Involvement of Angiopoietins in Cancer Progression in Association with Cancer Cell – Fibroblast Interaction

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Abstract. Purpose: The role of angiopoietins (ANGs) in cancer progression is a new field of research. We examined the patterns of expression of ANGs in human renal cancer tissues (n=12) and investigated their roles in cancer progression in vitro and in vivo.

Methods and Results: In normal renal tissue, the expression of ANG-1 was apparent in the glomerular capillaries and podocytes as well as in the endothelial cells of vessels, whereas we observed no expression of ANG-2. In contrast, in tumor tissue, dense, diffuse expression of ANG-1 was apparent in the fibroblasts, but not in the cancer cells. Intense ANG-2 expression was observed in the cancer cells themselves as well as in the endothelial cells, where its expression was restricted to small vessels or neoplastic capillaries and not mature vessels. Secondly, we investigated the influence of fibroblasts on the production of angiopoietins in cancer cells, using the human renal cancer cell lines SN12 and SN-PM6 and a human dermal fibroblast cell line (HDF). We examined the in vitro production of angiopoietins in these cell lines, in either monoculture of each cell line or co-culture of cancer cells and fibroblasts. Immunohistochemical study demonstrated marked production of ANG-1 in fibroblasts and ANG-2 expression in cancer cells when we performed co-culture, but no expression of either in each monoculture. Western blot analysis confirmed these results, showing marked expression of ANGs in co-cultured cells, but not in each monoculture. Conclusion: Fibroblasts may influence cancer progression by promoting neoplastic angiogenesis, and ANGs are profoundly involved in this process through their association with the carcinoma cell-fibroblast interaction.

Angiogenesis is required for a variety of physiological processes and for tumor growth and metastasis. Poorly vascularized tumors grow slowly or regress, but once tumors become vascularized they grow rapidly and aggressively. Among the various angiogenic factors, members of the angiopoietin (ANG) family are now considered to be essential molecules for cancer progression, in cooperation with vascular endothelial growth factor (VEGF). ANG was first reported by Davis et al. in 1996 (1) as a ligand for tyrosine kinase receptor-2 (TIE-2). The ANGs are a new family of growth factors that act specifically on vascular endothelial cells (1–3) and are involved in vascular maturation and/or remodeling. On the basis of structural disparity, four different members, ANG-1 to ANG-4, have been found in the ANG family. These molecules function via the TIE-2 receptor on endothelial cells, composing the so-called "Angiopoietin-TIE-2 receptor formation (1-3)." ANG-1 seems to be widely expressed in normal adult tissue, and is produced by periendothelial cells in the systemic vessels. It functions to promote vessel stabilization and maturation (2) by tightening the junction among endothelial cells, smooth muscle cells and pericytes (4, 5). In contrast, ANG-2, produced principally by endothelial cells, is dramatically induced only at sites of vascular remodeling, such as in the female reproductive tract. ANG-2 is known as a natural antagonist of ANG-1, acting as a competitive inhibitor at the TIE-2 receptor site (3). ANG-2 promotes destabilization of the vessel wall by loosening the junction between endothelial cells and surrounding cells. It disrupts blood vessel maturation, acting as a robust promoter of angiogenesis in cancer tissues in combination with other angiogenic factors such as VEGF (6, 7). ANG-3 is an agonist of ANG-1 and induces tyrosine phosphorylation of the TIE-2 receptor along with ANG-1. ANG-4, like ANG-2, is an antagonist of ANG-1.

The distinct roles of ANG-1 and ANG-2 – counteracting ligands for endothelium-specific TIE-2 receptor – in tumor development and progression remain poorly understood.

Key Words: Cytoplasm, fibroblast, angiopoietin, ANG-1, ANG-2.
although there have been several reports of ANGs and their expression and/or function in various neoplastic tissues. Sugimachi et al. (8) reported that expression of ANG-2 was observed in the more poorly-differentiated cells in human hepatocellular carcinomas in association with high levels of expression of TIE-2 in vascular endothelial cells and pericytes in the tumors, whereas ANG-1 expression was observed in normal hepatocytes. Hawighorst et al. (9) reported that expression of ANG-2, but not ANG-1, was up-regulated in angiogenic tumor vessels in the early stages of skin carcinogenesis, and was also observed strongly in squamous cell carcinoma (SCC) tissue. Moreover, stable overexpression of ANG-1 due to gene transfection in human A431 SCCs resulted in more than 70% inhibition of tumor growth as compared with control cells. This tumor inhibition was highly associated with enhanced TIE-2 phosphorylation levels in ANG-1-transfected SCC cells, whereas these levels were low in the controls. During early-stage epithelial tumorigenesis, ANG-2 may contribute to triggering of the angiogenic switch by counteracting the specific vessel-stabilizing effects of ANG-1.

We examined the expression of ANGs in human renal cancer tissue and investigated the influence of fibroblasts on the production of ANGs in cancer cells. We used human renal cancer cell lines SN12 and SN-PM6 and a normal human dermal fibroblast cell line (HDF). Our results indicated that ANGs may be profoundly involved in the process of cancer progression in association with the carcinoma cell – fibroblast interaction, and that modulation of ANG activity, which has been under investigation in our laboratory, may be a novel candidate strategy for cancer therapy.

**Materials and Methods**

*Patients.* Twelve patients – 7 men and 5 women – underwent radical nephrectomy between February 2001 and October 2002 at Osaka Medical College, Japan. The patients ranged in age from 28 to 70 years (average 55 years). According to the revised 1997 TNM classification, post-operative pathological assessment revealed pT1a in 5 patients, pT1b in 5, pT3a in one and pT3b in one. No patients had lymph node or distant metastasis. Cellular classification indicated that all patients had adenocarcinomas – 7 clear cell type and 5 granular cell type – and the tumors were all of histological grade 2.

*Immunohistochemistry.* The parts of the kidneys containing the tumors were frozen and stored at –80°C. Briefly, cryostat sections (about 10 μm thick) were air-dried and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.5, for 10 min at room temperature, and then washed twice with PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 15 min. The sections were then washed three times with PBS and incubated for 30 min with 5% bovine serum albumin in PBS. Excess blocking reagent was drained off, and the sections were incubated with polyclonal antibodies against ANG-1 or ANG-2 (1:50 dilution in 5% bovine serum, Santa Cruz, LA, USA) overnight at 4°C. The primary antibody was recognized by incubation of sections with HRP-conjugated anti-goat secondary antibody (1:100 dilution) for 2 h. The sections were rinsed with PBS and then incubated with amino ethyl carbazole (AEC) (Vector Laboratories Inc, Burlingame, CA, USA) for 15 min. The sections were counterstained with aqueous hematoxylin and then examined under a light microscope (Nikon, Tokyo, Japan).

*Immunoelectron microscopy analysis.* For immunoelectron microscopy, parts of both the normal renal tissues and the renal cancers were fixed in a solution containing 2% paraformaldehyde and 0.025% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2 h at 4°C. The specimens were then frozen with liquid nitrogen, thawed in 20% sucrose, and cut into 70-mm-thick serial sections with a microlicer (Dosaka, Kyoto, Japan) at 4°C. The sections were quenched with endogenous peroxidase with 0.05% H2O2 containing 0.1% gelatin and then stained by the indirect immunoperoxidase technique. After exposure for 5 min to a solution containing 0.05% 3,3’-diaminobenzidine tetrahydroxychloride and 0.01% H2O2 in Tris-HCl buffer at pH 7.6, sections were fixed with 1% osmium tetroxide in 0.1 M PB for 1 h, stained further with 1% uranyl acetate in 70% ethanol for 45 min, and then dehydrated and embedded in epoxy resin. Ultrathin sections (60 to 70 nm thick) were cut with an ultramicrotome equipped with a diamond knife. All samples were observed under an electron microscope (H-7100, Hitachi Ltd., Tokyo, Japan).

*Cell culture and immunohistochemical study.* We used two different human renal cancer cell lines, SN-12 and SN-PM6, and the HDF line. These cell lines were grown in culture medium (growth medium) containing high-glucose Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 2mL-glutamine (Gibco, Grand Island, NY, USA) non-essential amino acids, and penicillin-streptomycin and 10% fetal bovine serum (FBS). We performed either monoculture of each cell line (SN-12, SN-PM6, or HDFs) or co-culture of cancer cells (SN-12 or SN-PM6) and HDFs, in a cancer cells to HDF ratio of one to two. All cultures were maintained at 37°C in 5% CO2. The medium was replenished every 2 days at 80% confluence (approximately 4 or 5 days after co-culture).

*Immunocytochemistry in cultured cells.* Cultures were fixed in 5% formamide solution and subjected to immunocytochemical study using polyclonal antibodies against ANG-1, ANG-2 (1:100 dilution in 5% bovine serum, Santa Cruz), fibroblasts (1:200, Dako-Japan, Kyoto, Japan) and β-actin (1:200, Sigma, Tokyo, Japan) as primary antibodies. The expression of those molecules was detected as described above.

*Western blot analysis in cultured cells.* Cells were washed twice with PBS and were scraped. Cell lysates were sonicated three times on ice in radio-immunoprecipitation assay (RIPA) buffer. The protein concentration was determined by bichinchoninic acid (BCA) assay (BCA protein assay; Pierce, Rockford, IL, USA), and the proteins were separated by sodium dodecy1 sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). In all experiments, gels were stained...
with Coomassie blue to check the amount of proteins loaded on the gel and the homogeneity of the transfer. Immunodetection was performed after the blocking of membranes overnight at 4°C in blocking solutions [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Triton-X, and 3% bovine serum albumin], and samples were blotted with polyclonal antibodies against ANG-1, ANG-2 (1:100 dilution in 5% bovine serum, Santa Cruz) and β-actin (1:200, Sigma) for 1 h at room temperature. Immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) blotting system (Amersham Pharmacia Biotech, Uppsala, Sweden).

**Results**

**ANG expression in human tissue**

**ANG-1 expression**: In the normal renal tissues, expression of ANG-1 was observed in glomerular capillaries and podocytes in the glomeruli, as well as in the stromal vascular endothelial cells (Figure 1A-a). In contrast, expression of ANG-1 in renal cancer tissues was widespread in the stromal cells, but no immunoreactivity was observed in the cancer cells themselves (Figure 1A-b). The results of immunoelectron microscopic analysis basically corroborated those of the immunohistochemical analysis. In normal tissues, ANG-1 was expressed in the endoplasmic reticulum of glomerular endothelial cells (Figure 1A-c) and podocytes as well as stromal vascular endothelial cells. In contrast, ANG-1 expression in cancer tissues was observed in the endoplasmic reticulum of fibroblasts but not of cancer cells (Figure 1A-d).

**ANG-2 expression**: In the normal renal tissues, we observed no expression of ANG-2 (Figure 1B-a). In contrast, ANG-2 expression was observed in the cancer tissues, especially in the cancer cells themselves, and also in the endothelial cells of small vessels and neoplastic capillaries (Figure 1B-b). It was not apparent in large or mature vessels (Figure 1B-c). Immunoelectron microscopic analysis supported the immunohistochemical results. There was no marked ANG-2 expression in normal renal tissues. However, subcellular expression of ANG-2 was observed on the membranes of vesicles, which were abundant in the cytoplasm of cancer cells (Figure 1B-d).

The patients’ sex, age, tumor size, cancer cell type, or stage of disease did not significantly affect the expression patterns of ANG-1 and –2.

**Influence of fibroblasts on ANG expression in cancer cells in vitro**

To investigate the influence of fibroblasts on the production of ANGs in cancer cells, we examined the in vitro production of ANGs in human renal cancer cells (SN12 and SN-PM6 cells) as well as in fibroblasts. The cell lines were cultured in monoculture or in co-culture of either of the cancer cell lines together with the fibroblast line and then examined by immunohistochemistry and Western blot analysis. In the immunohistochemical analysis, we did not observe expression of ANG-1 or ANG-2 in cancer cells (SN12 or SN-PM6 lines) or fibroblasts (HDF cells) under monoculture (Figure 2A-a, -b, -c and -d). However, we found abundant expression of ANG-1 around the HDFs when we co-cultured either of the cancer cell lines and fibroblasts, especially in areas where the cancer cells and fibroblasts contacted each other. We did not observe ANG-1 expression in the cancer cells themselves (Figure 2B-a and -b). In contrast, ANG-2 was strongly expressed mainly in the cancer cells, but not in the HDFs, and its expression was intense in the areas where cancer cells and HDFs were in contact (Figure 2B-c and -d). Western blot analysis basically corroborated the results of the immunohistochemical study (Figure 2C). We did not find production of ANG-1 or ANG-2 in any of the monocultured cell lines (SN-PM6, SN-12, or HDFs). However, we found marked production of ANG-1 as well as ANG-2 in the cells when we performed co-culture of cancer cells and fibroblasts (SN-12 + HDFs and SN-PM6 + HDFs). These findings suggest that fibroblasts influence the production of ANGs in the cancer cells as well as in the fibroblasts themselves, and this may support our data derived from the human samples.

**Discussion**

Angiogenesis is essential for tumor growth and metastasis (10). It is a multifactorial phenomenon: many factors have been reported to be involved in this complicated process (11). The ANG-TIE-2 system, which includes two major mediators, ANG-1 and ANG-2, is a new, important family for the regulation of angiogenesis and vascular integrity (1–3, 9). Although only a few reports are available on the role of ANGs in neoplastic angiogenesis (7, 12, 13), their roles in embryonic development or normal tissues have been well described in several papers (3, 8, 14, 15). ANG-1 is expressed mainly in periendothelial cells (smooth muscle cells or pericytes). It binds the TIE-2 receptor, which is specifically expressed in endothelial cells, resulting in its autophosphorylation (1). In contrast, ANG-2, which is a natural antagonist of ANG-1, is expressed mainly in endothelial cells. It acts at the sites of vascular remodeling in adults and disrupts blood vessel maturation or stability of formation, whereas ANG-1 recruits and sustains periendothelial supporting cells (3).

Despite the fact that there have been several clinical studies of ANG expression in a variety of malignant tumors, such as neuroblastoma (16), breast carcinoma (4), colon carcinoma (5) and thyroid carcinoma (17), the exact localization and roles of ANG are not yet fully understood. Stratmann et al. (6) demonstrated that ANG-1 was expressed in almost all glioblastoma cells. In contrast, ANG-2 expression was restricted to vessels – especially small or immature vessels or neoplastic capillaries – and was not
Figure 1. Expression of angiopoietins in human renal tissue.
A. Representative pattern of ANG-1 expression in human renal tissue, analyzed by immunohistochemistry (a and b) and immunoelectron microscopy (c and d).

a: ANG-1 expression in normal renal tissue was present in glomerular capillaries and podocytes, as well as in the endothelial cells of vessels.
b: ANG-1 expression in cancer tissue was localized in the stromal cells, and especially in the fibroblasts, but not in the cancer cells.
c: ANG-1 expression (arrows) in normal tissue observed in the endoplasmic reticulum of glomerular endothelial cells. E: endothelial cells, L: capillary lumen, P: protuberance of podocyte.
d: ANG-1 expression in cancer tissue observed in endoplasmic reticulum of fibroblasts (arrow). Original magnification: x100 in a and b; x19,000 in c and d. Bar=1 μm

B. Representative pattern of ANG-2 expression in human renal tissue, analyzed by immunohistochemistry (a–c) and immunoelectron microscopy (d).

a: No expression of ANG-2 in normal renal tissue.
b: Intense ANG-2 expression in cancer cells and in the endothelial cells of small vessels or neoplastic capillaries in the tumor tissue.
c: No expression of ANG-2 in large or mature vessels in tumors.
d: Strong expression of ANG-2 on vesicles in the cytoplasm of cancer cells (arrows). Original magnification: x400 in a and b, x100 in c, and x29,000 in d. Bar=1 μm
Figure 2. In vitro production of angiopoietins in cancer cells and fibroblasts when cells were cultured in either monoculture or co-culture.

A. Immunohistochemical findings in each monoculture.
   (a). No expression of ANG-1 in HDF monoculture.
   (b). No expression of ANG-2 in HDF monoculture.
   (c). No expression of ANG-1 in SN-PM6 monoculture.
   (d). No expression of ANG-2 in SN-PM6 monoculture.

Original magnification: x200 in a–d

B. Immunohistochemical findings in co-cultured cells.
   (a, b). Marked production of ANG-1 around HDFs in co-culture of SN-PM6 cells and HDFs, especially in areas where cancer cells and fibroblasts were in contact with each other.
   (c, d). Marked expression of ANG-2 in SN-PM6 cancer cells but only faint expression in HDFs in a co-culture of SN-PM6 cells and HDFs, in the area where these cells were in contact.

Original magnification: x200 in a and c, x400 in b and d.
found in large, mature vessels. Moreover, they described a relationship between expression of ANGs and tumor grade. The expression of both ANG-1 and ANG-2 was frequently observed in high-grade, aggressive gliomas, but less often in low-grade gliomas (6).

Recently, several papers have reported that ANG-1 acts as an inhibitor of cancer progression, presumably by stabilizing vessel formation (4, 5). It may promote maturation of vessels, leading to inhibition of the intense and continuous new vessel development that is seen in tumor angiogenesis. In contrast, ANG-2 may promote cancer progression by destabilizing vessel walls. It may disrupt blood vessel maturation, acting as a robust promoter of angiogenesis and thus lead to cancer progression (4–7).

In our study, ANG-1 was diffusely expressed in the tumor tissues, and was primarily derived from fibroblasts, whereas ANG-2 was localized in the endothelial cells of small vessels or neoplastic capillaries, as well as in the cancer cells themselves. Our results may support the concept that ANGs are profoundly involved in the process of cancer progression in association with the carcinoma cell – fibroblast interaction, and that ANG-2 contributes to neoplastic angiogenesis and tumor expansion.

The growth of primary or metastatic carcinomas requires interaction between various cell types within the tumor, including neighboring normal epithelial and stromal cells (18, 19). Tumor cell proliferation may be affected by (a) direct fibroblast–tumor cell interactions, (b) fibroblast-derived soluble factors, (c) signals transduced by cell–matrix interactions, and (d) the extra cellular matrix (ECM) acting as a reservoir of several growth factors (19). The tumor stroma is considered to be an integral part of a neoplasm, with a major role in several steps in the metastatic cascade. Fibroblasts are the most abundant stromal host-cell type in desmoplastic tumors and the main source of various ECM components and modulators (19, 20). However, the role of fibroblasts in tumor growth and progression is controversial (21). For instance, there is an inverse relationship between host stromal response and lung metastasis in a metastatic animal model using Lewis lung carcinoma cells (22).

Alternatively, tumor growth could be explained by the presence of a tumor-cell-controlled mechanism for acquiring neovascularization. In this case, the stroma would play a favorable role for the growth of the neoplastic cells. Other studies have suggested that tumor-derived fibroblasts may induce tumor cell proliferation, probably because of a substantial phenotypic transformation (23, 24). In our present study, we investigated the influence of fibroblasts on the production of ANGs in cancer cells, using human renal cancer cell lines (SN12 and SN-PM6) and HDF cells. We examined the in vitro production of ANGs in cancer cells as well as in fibroblasts, in either monoculture or co-culture of both cell types. In our immunohistochemical study, we found marked production of ANG-1 around the fibroblasts and of ANG-2 in cancer cells when we performed co-culture, despite the lack of production of ANGs in each monoculture. Western blot analysis corroborated the results of our immunohistochemical study, showing marked production of ANGs in co-culture of cancer cells and fibroblasts, but no production of ANGs in monoculture. These findings suggest that fibroblasts influence the production of ANGs in the cancer cells as well as in the fibroblasts themselves. This may support the concept that fibroblasts exert agonistic forces on cancer progression by promoting neoplastic angiogenesis, and that ANGs are profoundly involved in this process through their association with the carcinoma cell – fibroblast interaction.

We found no significant correlation between the expression pattern of ANGs and histological grade or clinical stage of the tumors in this study. However, several reports have demonstrated that expression of ANG-2 is more frequently observed in poorly-differentiated carcinoma than in low-grade carcinoma in a variety of cancer tissues (4–7, 16, 17).

In conclusion, fibroblasts may influence cancer progression by promoting neoplastic angiogenesis, and ANGs are profoundly involved in this process through their association with the carcinoma cell – fibroblast interaction. Although further investigation is necessary, modulation of
ANG activity (e.g. inhibition of ANG-2 activity), which has been under investigation in our laboratory, may inhibit cancer growth or metastasis, and may be a novel candidate strategy for cancer therapy.

References


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