Increased Serum Angiogenin Concentration in Colorectal Cancer Is Correlated with Cancer Progression

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ABSTRACT

We have previously demonstrated that the increased expression of angiogenin (ANG) in pancreatic cancer is related to cancer aggressiveness; however, the relationship between ANG expression and its clinical relevance in colorectal cancer has not been demonstrated. We therefore investigated the correlation between serum ANG (sANG) concentration and colorectal cancer progression or the changes in sANG concentrations before and after cancer resection. To determine sANG concentration by ELISA, sera were obtained from colorectal cancer patients (the cancer group) preoperatively (n = 34) and postoperatively (n = 25), from hernia patients (the nonneoplastic group) preoperatively (n = 9) and postoperatively (n = 4), and from 23 healthy volunteers. The amount of ANG in the colorectal cancer tissues (n = 19) was determined by the same method. Before surgery, the mean sANG concentration in the cancer group (411.8 ± 106.3 ng/ml) was significantly higher than that in both the nonneoplastic group (344.0 ± 60.7 ng/ml; P = 0.04) and in the healthy volunteers (321.7 ± 59.7 ng/ml; P = 0.0001). The degree of elevation of sANG concentration in the cancer group was more significant in the more progressed subgroups as compared with that in the normal group (versus T1, P = 0.01; versus T2, P = 0.002; versus stage 0 + I cancer, P = 0.02; versus >stage III cancer, P = 0.001; versus Dukes’ A cancer, P = 0.02; versus Dukes’ C cancer, P = 0.006). After cancer resection, the mean sANG concentrations in each subgroup decreased to the same levels as those of the normal group; the degrees of reduction were more significant in the more progressed subgroups. The tissue ANG amount correlated significantly with sANG concentration (P = 0.007). These results suggest that the increased concentration of sANG that is derived from colorectal cancer correlates with cancer progression.

INTRODUCTION

Tumor angiogenesis is one of the most important biological features that is closely related to carcinogenesis (1), tumor growth (2), and tumor metastasis (3). Clinically, neovascularization was closely related to cancer aggressiveness in many human cancers (4). ANG2 is a potent blood vessel inducing protein that was originally purified from conditioned media of the human colon carcinoma cell line HT-29 (5). It was later found in normal human serum at a concentration of 60–480 ng/ml (6–8), and its mRNA was found to be expressed in human tumor cells (7–10) as well as nonmalignant cells such as peripheral blood cells, vascular endothelial cells, fibroblasts, and colon epithelial cells (7, 10, 11). However, the relationship between ANG expression in human cancers and its clinical relevance has not been thoroughly investigated. Very recently, we have demonstrated for the first time that the increased expression of ANG mRNA and higher concentration of sANG in pancreatic cancer patients correlate with cancer aggressiveness (7). These results indicate that the detection of ANG in sera or in tissues may help to evaluate potential tumor aggressiveness. However, in a review of the literature, there have been no investigations concerning the sANG concentration and its clinical relevance in colorectal cancer. In the present study, we demonstrate that the sANG concentration in colorectal cancer patients is significantly higher than those in nonneoplastic disease patients and in healthy volunteers, and that it decreases to these same levels after cancer resection. We also demonstrate that the sANG concentration in colorectal cancer patients correlates with cancer progression.

PATIENTS AND METHODS

Patients, Tissue Samples, and Sera. This study comprised 34 patients with colorectal cancer (the cancer group; male:female, 21:13; mean age, 62.3 ± 9.2 years), 9 hernia patients (the nonneoplastic group; male:female, 8:1; mean age, 55.9 ± 18.2 years), and 23 adult healthy volunteers (the normal group; male:female, 14:9; mean age, 33.6 ± 9.0 years). In the cancer group, 32 patients underwent curative resection, and 2 patients did not undergo resection because of recurrent disease. In the nonneoplastic group, eight patients had an inguinal hernia, and one patient had an abdominal incisional hernia. The depth of invasion and stage grouping of cancer were classified according to the tumor-node-metastasis (TNM) classification and Dukes’ classification (12). Detailed patient distributions for each subgroup are demonstrated in Table 1. The two recurrent cancer patients were assigned to the >stage III subgroup, but they were excluded from the tumor classification and Dukes’ classification (Table 1). All patients were admitted to our hos-
hospital between October 1997 and August 1998, and all serum samples were collected during this period. The sera were obtained preoperatively from the cancer patients and nonneoplastic patients and from healthy volunteers. Postoperative serum samples were obtained from 25 patients in the cancer group and 4 patients in the nonneoplastic group on the day after surgery to investigate the influence of surgery itself on the postoperative sANG profile. Colorectal cancer tissues were obtained from 19 patients. Most of them were advanced cases, but three patients had T2 cancer, two patients were in stage I, and two patients were classified as Dukes’ A. The tissues were snap-frozen immediately after resection and stored at −80°C.

**Protein Lysates.** Protein lysates were extracted following the procedures described previously (7, 13, 14). In brief, frozen colorectal cancer tissues were lysed in the lysis buffer and homogenized by a Teflon homogenizer. The lysis buffer consisted of 50 mM Tris-HCl, 150 mM sodium chloride, 10 mM sodium orthovanadate (Sigma), and 100 mM phenylmethylsulfonyl fluoride (Sigma), and 1% IGEPAL-CA630 (Sigma), respectively.

**Table 1 Mean sANG concentrations and mean tissue ANG amounts in the cancer group**

<table>
<thead>
<tr>
<th>Tumor classificationa</th>
<th>Mean sANG concentration (ng/ml)</th>
<th>Mean tissue ANG amounts (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>preOp (n = 34)</td>
<td>postOp (n = 25)</td>
</tr>
<tr>
<td>T_1 + T_2</td>
<td>368.9 ± 58.3 (14)b</td>
<td>336.7 ± 83.5 (9)</td>
</tr>
<tr>
<td>T_3 + T_4</td>
<td>433.0 ± 121.8 (18)</td>
<td>323.6 ± 77.2 (16)</td>
</tr>
<tr>
<td>Cancer stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 0 + I</td>
<td>366.2 ± 60.2 (12)</td>
<td>336.7 ± 83.5 (9)</td>
</tr>
<tr>
<td>Stage II</td>
<td>426.9 ± 138.2 (8)</td>
<td>291.3 ± 59.2 (7)</td>
</tr>
<tr>
<td>&gt; Stage III</td>
<td>442.4 ± 110.8 (14)</td>
<td>348.7 ± 83.2 (9)</td>
</tr>
<tr>
<td>Dukes’ classificationa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dukes’ A</td>
<td>366.2 ± 60.2 (12)</td>
<td>336.7 ± 83.5 (9)</td>
</tr>
<tr>
<td>Dukes’ B</td>
<td>426.9 ± 138.2 (8)</td>
<td>291.3 ± 59.2 (7)</td>
</tr>
<tr>
<td>Dukes’ C</td>
<td>429.1 ± 107.2 (12)</td>
<td>348.7 ± 83.2 (9)</td>
</tr>
</tbody>
</table>

a The two patients with recurrent disease were excluded from these subgroups.
b Numbers in parentheses indicate the number of patients examined.

**Statistical Analyses.** The differences in the mean values were tested by paired and unpaired Student’s t tests. Welch’s test was also applied, if necessary. Pearson’s correlation coefficient (r) was tested by the F test. All calculations were performed using Macintosh StatView Software. A probability (P) of < 0.05 was considered significant.

**RESULTS**

**Distributions of sANG Concentration before and after Surgery.** Distributions of sANG concentrations in the nonneoplastic group and the cancer group before and after surgery as well as that of the normal group are demonstrated in Fig. 1. Before surgery, the mean sANG concentration in the cancer group (411.8 ± 106.3 ng/ml) was significantly higher than that in both the nonneoplastic group (344.0 ± 60.7 ng/ml; P = 0.04) and the normal group (321.7 ± 59.7 ng/ml; P = 0.0001; Fig. 1). On the other hand, a comparison of the mean sANG concentration between the nonneoplastic group and the normal group showed no statistical differences (P = 0.18). Postoperatively, the mean sANG concentration in the cancer group (328.3 ± 78.1 ng/ml) showed no significant difference as compared with those in the nonneoplastic group (376.3 ± 86.7 ng/ml) and the normal group (Fig. 1). A significant decrease in the mean sANG concentration could be observed by cancer resection in the cancer group [P = 0.0008 (unpaired t test) and P = 0.0001 (paired t test)], whereas no change in mean sANG concentrations could be observed after surgery in the nonneoplastic group.

**Elevation of sANG Concentrations and Their Reduction after Cancer Resection Correlating with Cancer Progression.** The preoperative and postoperative mean sANG concentrations in the cancer group, as subdivided according to the tumor classification, cancer stage, and Dukes’ classification, are demonstrated in Fig. 2, a–c. Before cancer resection, the mean sANG concentration increased according to the progression of cancer, such as the progression of the tumor classification (Fig. 2a), cancer stage (Fig. 2b), and Dukes’ classification (Fig. 2c). The mean sANG concentrations were significantly higher in the T_1 + T_2 (368.9 ± 58.3 ng/ml; P = 0.01) and the T_3 + T_4 (433.0 ± 121.8 ng/ml; P = 0.002) subgroups (Fig.
DISCUSSION

Tumor angiogenesis is thought to play an important role in the biological behaviors of tumors, including carcinogenesis, invasion, and metastasis (1–4). Although many angiogenic factors including ANG have been reported, there have been only a few investigations concerning ANG expression in human cancers (7–9) or in malignant cell lines (10). Furthermore, very little is known about the relationship between ANG expression and its clinical relevance in human cancers. We have recently demonstrated for the first time that both increased ANG mRNA expression and higher sANG concentration in pancreatic cancer correlate with cancer aggressiveness (7). In addition, Barton et al. (8) have demonstrated that the sANG concentration is increased in advanced ovarian cancer patients. Consistent with these previous reports, we demonstrate in the present study that sANG concentration is elevated in colorectal cancer patients and that the degree of its elevation correlates with the degree of cancer progression. To the best of our knowledge, this is the first report concerning sANG concentration and its clinical relevance in colorectal cancer and the reduction of sANG concentration after cancer resection.

In the present study, we did not definitively elucidate the source of sANG in our series of samples. The mean sANG concentration in the normal group was at the same level as that reported previously (7, 8). Furthermore, in the cancer group, the rate of patients with elevated sANG concentration as compared with sANG distribution in the normal group was also nearly the same as that reported previously (7). Because many types of nonmalignant cells such as peripheral blood cells, vascular endothelial cells, fibroblasts, and normal colon epithelial cells express ANG mRNA (7, 10, 11), these cells could be the source of sANG in colorectal cancer patients as well as in healthy volunteers and in nonneoplastic patients. However, it is probable that colorectal cancer cells can contribute to the increased sANG concentration. This hypothesis is supported both by the previous findings of increased mRNA expression in a relatively smaller number of colorectal cancer tissues (9) or of the ANG secretion ability of tumor cells (15) and by our present findings of a reduction in sANG concentrations after cancer resection to the same level as that in the normal group. In addition, it should be noted that the ANG was detected in the cancer tissue lysates and that tissue ANG amounts correlated significantly with sANG concentration. Finally, the decrease in sANG concentration after cancer resection was more significant in the more progressed colorectal cancer patients. Furthermore, the reduction of sANG concentration in the cancer group after surgery was not due to the surgery itself but was due to the effects of cancer resection, because no changes in mean sANG concentration after surgery could be observed in the nonneoplastic group. However, it remains possible that noncancerous cells in the surrounding tissue are other sources of ANG expression because our previous reports demonstrated ANG mRNA expres-
Fig. 2  Preoperative and postoperative mean sANG concentrations of (a) the T₁ + T₂ + T₃ and the T₄ + T₅ subgroups, (b) the stage 0 + I, the stage II, and the >stage III subgroups, and (c) the Dukes’ A, Dukes’ B, and Dukes’ C subgroups as well as that of the normal group. P is expressed by an unpaired t test. SDs are expressed by lines. preOp, preoperatively; postOp, postoperatively. *, P measured using Welch’s test.
tion in the fibroblasts or noncancerous epithelial cells in the cancer microenvironment (7, 14).

The reasons why colorectal cancers with higher sANG concentrations were more progressed remain unclear. Several investigations have provided evidence concerning the unique biological characteristics of ANG. ANG can bind to cultured endothelial cells (16) or extracellular matrix molecules (17) through the cell surface actin-like receptor (18). The ANG-actin complex could subsequently activate cell-associated proteases (19). This activation can be considered a rational biological phenomenon because the extracellular matrix breakdown is a prerequisite for endothelial cell migration during physiological angiogenesis (3). Indeed, ANG-actin complexes could strongly induce endothelial cells to invade through the artificial matrix in vitro (20). Likewise, the breakdown of some extracellular matrix molecules in the cancer stroma has been also demonstrated (21). Therefore, the increased ANG expression in cancer cells and the subsequent increase in sANG concentration in cancer patients could allow cancer cells to migrate through the extracellular matrix by their proteolytic activity and facilitate cancer cell invasion at the primary and metastatic sites. This hypothesis is supported by the in vivo findings that the monoclonal anti-ANG antibody and ANG antagonists can significantly suppress the establishment of HT-29-induced tumors in athymic mice (15, 22). Based on these previous findings, sANG could be useful as a diagnostic factor for stage grouping and tumor or Dukes’ classification as a result of cancer cell invasion. In the same sense, examination of sANG concentration could be potentially useful for long-time follow-up of patients as an early diagnostic marker of recurrence. Although we cannot discuss the relationship between sANG concentration and survival in the present study because of the insufficient follow-up period,

the reverse correlation between sANG concentration and survival has been demonstrated in pancreatic cancer (7).

Very recently, another type of $M$, 170,000 ANG receptor has been reported by Hu et al. (23), and the ANG that binds to this receptor can stimulate the growth of human endothelial cells under a specific, cell sparse condition. From these findings, they speculated that the expression of two types of ANG receptor depended on the endothelial cell density and that the dual biological properties of ANG-proteolytic activity and stimulation of endothelial cell growth depended on the two types of receptors to which ANG could bind. Therefore, it is conceivable that the inducible proteolytic activity by ANG that favors cancer cell invasion depends on the type of ANG receptor surrounding ANG-expressing cells. In the future, analyses of the expression of ANG and the types of ANG receptor as well as the usefulness of ANG as a diagnostic/prognostic marker including a postoperative follow-up period will be required in many other human cancers to elucidate the roles of ANG in cancer progression.

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