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ANGIOGENIN EXPRESSION IN SUPERFICIAL BLADDER CANCER

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The concentration of angiogenin in the tumor tissues and corresponding normal tissues of 20 superficial bladder cancer patients was measured using a sandwich enzyme immunoassay (ELISA). In addition, immunohistochemical assays were performed in order to clarify the localization of angiogenin expression in bladder tissue. The mean concentration of angiogenin in the carcinoma tissues was significantly lower than that in the corresponding normal tissues ($P<0.001$). Angiogenin expression was weak in the bladder cancer cells. The present results show that the expression of angiogenin is lower in superficial bladder cancer tissues than in corresponding normal tissues. The biological role of angiogenin in carcinogenesis of bladder cancer may be different from those of other angiogenic factors.

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Key words : Bladder cancer, Angiogenin, Angiogenesis

INTRODUCTION

Angiogenesis is one of the most important biological processes underlying organogenesis in general and tumorigenesis in particular[1]. It is essential for the successful growth of solid tumors and their acquisition of metastatic and invasive ability, and it is accomplished in multiple steps including migration, proliferation and differentiation of endothelial cells, in addition to proteolysis of the extracellular matrix[2,3]. To date, several angiogenic factors have been identified, and it has been determined that they control angiogenesis in concert.

Human angiogenin, a protein with a potent ability to induce new blood vessel growth, was initially isolated from conditioned medium of the human colonic adenocarcinoma cell line HT29 using an assay based purely on its function in angiogenesis[4]. Angiogenin promotes vascularization in the chicken chorioallantoic membrane and rabbit cornea[4]. However, it is not a tumor-specific product, and has also been isolated from non-malignant cells including human peripheral blood cells, vascular endothelial cells, fibroblasts, colon epithelial cells and urothelial cells[5-7] It is a protein which consists of a single chain: a 123-amino-acid polypeptide with a molecular weight of 14.2 kDa[8] Its sequence is homologous to the ribonuclease (RNase) A superfamily, although its ribonucleolytic activity is limited in comparison to that of RNases[8-10] Its ribonucleolytic activity seems to be necessary but not sufficient for angiogenic activity[11], and it seems to interact with endothelial cells via specific molecules[12] A series of studies has highlighted the interactions between angiogenin and plasminogen, plasminogen activators, actin and other factors, all of which have been implicated as part of a potential regulatory system for angiogenesis[13-15] The binding of angiogenin to endothelial cell surfaces is mediated by actin[13,15] The binding to actin is the first step in a process of internalization, nuclear translocation and nucleolar accumulation that leads to angiogenesis[16] Angiogenin is involved in nearly all steps and phases of angiogenesis. It binds to endothelial cells[17] and activates second messengers[12,18], thus mediating activation of basement membrane proteases which facilitate cell invasion[15,19], cell adhesion[20], construction of tubular structures from endothelial cells[21], and cell proliferation through mitogenesis[22] Although the physico-chemical properties of angiogenin and the mechanisms of signal transduction and receptor binding in vitro have been described, little is known about these processes in normal or pathological cellular environments.

As with other carcinomas, angiogenesis is crucial for the growth and metastasis of human bladder cancer[7]. In fact, human bladder cancer pathologically overexpresses several angiogenic factors, including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and platelet-derived endothelial cell growth factor (PD-ECGF), all of which are important in modulating tumor-host interactions that result in tumor neovascularization[23-26] However, there have been very few reports on angiogenin expression in human bladder cancer[7]

Studies of cancer of the colorectum, pancreas, ovary, breast and urothelium have shown that the serum angiogenin concentration is higher in cancer patients than in normal subjects[7,22-30] However, there was no correlation between serum concentra-

tion and tissue concentration of angiogenin in breast cancer patients[29] In the present study, we examined concentrations of angiogenin in tumor tissues and corresponding normal tissues of bladder cancer patients, in order to clarify its function in pathological cellular processes.

PATIENTS AND METHODS

The subjects were 20 patients with superficial bladder cancer pathologically diagnosed as transitional cell carcinoma. All patients were admitted to Nagasaki University Hospital between June, 1997, and August, 2000, and all samples (tumor tissues and corresponding normal tissues) were obtained during this period by cold cup biopsy. Adjuvant intravesical chemotherapy was administered to 10 patients, and immunotherapy was administered to 6 patients. Detailed clinicopathological features of patients are shown in Table 1. Pathological staging and histologic grade were diagnosed according to Union Internationale Contrele Cancer (UICC) and World Health Organization classification criteria, respectively[31,32] Informed consent was obtained from all 20 patients before this study was begun.

Immediately after cold cup biopsy, tumor tissues and corresponding normal tissue were immediately frozen in liquid nitrogen and stored at $-130°C$ until assayed. Protein lysates were extracted using the following procedure. Briefly, frozen tissues were lysed in 300 μl of ice-cold lysis buffer and homogenized with a sonicator. The lysis buffer consisted of 50 mM Tris-HCl (pH 7.5), 0.6 M NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM PMSF, 2.5 mM EDTA, 2.5 mM EGTA and 20 U aprotinin. After centrifugation for 15 min at 1,500×g, the supernatant was used for the angiogenin assays. An ELISA kit (R & D Systems, Inc., Minneapolis, MN, USA) was used for quantitative determination of tissue angiogenin concentration. Briefly, each protein lysate was diluted 1 : 150 and added to

microtiter wells precoated with murine monoclonal anti-human angiogenin antibody, and then incubated with horseradish peroxidase. After complete washing, a substrate solution (tetramethyl benzidine-H_2O_2 mixture) was added to the wells, and color intensity developed in proportion to the amount of bound angiogenin. Color development was stopped with sulfuric acid, and the intensity of the color was measured with a spectrophometer set to 450 nm. The angiogenin value was expressed as ng/mg protein.

Tissue samples were embedded in paraffin, and sections were cut from the paraffin blocks thus produced. These sections were incubated with goat polyclonal anti-angiogenin antibody (R & D Systems, Inc., Minneapolis, MN, USA) diluted at 40 ng/ml in PBS with 0.03% BSA and 0.05% N_2Na. Sections were then stained using the DAB immunoperoxidase method and counterstained with Mayer's hematoxylin, to visualize cell nuclei. The negative controls were sections from the same tissue block, that were incubated with goat IgG diluted 1/10,000 as the primary antibody. Immunohistochemical results were interpreted blinded to the results of the angiogenin expression assays (ELISA) and the clinicopathological features.

Differences in mean values were tested using unpaired Student's tests. Welch's test was also applied, where necessary. Pearson's correlation coefficient (r) was tested using the F test. All calculations were performed using Macintosh Stat View Software. A probability (P value) of <0.05 was considered to indicate significance.

RESULTS

1. ELISA

Fig. 1 shows the results of ELISA assay for angiogenin concentration. Angiogenin was detected in all of the bladder carcinomas and corresponding normal tissue samples. The angiogenin concentrations varied markedly among the patients. Quantitative evaluation by ELISA revealed that the average angiogenin concentration was 6.4±4.5 S.D. ng/mg protein (1.4–16.5 ng/mg protein) in bladder cancer tissues and 14.5±6.6 S.D. ng/mg protein (4.5–27.0 ng/mg protein) in normal tissues. Thus, the mean angiogenin concentration in normal tissue was significantly higher than that in carcinoma tissues ($P<0.001$). The angiogenin concentration showed no correlation with gender or age. In both tumor tissue and corresponding normal tissues, there was no significant correlation between angiogenin concentration and T stage (Ta or T1), main tumor size, history (primary or recurrent tumors) or multiplicity (solitary or multiple tumors) (Tables 2 and 3). Only 2 of the 20 patients, all of whom had undergone a complete resection, were found to have

Table 1. Clinicopathological features of 20 superficial bladder cancer patients

Age (y.o.)	mean±S.D. (range)	64±11 (33–84)
Gender	male	17
	female	3
Tumor stage	Ta	3
	T1	17
Tumor grade	G1	10
	G2	9
	G3	1
History	primary	15
	recurrence	5
Multiplicity	solitary	10
	multiple	10
Operation	TUR-Bt	17
	partial cystectomy	3

(a)

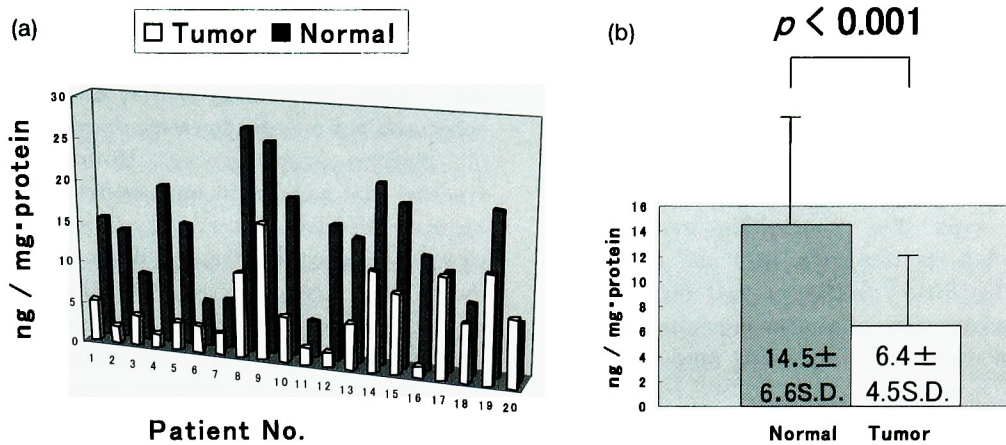□ Tumor ■ Normal

(b)

$p < 0.001$

Patient No.

Fig. 1. Assay of tissue angiogenin concentration by ELISA. (a) Expression of angiogenin was detected in all assayed bladder carcinoma tissues and corresponding normal urothelial tissues. In 18 of the total 20 patients, angiogenin concentrations detected in normal tissues were higher than those detected in cancer tissues. (b) The mean angiogenin concentration in carcinoma tissues was lower than that in the corresponding normal tissues ($P<0.001$).

Table 2. Angiogenin concentration in tumor tissues of 20 superficial bladder cancer patients

Feature		Mean±S.D. (ng/mg · protein)	P value
Tumor grade	G1	4.8±3.3	P=0.115
	G2, G3	8.0±5.2	
History	primary	6.4±3.9	P=0.987
	recurrence	6.4±6.8	
Multiplicity	solitary	6.6±4.9	P=0.820
	multiple	6.2±4.4	

Table 3. Angiogenin concentration in normal tissues of 20 superficial bladder cancer patients

Feature		Mean±S.D. (ng/mg · protein)	P value
Tumor grade	G1	13.5±6.0	P=0.510
	G2, G3	15.6±7.3	
History	primary	13.4±5.4	P=0.188
	recurrence	18.0±9.2	
Multiplicity	solitary	14.9±5.6	P=0.832
	multiple	14.2±7.7	

recurrent tumors during the follow-up period. Tumors recurred in those 2 patients at 26 and 9 months after resection, respectively. The angiogenin concentrations in carcinoma tissues of the 2 patients with recurrent tumors were 3.3 and 5.6 ng/mg

protein, and concentrations in the corresponding normal tissue were 5.5 and 15.0 ng/mg protein, respectively. There was no correlation between angiogenin concentration (in cancer or normal tissue) and tumor recurrence.
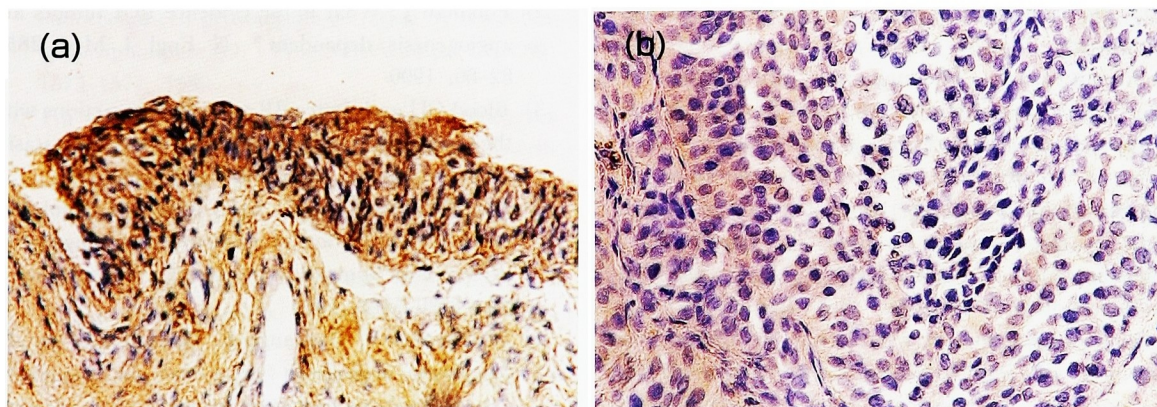

Fig. 2. Immunohistochemical staining of angiogenin in human bladder cancer and corresponding normal tissue, using an anti-angiogenin antibody. In normal tissue, angiogenin immunodetection showed a typical staining pattern in which angiogenin was confined to the cytoplasm of the urothelial cells and interstitial cells. Expression was noticeably weak in the bladder cancer tissue. (a) Normal tissue, ×200. The staining pattern indicated strong immunoreactivity in the cytoplasm. A small number of interstitial cells were weakly stained. (b) Bladder cancer, ×200. The pattern of angiogenin staining was significantly weaker than that of normal tissue.

2. Immunohistochemistry

The angiogenin staining pattern was homogeneous, and showed strong immunoreactivity in the cytoplasm of normal urothelial cells (Fig. 2(a)). A small number of stromal cells stained weakly. In carcinoma tissues, the angiogenin staining pattern was very weak (Fig. 2(b)). Our immunohistochemical data is consistent with the results we obtained by ELISA assay, in that both showed angiogenin concentrations in carcinoma tissues to be lower than those in corresponding normal tissues.

DISCUSSION

Because angiogenin is one of the most potent angiogenic factors, there have been several studies on the relationship between angiogenin and neoplasms. However, there have been few reports of studies of angiogenin in bladder cancer patients[7] In the present study, we used ELISA to evaluate the concentration of angiogenin in carcinoma tissues and corresponding normal tissues of superficial urinary bladder carcinoma patients, and used immunohistochemical analysis to investigate the localization of angiogenin in urothelial cells. Miyake et al. previously reported that serum levels of angiogenin in patients with invasive urothelial carcinoma were significantly higher than angiogenin levels in healthy controls and patients with superficial carcinoma[7] They found that overall survival of patints with elevated serum levels of angiogenin was lower than that of patients with normal levels. As many previous studies have shown, an elevated serum level of angiogenin is an independent prognostic predictor for a variety of carcinomas. However, in the present study, expression of angiogenin was detected in all of the assayed bladder carcinoma tissues and corresponding normal urothelial tissue, and the mean angiogenin concentration was significantly higher in normal tissues than in carcinoma tissues. The angiogenin concentration in tissue in the present study is different from the serum concentration reported by many other authors. Montero et al. observed no correlation between serum concentrations and tissue concentrations of angiogenin in breast cancer patients[29] Furthermore, when they compared tissue levels of angiogenin with clinicopathological factors, they found that an elevated angiogenin concentration in tissue was associated with smaller tumors and carcinomas of low or moderate histological grade, implying an association between expression of angiogenin and reduced aggressiveness of tumors[29] Similarly, the present results suggest a lack of association between angiogenin concentration in serum and tissue in bladder cancer. In our immunohistochemical assay, the staining pattern for angiogenin was homogeneous and showed that there was stronger cytoplasmic

immunoreactivity in normal urothelial cells and stromal cells than in superficial urothelial carcinoma cells. These results suggest that tissue angiogenin concentrations may be down-regulated in the course of bladder carcinogenesis. However, Miyake reported that angiogenin was strongly expressed in invasive bladder cancer[7] Two hypotheses are clearly suggested by these findings. One is that during the course of tumor progression, angiogenin expression may be up-regulated by genetic factors. Another is that there are two distinct angiogenic pathways involved in different stages of bladder cancer (superficial or invasive tumors)[33] When we examined for relationships between angiogenin concentration and clinicopathological factors, we found no correlation between angiogenin levels and T stage (Ta or T1), grade, main tumor size, history or localization. Other important angiogenic factors such as VEGF and PD-ECGF may be involved in bladder carcinogenesis[23-26] It has been reported that these angiogenic factors are expressed at higher levels in tumor tissues than in surrounding normal tissues. Our results suggest that the role of angiogenin may be different from those of other angiogenic factors in the superficial growth phase. Further detailed research will help clarify the role of angiogenin in the adhesion of tumor cells.

ACKNOWLEDGMENTS

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和文抄録

表在性膀胱腫瘍における Angiogenin の発現

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血管新生は腫瘍の発育，進展，浸潤，転移に不可欠な役割を担っていると考えられており，とくに腫瘍の発育には様々な血管新生因子が関与している．今回われわれは，表在性膀胱腫瘍患者（移行上皮癌）を対象に，血管新生因子の1つである angiogenin の組織内濃度について検討を行った．

20名の表在性膀胱腫瘍患者の腫瘍組織内と正常組織内それぞれの angiogenin 濃度をサンドウィッチ ELISA 法を用い測定した．また膀胱組織内における angiogenin の局在を明らかにする目的で免疫染色を行った．

腫瘍組織内の angiogenin 濃度は正常組織内の濃度と比較して有意に低下していた（$P < 0.001$）．また angiogenin の発現は正常移行上皮に高く，腫瘍組織内において明らかに低下していた．

膀胱腫瘍患者の組織内での angiogenin の発現は正常組織と比較すると腫瘍組織内で有意に低下していた．この結果は，膀胱腫瘍の発育の過程では angiogenin は他の血管新生因子とは異なる役割を担っている可能性が示唆された．

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