN6a has not been reported previously.

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4 The abbreviations used are: MTS, metastasis/metastases; EDG, endoglin; Ab, antibody; mAb, monoclonal Ab; VEGF, vascular endothelial growth factor; CEA, carcinoembryonic antigen; TGF, transforming growth factor; RPCI, Roswell Park Cancer Institute.
Epitope Comparison. A competitive binding assay was performed to compare the epitopes defined by SN6a and SN6h. The assay was carried out as described previously (31).

Antigen-binding Avidity of mAbs. The purified mAbs SN6a and SN6h were individually radiolabeled with 125I using Bolton-Hunter reagent, a 125I-labeled acetylating agent (32) as described previously (33). This reagent allows us to radiolabel antigen groups of the proteins, whereas tyrosine of the proteins is primarilradiolabeled by other commonly used radiolabelling methods such as the Iodo-Gen method and the chloramine-T method (33). In general, Ab activity is less affected by radioiodination of antigen groups compared with radioiodination of tyrosine residues. The radiolabeled SN6a and SN6h were determined to contain 1.67 and 1.07 iodine atoms per IgG molecule on the average, respectively. Titration experiments that were carried out using a fixed amount (0.1 μg) of each 125I-mAb and 2-fold serial increments of EDG-expressing KM-3 cells showed that 66.8 and 45.1%, respectively, of the labeled SN6a and SN6h retained antigen-binding activity. In the analyses of the binding data for SN6a and SN6h, corrections were made for the above numbers. Scatchard analysis of the binding data were carried out as described previously (34). An equilibrium constant and an average maximal number of mAb bound/cell were estimated by this analysis.

Immunohistochemical Staining of Tissues. Tissues were frozen, air-dried, and fixed with acetone (9). Fixed tissues were cut on a cryostat and stained using DAKO LSAB+ kits following the supplier’s recommended procedure. Counterstaining was performed with hematoxylin.

Double-Ab Sandwich Assay. EDG in serum was measured using a double-Ab sandwich assay. The procedure is briefly described below. MaxiSorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with mAb SN6h at a concentration of 10 μg/ml in PBS (100 μl/well). The concentration of 10 μg/ml was determined by preliminary titration experiments. As a control, the plate wells were coated with an isotyped control IgG instead of SN6h. The SN6h- or control IgG-coated plates were washed twice with blocking buffer (PBS containing 1% BSA and 0.02% sodium azide) and subsequently incubated with the blocking buffer for 1 h at room temperature. After the plates were emptied of blocking buffer by inverting and flicking, 50 μl of serum samples, diluted with an equal volume of blocking buffer, were added to the SN6h-coated wells, and the plates were incubated overnight at 4°C. The next day, the wells were washed three times with washing buffer (PBS containing 0.05% Tween 20) followed by the addition of 125I-labeled control IgG (1 x 10^5 cpm/well) in 100 μl of blocking buffer; mAb SN6a was radiolabeled with carrier-free 125I by the Iodo-Gen method (14, 33). The plates were incubated for 2 h at 4°C. Then, the wells were washed three times with the washing buffer, and the radioactivity of each well was counted in a Cobra Series Auto-gamma Counter (Packard Instrument Company, Meriden, CT). Serial dilutions of an isolated EDG preparation (see above), instead of serum samples, were added to the assay to generate a standard curve. The standard curve is constructed by generating a simple linear regression line using the assay results and StatView 4.5 software (Abacus Concepts Inc., Berkeley, CA). The measured serum EDG values were presented as the mean ± SD. In additional
tests, a single-Ab sandwich assay was performed to compare with the double-Ab sandwich assay. The single-Ab assay was carried out by coating the plate wells with either SN6h or SN6f (17) as described above, and using 125I-labeled SN6h and 125I-labeled SN6f, respectively, in detection of the antigen bound to the plate wells. The plate was also coated with an isotype-matched control IgG (IgG1-κ) instead of the mAb in the assay.

Statistical Analysis. The Mann-Whitney U test and two-tailed Student t test were used to analyze the significance of any differences between different groups. P < 0.05 were considered as statistically significant.

RESULTS

Generation and Characterization of mAbs. Anti-EDG mAbs that were generated by immunizing mice with an isolated EDG preparation were initially identified by testing against a variety of hematopoietic cell lines in a cellular RIA and by immunoprecipitating EDG as described previously (14, 17). In addition, the generated mAbs were tested against various malignant tissues by immunohistochemical staining. We present data of anti-EDG mAbs D4–2G10 (or termed SN6a) and G4–2C2 (SN6h); SN6a and SN6h were determined to be IgG2a-κ and IgG1-κ antibodies, respectively. The prototype anti-EDG mAb SN6 (14, 21) was included in the various tests of SN6a and SN6h as a reference. Both SN6a and SN6h as well as SN6 (IgG1-κ Ab) reacted with all five of the immature B-lineage leukemia cell lines tested (KM-3, NALM-16, REH, NALM-1, and NALM-6) and all three of the myelo-monocytic leukemia cell lines tested (ML-2, HL-60, and U937). However, they did not react with any of the seven mature B-lineage leukemia lymphoma cell lines (BALL-1, BALM-2, Daudi, Ramos, U698M, BALM-6, and SU-DHL-4), any of the seven T-leukemia cell lines (MOLT-4, JM, CCRF-CEM, CCRF-HSB2, Ichikawa, HPB-MLT, and HUT-78) or any of the three EBV-transformed B-cell lines (CCRF-SB, RPMI 1788, and RPMI 8057). This pattern of reactivity is typical for anti-EDG mAbs (14, 17). In addition, SN6a and SN6h reacted strongly with vascular endothelium of all of the tested malignant tissues as described above, and using 125I-labeled SN6h and 125I-labeled SN6f, respectively, in detection of the antigen bound to the plate wells. The plate was also coated with an isotype-matched control IgG (IgG1-κ) instead of the mAb in the assay.

Epitope Comparison. Epitopes defined by SN6a and SN6h were compared by a competitive binding assay. Isotype-matched control IgGs [MOPC 195 variant (IgG1-κ) and IgG2a-κ] were included in the assay. The results are shown in Fig. 2. Preincubation of EDG-expressing KM-3 cells with SN6h could block the subsequent binding of 125I-labeled SN6h completely, whereas preincubation of KM-3 cells with an isotype-matched control IgG (MOPC 195 variant) showed no significant blocking (Fig. 2A). The results indicate that the blocking is specific. SN6a did not show any significant blocking of the subsequent binding of 125I-labeled SN6h (Fig. 2A). On the other hand, preincubation of KM-3 cells with SN6h blocked the subsequent binding of 125I-labeled SN6h up to 90%, whereas preincubation with either control IgG (RCP-5) or SN6h showed no significant blocking (Fig. 2B). These results show that the epitope defined by SN6a (SN6a epitope) is in a distant position from SN6h epitope. Recent epitope mapping studies using eight recombinant fragments of EDG showed that SN6a epitope and SN6h epitope are at the widely separated positions in the primary structure of EDG.

Ab Avidity and Number of Available Epitopes on EDG-expressing Cells. Scatchard plot analyses of direct binding of radiolabeled SN6a and SN6h to KM-3 cells were carried out (Fig. 3); the results showed equilibrium constants (K) of 2.85 × 10^3 and 1.38 × 10^11 liters/mol, respectively. In the same analysis, the average number of Ab molecules bound per cell was estimated to be 1.44 × 10^4 and 1.45 × 10^4, respectively, at Ab saturation. Because both SN6a (IgG2a) and SN6h (IgG1) are bivalent Abs, the average number of antigen on these cell specimens is probably 1- to 2-fold greater than the Ab number. The present results show that SN6h possesses an exceedingly

5 X. She and B. K. Seon. Epitope mapping of EDG, manuscript in preparation.
high binding avidity to EDG-expressing cells, whereas SN6a possesses a good binding avidity. It should be noted that SN6h shows an excellent binding activity to denatured as well as native EDG molecules (Ref. 21 and the present results).

**Source of Serum Samples.** We determined quantities of EDG in serum samples from 101 patients with active solid tumors that consisted of 34 colorectal cancer, 16 breast cancer, and 24 lung cancer, and 27 other cancers (8 prostate cancer, 2 bladder cancer, 2 nonseminomatous germ cell tumor, 2 metastatic adenocarcinoma of unknown primary, 1 hemangiopericytoma of the brain, 1 desmoplastic small round cell carcinoma, 1 esophageal cancer, 1 extraskelatal osteosarcoma, 1 head and neck cancer, 1 ovarian cancer, 1 pancreaticobiliary tract cancer, 1 perianal squamous cell cancer, 1 pleural mesothelioma, 1 renal cell carcinoma, 1 soft tissue cancer, 1 tongue cancer, and 1 angiosarcoma of the breast). Patient characteristics are listed in Table 1. Those patients in various therapeutic statuses were divided into two groups based on the presence or absence of MTS (Table 1). In addition, we determined EDG in serum samples from eight patients with benign diseases, consisting of two colon adenomas, one anal hemorrhage, one hamartoma of the lung, one pulmonary coccidioidomycosis, one sarcoidosis, one asthma, and one acute pancreatitis. Control serum samples from 31 healthy volunteers were included in the assays.

**Application of Double-Ab Sandwich Assay and Comparison with Single-Ab Assay.** A standard curve for the double-Ab sandwich assay was constructed using serial dilutions of an isolated EDG preparation (see "Materials and Methods" for details). An example is shown in Fig. 4. The measured values of radioactivities in the assay of the test samples were converted into ng EDG/ml using the standard curve. For a comparison, a single-Ab sandwich assay was performed by coating 96-well microtiter plate wells with SN6h, SN6f, or an isotype-matched control IgG (see "Materials and Methods" for details). After the test samples containing EDG were incubated in the mAb- or control IgG-coated wells, and the wells were washed, 125I-labeled SN6h did not show any significant binding to either SN6h-coated wells or control wells; however, 125I-labeled SN6f showed a strong binding to SN6h-coated wells. Furthermore, 125I-labeled SN6a showed significant binding to the samples in SN6h-coated wells and SN6f-coated wells but no significant binding to the

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**Table 1** Characteristics of solid tumor patients

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of patients (male/female)</th>
<th>Age range (median)</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancer</td>
<td>34 (21/13)</td>
<td>26–95 (65.5)</td>
<td>+ 19</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>16 (0/16)</td>
<td>37–84 (52.0)</td>
<td>11 5</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>24 (11/13)</td>
<td>50–79 (65.5)</td>
<td>12 12</td>
</tr>
<tr>
<td>Other cancers</td>
<td>27 (19/8)</td>
<td>23–83 (60.0)</td>
<td>21 6</td>
</tr>
</tbody>
</table>

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**Fig. 2** Competitive binding between SN6a and SN6h as measured by a cellular RIA. A, EDG-expressing KM-3 leukemia cells (2 × 10^5 cells) were incubated with serial dilutions of purified SN6a, SN6h, or MOPC 195 variant for 1 h at 4°C in individual wells of 96-well microtiter plates. 125I-labeled SN6h (4 × 10^4 cpm) was then added, and the incubation was continued for an additional 1 h. Cells were washed three times, and the radioactivity in the washed cell pellets was determined in an auto-gamma counter. B, in the tests, MOPC 195 variant and 125I-SN6h of A were replaced with RPC-5 and 125I-SN6a, respectively.

**Fig. 3** Binding of 125I-labeled SN6a and SN6h to EDG-expressing KM-3 cells. A, the number of Ab molecules bound to one cell at a given dilution. A (in the formula), the molar concentration of total Ab; x, the molar concentration of bound Ab; (A – x), the molar concentration of the free Ab. The slope of the binding curve gives the equilibrium constant for the reaction (K, expressed in liters per mole); n, the maximum number of Ab molecules that can be bound to a single cell.

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**Fig. 4** Binding of 125I-labeled SN6a and SN6h to EDG-expressing KM-3 cells. r, the number of Ab molecules bound to one cell at a given dilution. A (in the formula), the molar concentration of total Ab; x, the molar concentration of bound Ab; (A – x), the molar concentration of the free Ab. The slope of the binding curve gives the equilibrium constant for the reaction (K, expressed in liters per mole); n, the maximum number of Ab molecules that can be bound to a single cell.
EDG Levels in the Serum Samples from Patients with Different Diseases. EDG values in the serum samples from normal donors, patients with benign diseases, and all cancer patients were 41.0 ± 15.0, 28.3 ± 13.2, and 51.4 ± 59.7 ng/ml, respectively (Fig. 5). The values in the serum samples from patients with colorectal cancer, breast cancer, lung cancer, and other cancers were 45.0 ± 37.8, 66.0 ± 63.6, 37.8 ± 43.9, and 63.0 ± 85.8 ng/ml, respectively (see “Results”). Serum from a patient with metastatic angiosarcoma showed the highest value (i.e., 416 ng/ml). Variation in the EDG values among the different donors of the normal group may involve several factors that include menstruation of female donors at the time when the serum samples were collected (see “Discussion”).

Association of MTS with Increased Serum EDG in Cancer Patients. A scatterplot of the test results of 140 serum samples is shown in Fig. 5. Serum EDG level was compared between 42 MTS-negative patients (group A) and 59 MTS-positive patients (group B). In addition, the comparison was made between the group A and 39 patients whose MTS was found after cancers had been diagnosed (postdiagnostic MTS, group C). It should be noted that MTS in the remaining 20 patients with solid tumors. Diseases of the cancer patients are described in Table 1 and “Results.” The difference in the serum EDG concentration is statistically significant between the 59 MTS-positive patients and the 42 MTS-negative patients, as well as between the 39 patients with postdiagnostic MTS and the MTS-negative patients. In the patients with postdiagnostic MTS, MTS was detected after cancer had been diagnosed. All of the six highest EDG values (>200 ng/ml) were detected in the serum samples from patients with postdiagnostic MTS.

Colorectal Cancer Patients. Serum EDG values of 34 (total) colorectal cancer patients, 19 MTS-negative patients (group A) and 15 MTS-positive patients (group B) were 45.0 ± 37.8, 32.1 ± 18.4, and 61.3 ± 49.2 ng/ml, respectively (Fig. 6). All of the eight highest EDG values (>70 ng/ml) were detected in serum samples from the MTS-positive patients among the 34 colorectal cancer patients. The 15 MTS-positive patients (group B) were divided into two groups, i.e., 8 patients with postdiagnostic MTS (group C) and 7 patients with prediagnostic MTS (group D; this group is not shown in Fig. 6). Serum EDG values were 62.6 ± 61.1 and 59.8 ± 35.8 ng/ml, respectively, for groups C and D. Difference between MTS-positive patients (group B) and normal control was statistically significant (P < 0.05). Difference between the MTS-positive patients and MTS-negative patients (group A) was also statistically significant (P < 0.03).

Breast Cancer Patients. Serum EDG values of 16 (total) breast cancer patients, 5 MTS-negative patients (group A), and
11 MTS-positive patients (group B) were 66.0 ± 6.6, 63.6, 32.5 ± 14.8, and 81.3 ± 71.9 ng/ml, respectively (Fig. 7). All of the five highest values (≥33 ng/ml) among the EDG values of the 16 breast cancer patients were detected in serum samples from the MTS-positive patients.

11 MTS-positive patients (group B) were divided into two groups, i.e., 7 patients with postdiagnostic MTS (group C) and 4 patients with prediagnostic MTS (group D; this group is not shown in Fig. 7). Serum EDG values were 94.8 ± 87.0 and 57.6 ± 30.2, respectively, for groups C and D. Differences between the normal control and the total breast cancer patients, the MTS-positive patients (group B) or patients with postdiagnostic MTS (group C) were all statistically significant (P < 0.05, < 0.005, and < 0.002, respectively).

**Lung Cancer Patients.** Serum EDG values of 24 (total) lung cancer patients, 12 MTS-negative patients (group A), and 12 MTS-positive patients (group B) were 37.8 ± 43.9, 29.3 ± 28.6, and 46.3 ± 51.1 ng/ml, respectively. Although serum EDG level is higher in MTS-positive patients than in MTS-negative patients, this difference is not sufficient to be statistically significant. The reasons for the relatively small difference may involve effects of chemotherapy (see "Results") and other factors (see "Discussion").

**Suppressive Effects of Chemotherapy on Serum EDG.** We examined the effect of chemotherapy on serum EDG by comparing chemotherapy-receiving [chemotherapy(+) group] patients with chemotherapy(−) patients (Fig. 8). Drugs used in the chemotherapy include many common anticancer agents such as 5-fluorouracil, Adriamycin, paclitaxel (Taxol), and cisplatin. Of the 59 MTS-positive patients with various solid tumors (see Table 1), 32 patients were chemotherapy(+) whereas 22 patients were chemotherapy(−); chemotherapy status (i.e., time schedule of drug administration) of the remaining 5 patients was not clear and was not included in the analysis. Serum EDG was elevated in the majority (13 of 22 patients) of the MTS-positive patients who were not receiving chemotherapy. In contrast, serum EDG is low in the majority of the chemotherapy-receiving patients, although there are some exceptions (see "Discussion"). Difference in serum EDG between the chemotherapy(+) group and chemotherapy(−) group is statistically significant.
between chemotherapy(+) and chemotherapy(−) groups is statistically significant \((P < 0.03)\). The results indicate that chemotherapy of solid tumor patients decreases serum EDG.

Among the chemotherapy(+) group, the highest EDG value (236 ng/ml) was detected in a serum sample from a 42-year-old breast cancer patient who was undergoing chemotherapy when blood was taken for the assay. This high EDG value was consistent with the patient’s medical record, which described an aggressively growing breast cancer despite the chemotherapy. The elevated serum EDG in this patient can be explained as follows: conventional chemotherapy regimens suppress endothelial cells in tumor vasculature \((35, 36)\) and consequently will inhibit the release of EDG from endothelial cells. On the other hand, tumor cells release various angiogenic factors \((e.g., \text{VEGF})\) that stimulate proliferation of endothelial cells and promote angiogenesis \((26)\). In aggressively growing tumors such as the tumor in the above patient, the tumor-derived angiogenic factors may overcome the cytotoxic effects of chemotherapeutic drugs, and the balance may favor endothelial cell proliferation and release of EDG from the proliferating endothelial cells.

**DISCUSSION**

Early detection of MTS is extremely important because MTS is the most lethal attribute of human cancers \((1−4, 27)\). Angiogenesis-associated antigens and other tumor-associated antigens in the blood of cancer patients may be candidates for a convenient marker for detection and monitoring of MTS. An example of such serum markers is CEA in colorectal cancer patients. There is no role for CEA assessment as a screening tool for colorectal cancer, but routine CEA monitoring postresection of colon cancer may be useful for early detection of metastatic disease \((37, 38)\). The potential advantages of angiogenesis-associated serum antigens as a MTS marker could be its wide applicability to different types of solid tumors. VEGF is a major angiogenesis factor that can be detected in the blood \((39, 40)\). A number of investigators reported varying relevance of serum or plasma VEGF to cancer. Recently, Adams et al. \((41)\) carried out a detailed study of plasma and serum VEGF and reported that the highest plasma and serum VEGF concentrations were detected in cancer patients in remission compared with cancer patients with other disease status including MTS-positive patients. Therefore, plasma and serum VEGF may not be a useful marker for MTS.

Recently, Li et al. \((29)\) reported a correlation of plasma EDG (CD105) with MTS in breast cancer patients. They used a single-Ab sandwich ELISA in measuring plasma EDG. In our studies, a double-Ab sandwich assay was superior to a single-Ab sandwich assay in quantitating serum EDG (see “Materials and Methods” and “Results”). The comparison was made using multiple anti-EDG mAbs. Li et al. \((29)\) may have been able to measure plasma EDG by amplifying a weak signal in the single-Ab assay using Amerlite. Using the double-Ab sandwich assay, we found association of the increased serum EDG with MTS in 101 cancer patients with various defined solid tumors \((see \text{ Fig. } 5 \text{ and “Results”})\). The association was detected in patients with colorectal and breast carcinomas when the analysis was performed with individual cancers. Additional studies showed that chemotherapy of patients suppresses serum EDG. The results suggest that serum EDG may be useful for monitoring early signs of MTS and cancer relapse in a long-term follow-up of solid-tumor patients such as colorectal and breast cancer patients. Although serum EDG was increased in the MTS-positive patients with lung carcinoma compared with the MTS-negative lung cancer patients, the difference was not sufficient to be statistically significant. Besides the suppressive effect of chemotherapy, the potential reasons for the relatively small difference in lung cancer patients could be as follows: one factor could be the effect of the primary tumors on serum EDG. The primary tumors were resected in only 9 of the 24 lung cancer patients whose serum samples were evaluated in the present studies. It should be noted that in the case of colorectal and breast cancer patients, the primary tumors were resected in 29 of the 34 colorectal cancer patients and in 15 of the 16 breast cancer patients. The 15 lung cancer patients whose primary tumors were not resected consisted of 7 MTS-negative patients and 8 MTS-positive patients. Serum samples from two of the 7 MTS-negative patients showed the two highest EDG values \((i.e., 97.5 \text{ and } 70.8 \text{ ng/ml})\) of the values of all of the 12 MTS-negative lung cancer patients. It is likely that these elevated EDG values originated from the progressive primary tumors in the patients. In the case of the 8 MTS-positive patients whose primary tumors were not resected, differentiation of the primary tumor effect on serum EDG from the MTS effect is difficult. Nevertheless, evaluation of the present data including those of breast, colorectal, and other cancers lead us to believe that generally MTS exerts a stronger effect on serum EDG than primary tumors. However, the effect of primary tumors on serum EDG will be strongly influenced by the progressive status \((i.e., \text{vascular status})\) of the primary tumors. At any rate, the effect of the primary tumors on serum EDG may have obscured the statistical significance in the difference between the MTS-negative and MTS-positive lung cancer patients. Another factor could be aberrant growth of tumors in some lung cancer patients. Recently, Pezzella et al. \((42)\) reported that tumor growth occurred without morphological evidence of neangiogenesis in some \((≈16\%\) patients with non-small cell lung carcinoma. We cannot exclude the possibility that our serum samples from lung cancer patients may have contained samples from this population of patients, and such samples may have influenced our test results of lung cancer patients.

EDG is a proliferation-associated antigen on endothelial cells \((18, 20)\) and essential for angiogenesis \((26)\). Expression of EDG in tumor tissues was restricted to endothelium and not detected in tumor cells per se \((18, 24)\). Therefore, we conclude that the increased serum EDG in cancer patients with metastatic diseases originates from the neovascularure of metastatic tumors but not from tumor cells. The mechanisms by which EDG is released from the neovascularure remain to be studied. MTS is dependent on angiogenesis \((26−28)\). Therefore, we anticipate that serum EDG will be increased in patients with metastatic diseases of most forms of solid tumors. The present data showed that chemotherapy of cancer patients exerts suppressive effect on serum EDG. In this regard, serum EDG was elevated in the majority of MTS-positive patients who were not receiving chemotherapy. Dividing endothelial cells in newly forming tumor blood vessels should render such endothelial cells, in con-
trast to their quiescent counterpart in normal adult tissues, sensitive to the cytotoxic effects of chemotherapeutic drugs in a manner similar to dividing bone marrow cells (36). Brower et al. (35) reported that cyclophosphamide induced apoptosis of endothelial cells within tumors. Klement et al. (36) reported that vinblastine strongly inhibited dividing endothelial cells. Other chemotherapeutic drugs that inhibit angiogenic endothelial cells include a purine analogue, 6-methylmercaptopurine riboside (43) and camptothecin analogues (44). Combination chemotherapy using multiple drugs is likely to decrease serum EDG. The suppressive effect of chemotherapy may present an obstacle in applying serum EDG for diagnostic purposes. However, this problem may be circumvented by using serum EDG as a marker for MTS and tumor recurrence in a long-term follow-up of cancer survivors who are not receiving chemotherapy. In addition, such a follow-up study of individual patients will obviate the problem of variation in serum EDG level among different individuals by establishing individually tailored baseline of serum EDG level.

An ideal baseline value would be the serum EDG value of healthy individuals before cancers develop. Such a baseline value would become available when a serum EDG assay is used for routine screening in medical check up of healthy individuals. At the present time, however, we will not be able to obtain such a baseline value. Alternatively, therefore, we may need to determine the baseline value by periodic (e.g., weekly) quantitation of EDG in the serum samples from patients before and after tumor resection and/or chemotherapy.

A special attention needs to be directed to young female patients because angiogenesis occurs at the corpus luteum during the menstrual period (45). Other factors that might influence serum EDG include chronic inflammation (e.g., rheumatoid arthritis and psoriasis), age and sex of donors. There is considerable evidence to suggest that angiogenesis and chronic inflammation are codependent (46).

In conclusion, the present results suggest that serum EDG may be a useful marker for monitoring early signs of MTS and cancer relapse in a long-term follow-up of solid-tumor patients.

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