Increased Angiogenin Expression in Pancreatic Cancer Is Related to Cancer Aggressiveness

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Abstract

We have investigated the expression of angiogenin (ANG) in pancreatic cancer and the relevance of ANG expression to the progression of pancreatic cancer. In comparison to normal pancreas, increased ANG mRNA expression was observed in 80.0% of the cases of pancreatic cancer by in situ hybridization, and increased ANG protein expression was observed in 86.7% of the cases of pancreatic cancer using Western blot analysis. The mean serum ANG concentration of pancreatic cancer patients (566.6 ± 191.9 ng/ml) was significantly higher (P < 2.0 x 10⁻⁸) than that of healthy volunteers (359.0 ± 59.9 ng/ml). Increased ANG mRNA expression as well as elevated serum ANG concentration correlated with poor prognosis. These findings suggest that ANG expression is up-regulated in pancreatic cancer patients and that ANG contributes to the aggressiveness of pancreatic cancer.

Introduction

Angiogenesis is a process necessary for the physiological and pathological growth of tissues. To date, many angiogenic factors, such as TGF-α, TGF-β, aFGF, and bFGF, have been reported, and tumor cells can produce these angiogenic factors (1, 2). Tumor angiogenesis has been thought to be closely related to carcinogenesis (3), tumor growth (4), and tumor metastasis (5). Neovascularization in the tumor makes tumor cells more likely to shed into the bloodstream and may result in the establishment of metastasis (5). Clinically, it has been well established that tumor angiogenesis is closely connected to metastasis in many kinds of human cancers (6). ANG is a Mr 14,100 polypeptide that was first isolated from conditioned medium from the human colon adenocarcinoma cell line HT-29 (7). It is an inducer of polypeptide that was first isolated from conditioned medium from the chick chorioallantoic membrane and rabbit cornea (7). Subsequently, ANG mRNA was detected in other malignant human cells and tissues (8, 9). However, to date, there are no studies about the expression of ANG and its clinical relevance in pancreatic cancer. In the present study, we demonstrate that both ANG mRNA expression and ANG protein expression are increased in pancreatic cancer as compared to normal pancreas, and that the sANG concentration is higher in pancreatic cancer patients than in healthy volunteers. We also demonstrate that pancreatic cancer patients with the increased ANG mRNA expression or higher sANG concentration exhibit a more rapidly progressive course in comparison to those with no ANG mRNA expression or a lower sANG concentration.

Materials and Methods

Tissue Samples and Sera. Pancreatic cancer tissues were obtained from patients who underwent surgical operations for pancreatic cancer. Forty-pancreatic cancer tissues (adenocarcinoma, n = 37; cystadenocarcinoma, n = 3) and 11 normal pancreas tissues were used for in situ hybridization. Fifteen pancreatic cancer tissues (adenocarcinoma, n = 14; cystadenocarcinoma, n = 1) and two normal pancreas tissues were used for Western blot analysis. The sera were obtained from 47 patients with pancreatic cancer (cancer group: mean age, 64.2 years; range, 40-86 years; 32 males and 15 females) and 16 healthy volunteers (normal group: mean age, 45.4 years; range, 19-80 years; 13 males and 3 females). The pancreatic cancer tissues were classified according to the TNM classification (10). A one-year survival rate was defined as the rate of pancreatic cancer patients who survived for more than 1 year after surgical operation.

cDNA Probe. The cDNA probe cocktail, which consists of an equimolar mix of three kinds of region-specific probes for human ANG (8), was obtained from British Biotechnology (Oxford, United Kingdom). The probes are single-stranded oligonucleotides that have been chemically synthesized and modified at the 5' and 3' end with digoxigenin. Although each cDNA probe has a different GC content (48% and 59%), they have the same melting temperature (86°C).

In Situ Hybridization. Five-μm sections were deparaffinized, digested by the proteinase K (10 μg/ml), prehybridized, and hybridized with the cDNA probe for ANG (200 ng/ml). To detect the digoxigenin signals, the sections were incubated with the alkaline phosphatase-conjugated antidigoxigenin antibody for 30 min, followed by additional incubation with the mixture of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 4 h. After visualization of the digoxigenin signal, the sections were mounted with cover glasses without counterstaining. To confirm the specificity of the hybridization signal, two different kinds of negative controls were made in the serial sections: (a) incubating the sections only with the prehybridization solution (e.g., without cDNA probe) and (b) treating the sections with excessive RNase A (Sigma) prior to hybridization.

To evaluate the degree of ANG mRNA expression, the staining intensity was divided into three groups (-, +, ++).

Western Blot Analysis. Pancreatic tissues were lysed and dissociated by sonication. Protein samples were boiled in SDS gel sample buffer for 5 min at 95°C (11). One hundred fifty μg/lane of proteins were electrophoresed in a 15% SDS-polyacrylamide gel with a 3% stacking gel (11). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a transblot apparatus (Phase, Lübeck, Germany). Nonspecific antibody bindings were blocked by preincubation of the membranes with 5% nonfat dry milk in PBS. The membranes were incubated for 1 h with the goat anti-human ANG antibody (Oncogene Science, Uniondale, NY) was used for a control protein expression.

ELISA. The ELISA kit (British Biotechnology) was used for the quantitative determination of the sANG concentration. Briefly, each sample serum diluted in 1:2000 was added to the microtiter wells precoated by mouse monoclonal anti-human ANG antibody and incubated for 1 h. After washing the wells three times, polyclonal anti-human ANG antibody conjugated with horseradish peroxidase was added to the wells and incubated for 1 h. After being washed three times again, the wells were incubated with a tetramethylbenzidine-H₂O₂ mixture for 20 min. The absorbance of each well was deter-
mained using a spectrophotometer with a 450-nm wavelength. The same procedure was performed simultaneously using an ANG standard solution of a serial 2-fold diluted series supplied in the kit to draw a standard curve.

Statistical Analyses. Student’s t test and Fisher’s exact test were used for statistical analyses. A P < 0.05 was considered to be significant.

Results

Overexpression of ANG mRNA in Pancreatic Cancer. The expression of ANG mRNA was observed in 32 cases (80.0%) of pancreatic cancer with varying staining intensity (+, 17 cases; ++, 15 cases; Fig. 1A). ANG mRNA was also detected in the fibroblasts surrounding the cancer cells (Fig. 1A). On the other hand, no ANG mRNA expression was detected in all cases of normal pancreas (Fig. 1B). The ANG signals in both the cancer cells and the fibroblasts disappeared by treating the sections with excessive RNase A (Fig. 1C).

Correlation between ANG mRNA Expression and Clinicopathological Parameters. Eight patients were omitted from 1-year survival analysis because these patients underwent surgical operation within 1 year from the time of this study. In comparison to the patients who showed no ANG mRNA expression, the patients with increased ANG mRNA expression (+ and ++) exhibited lower 1-year survival rates (P < 0.05). In addition, they had a tendency to have advanced stage cancers (P = 0.08; Table 1). There was no significant correlation between ANG mRNA expression and histological grade (Table 1) or any other parameters (TNM categories and resectability).

Overexpression of ANG Protein in Pancreatic Cancer. Western blot analysis revealed a large amount of protein expression at M₆ 14,000, which was detected by antihuman ANG antibody in 86.7% of the pancreatic cancer tissues. Of these, 76.9% had stronger protein expression at this molecular weight point (Fig. 2, lower panel, Lanes 1-6). The others had a little weaker protein expression (Fig. 2, lower panel, Lane 7), but it was still stronger than that of normal pancreatic tissues, which had a very weak protein band at this molecular weight point (Fig. 2, lower panel, Lanes 8 and 9). Actin protein was also detected in the same cases (Fig. 2, upper panel). Increased ANG protein expression was associated with increased ANG mRNA expression in the same patients.

Increased sANG Concentration in Sera of Pancreatic Cancer Patients. The mean ± SD and range of sANG concentration was 566.6 ± 191.9 (range, 270.6-1267.4) ng/ml in the cancer group and 359.0 ± 59.9 (range, 257.9-480.9) ng/ml in the normal group (Fig. 3). The difference in mean sANG concentrations between these two groups was statistically significant (P < 2.0 × 10⁻⁸). In the normal group, the sANG concentration was below 500 ng/ml in all cases, whereas it was over 500 ng/ml in 28 cases (59.6%) in the cancer group. The sANG concentration in pancreatic cancer patients became progressively higher according to the degree of ANG mRNA expression.

Correlation between sANG Concentration and Clinicopathological Parameters. In comparison to the patients who showed lower sANG concentrations (below 500 ng/ml), the patients with higher sANG concentrations (over 500 ng/ml) showed lower 1-year survival rates (P < 0.03; Table 1). In addition, they had a tendency to have larger cancers (tumor category, P = 0.06; data not shown). There was no significant correlation between sANG concentration and any other parameters (cancer stage, histological grade, node and metastases categories, and resectability).

Discussion

Tumor angiogenesis is thought to play a crucial role in the biological behaviors of malignant cells (3-6) and it is mediated by many angiogenic factors (1, 2). Although ANG is one of the angiogenic factors, nothing is known about the role of ANG in pancreatic cancer. In a review of the literature, we found a few investigations concerning the ANG mRNA expression in some other human cancers or in some cell lines (8, 9). ANG mRNA was detected in 80.0% of the cases of colon carcinoma (8), in 57.1% of the cases of gastric carcinoma (8), and in some human malignant cell lines to a various degree (9). Consistent with these previous reports, our present results indicate that ANG mRNA as well as ANG protein were overexpressed in a high percentage of pancreatic cancer patients, like colon carcinoma, as compared with normal pancreas.

Fig. 1. ANG mRNA expression by in situ hybridization in pancreatic cancer and in normal pancreas. A. ANG mRNA is detected in cancer cells as well as in fibroblasts around the cancer cells. B. ANG mRNA is not detected in normal pancreas. C. Same case as demonstrated in A. The section pretreated with excessive RNase A showed no specific staining; without counterstaining. A-C, ×400.
INCREASED EXPRESSION OF ANG IN PANCREATIC CANCER

Table 1 Correlation between clinicopathological parameters and ANG mRNA expression or ANG concentration

<table>
<thead>
<tr>
<th>Cancer stage (% of cases)</th>
<th>ANG mRNA expression</th>
<th>sANG concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stages I and II</td>
<td>-</td>
<td>+ and ++</td>
</tr>
<tr>
<td>Stages III and IV</td>
<td>62.5</td>
<td>90.6</td>
</tr>
<tr>
<td>Histological grade (% of cases)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Grades 1 and 2</td>
<td>87.5</td>
<td>65.6</td>
</tr>
<tr>
<td>Grade 3</td>
<td>12.5</td>
<td>34.4</td>
</tr>
<tr>
<td>1-yr survival rate (%)</td>
<td>75.0</td>
<td>33.3</td>
</tr>
<tr>
<td>Median survival (mo)</td>
<td>12.5</td>
<td>10</td>
</tr>
</tbody>
</table>

* ANG mRNA expression intensity is divided into three groups as described in "Materials and Methods."
* Tested using Fisher's exact test.
* N.S., not significant.

Fig. 2. Western blot analysis of pancreatic cancer tissues (Lanes 1~7) and normal pancreatic tissues (Lanes 8 and 9). The expression of actin protein and ANG protein was demonstrated in the upper and lower panels, respectively. Left, molecular weight in thousands.

The concentration of sANG was significantly higher in the sera of pancreatic cancer patients than in the sera of healthy volunteers. The findings that peripheral blood cells express ANG (9) could explain the existence of ANG in healthy human sera. These cells could be also the source of the sANG in the pancreatic cancer patients; however, it is probable that cancer cells can contribute to the higher sANG concentration since both ANG mRNA and its protein product were overexpressed in pancreatic cancer tissues. Indeed, the observation that human ANG was detected in the sera of mice with established HT-29 xenografts confirms the ability of HT-29 cells to secrete ANG (12).

Our in situ hybridization study demonstrated that ANG mRNA could also be detected in the fibroblasts of the cancer stroma. Recently, the mRNA of some growth factors and angiogenic factors has been detected in the fibroblasts from neoplastic tissues (13). It is likely that these stromal cells can influence the growth of neighboring tumor cells. Adams et al. (14) reported that conditioned medium from breast tumor-derived fibroblasts enhanced the growth of the breast cancer cell line MCF-7, whereas conditioned medium from normal breast-derived fibroblasts had an inhibitory effect on the growth of the cells. Although our present study did not clarify the reasons for increased expression of ANG mRNA in the fibroblasts, ANG from surrounding fibroblasts may amplify the biological action of ANG from the cancer cells, which has been stated as stromal epithelial interactions (14).

The properties of ANG are somewhat different from those of other angiogenic factors. aFGF and bFGF stimulate locomotion and proliferation of endothelial cells, whereas TGF-α has an effect on endothelial cell proliferation (15). In contrast, ANG seems to have no such known effects on endothelial cells (15). Likewise, some studies have provided evidence that ANG interacts with endothelial cells and ECM molecules, and that the biological activities of ANG are mediated through a cell surface receptor (16~20). ANG can bind to the cultured endothelial cells (16) and support their spreading (17). The adhesion of ANG to proteoglycans, a member of ECM, has been demonstrated recently (18). Furthermore, it has been reported that actin is an ANG-binding protein on the endothelial cell surface (19) and that the ANG-actin complex can lead to the activation of several protease cascades (20). We have reported recently that the expression of some ECM components is decreased in pancreatic cancer (21). Many proteolytic enzymes are involved in the breakdown of the ECM structure (22), a process of which is also a prerequisite for angiogenesis (5), and
this process allows cancer cells to penetrate the surrounding ECM. Therefore, it is possible that ANG on cancer cells can facilitate cancer cells’ invasion through the ECM, their attachment to the endothelial cells through a surface receptor, and, consequently, their entry into the bloodstream. Finally, the observations that both anti-ANG monoclonal antibody and ANG antagonists could delay the appearance of HT-29-induced tumor in vivo suggest the stimulatory effects of ANG on tumor growth (12, 23).

In this context, we investigated the relationship between the clinicopathological parameters of the pancreatic cancer patients and the degree of ANG mRNA expression or the level of the sANG concentration. In comparison to the patients with no ANG mRNA expression or a lower sANG concentration, the 1-year survival rate was significantly lower in the patients with increased ANG mRNA expression or an elevated sANG concentration. In addition, the more advanced stage cancers or the larger cancers were more frequent in these patients. From these observations, along with the biological activities of ANG as described above, it can be suggested that the overexpression of ANG may contribute to the aggressiveness of pancreatic cancer.

References
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