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Advances in Brief

Apoptosis and Tumor Angiogenesis in Cervical Cancer after Preoperative Chemotherapy

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Abstract

The correlation between apoptosis and tumor angiogenesis in uterine cervical cancer treated by preoperative intraarterial infusion chemotherapy (IAC) was investigated. Cervical cancer samples surgically obtained from 12 patients (stages Ib-IIIb) receiving IAC and from 10 patients (stages Ib-IIb) receiving no chemotherapy and biopsy specimens from the 12 patients before IAC were examined. The apoptotic index (AI) was determined with an *in situ* end-labeling assay. Intratumoral microvessel density (IMVD) and thymidine phosphorylase (dThdPase) expression were evaluated immunohistochemically using anti-CD34 and anti-dThdPase antibodies. AIs were higher in the 8 patients with complete or partial response to IAC than they were in the 4 nonchemoresponsive patients and in the 10 patients who received no chemotherapy ($P < 0.01$) and were inversely related to IMVDs ($r = 0.724$; $P < 0.01$). AIs and IMVDs after IAC were higher and lower than those before IAC ($P < 0.01$), respectively. The expression of dThdPase, which has angiogenic activity, was markedly decreased after IAC. These results suggest that the antitumor effects of IAC are closely associated with apoptotic cell death, which may be influenced in part by the extent of tumor angiogenesis inhibition.

Introduction

Apoptosis, often referred to as programmed cell death, is a physiological process for the elimination of specific types of cells, occurring extensively in embryonic development, metamorphosis, and differentiation (1). This process also plays a crucial role in both proliferation and cell turnover in various tumors. Recent studies revealed that cells from a wide variety of human malignancies generally responded to some physiological stimuli that may contribute to tumorigenesis, growth, and metastasis (2). Conversely, tumor cells undergo apoptosis under nonphysiological conditions, such as radiation and chemotherapy, leading to the suppression of tumor growth and micrometastases (2, 3).

Tumor angiogenesis is essential for tumor development. Prevascular tumors may remain dormant *in situ* for months or years, and switching from a subgroup of prevascular tumor cells to an angiogenic phenotype enables rapid growth, progression, and metastasis (4). Angiogenesis inhibitors can induce and sustain the dormancy of experimental primary tumors and micrometastases by elevating the incidence of apoptosis in tumor cells, although the proliferation rate remains unchanged (5, 6). Therefore, a correlation between apoptosis and tumor angiogenesis might be expected in human solid tumors.

Recent investigations have revealed that the incidence of spontaneous apoptosis in tumor cells is inversely related to the extent of neovascularization, and that tumor angiogenesis may contribute to a reduction of apoptosis in tumor cells (7). However, the correlation between apoptosis and angiogenesis in human solid tumors treated by chemotherapeutic agents has not yet been evaluated. In the present study, we investigated AI[2] and IMVD in uterine cervical cancer tissues from patients who underwent radical surgery after preoperative chemotherapy. In addition, immunohistochemical staining for dThdPase/PD-ECGF was conducted simultaneously to evaluate the relationship between apoptosis and the angiogenic activity of tumor cells treated by preoperative chemotherapy.

Materials and Methods

Patients and Tissue Samples. In the past 3 years, preoperative IAC was administered to 38 patients with locally advanced cervical cancer (the International Federation of Gynecology and Obstetrics stages Ib-IIIb) at our institution. Subjects with stage Ib and IIa carcinomas were required to have tumors larger than 4 cm in diameter. Among these patients, the 12 who underwent a radical hysterectomy after the completion of chemotherapy were enrolled in this study. The other 26 patients were treated further with radiotherapy. For IAC, 5-French polyethylene catheters were inserted through both femoral arteries by Seldinger's technique, and each catheter tip was placed in the anterior division of the internal iliac artery, just distal to the branching out of the superior gluteal artery. The regimen used was mitomycin-C (10 mg/m^2), therarubicin (10 mg/m^2), and cisplatin (100 mg/person). The course was repeated twice at an interval of 21 days. Fifteen days from the end of the second IAC course, a clinical response to IAC was evaluated by gynecological examination and magnetic resonance imaging. Clinical CR was defined as the complete disappearance of all objective clinical evidence of disease. PR was defined as a greater than 50% reduction in tumor volume. SBD was used for a decrease in tumor volume of less than 50%. Radical surgery was performed within 14 days after the evaluation of clinical response to IAC. Uterine samples surgically obtained from the 12 patients after IAC and from an additional 10 patients (the International Federation of Gynecology and Obstetrics stages Ib-IIb) with locally advanced cervical cancer receiving no preoperative chemotherapy and the 12 biopsy specimens obtained before IAC were fixed in 10% formalin and embedded in paraffin wax. Serial sections including the greatest diameter of the tumors from the operative specimens and including tumor cells from the biopsy specimens were used for the present study.

Determination of AI. DNA breaks were detected *in situ* by TUNEL according to the method of Gavrieli *et al.* (8) with some modifications as described previously (9). Paraffin sections were de-waxed, rehydrated through a graded alcohol series, and washed with PBS. Subsequently, the tissues were digested with 20 μg/ml proteinase K (Sigma, St. Louis, MO) for 15 min at room temperature and then washed with distilled water and subsequently with PBS. The tissues were incubated with a solution containing 2% H_2O_2 in PBS to inhibit endogeneous peroxidase activity and then washed with PBS. TdT buffer solution [100 mM potassium cacodylate, 2 mM cobalt chloride, and 0.2 mM DTT (pH 7.2)] containing 0.3 unit/μl TdT (Oncor, Gaithersburg, MD) and 0.04 nmol/μl digoxigenin-dUTP (Oncor) was added to cover the tissues, which were then incubated in a humidified atmosphere for 60 min at 37°C. The tissues were washed with buffer solution containing 300 mM sodium chloride and 30 mM sodium citrate for 30 min at 37°C to terminate the reaction and then washed with PBS. They were subsequently incubated with anti-digoxigenin-

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[2] The abbreviations used are: AI, apoptotic index; IMVD, intratumoral microvessel density; dThdPase, thymidine phosphorylase; PD-ECGF, platelet-derived endothelial cell growth factor; IAC, intraarterial infusion chemotherapy; CR, complete response; PR, partial response; SBD, stable disease; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated nick end labeling.

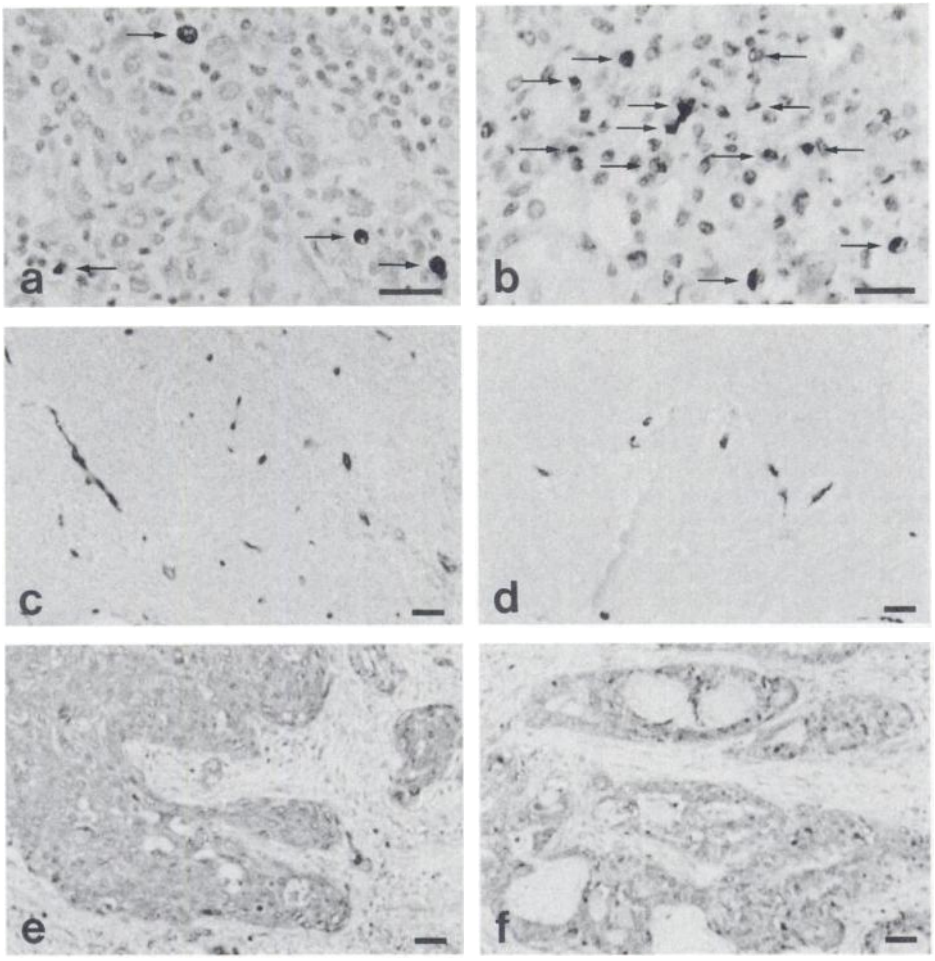

Fig. 1. TUNEL signals, intratumoral microvessels, and dThdPase expression in cervical cancer. Intense TUNEL signals are demonstrated in the nuclei of some tumor cells from both a patient who received no preoperative chemotherapy with an AI of 1.8% (*arrows* in *a*) and a chemoresponsive patient after IAC with an AI of 12.0% (*arrows* in *b*). Intratumoral microvessels are detected as consistent staining of endothelial cells using anti-CD34 antibody (*c* and *d*). The IMVDs, 52 in *c* and 12 in *d*, are shown for the area used for counting the AIs of *a* and *b*, respectively. Immunoreactivity of dThdPase is mainly identified in the cytoplasm of tumor cells. There is also a strong staining in squamous cell carcinoma (*e*) and adenocarcinoma (*f*). *Scale bars*, 30 μm.

Table 1 *Clinical features, AI, IMVD, and dThdPase expression of the 22 uterine cervical cancer tissue samples analyzed*

Case no.	Age (yr)	Stage[a]	Histological type[b]	Clinical response	AI[c] (%)	Average[d] (%)	IMVD[e]	Average[d]	dThdPase[f]
1	59	IIIb	NKL	CR	12.8 ± 2.6[g]	12.4 ± 2.1	10.2 ± 1.9[g]	16.0 ± 7.1	−
2	72	IIb	NKL	CR	12.0 ± 1.6		21.8 ± 5.1		−
3	55	Ib	AD	PR	9.2 ± 1.9	7.6 ± 1.8	24.2 ± 7.3	24.4 ± 7.4	1+
4	57	IIa	NKL	PR	5.8 ± 0.8		25.6 ± 8.6		−
5	63	IIb	NKL	PR	7.6 ± 1.8		25.2 ± 7.7		1+
6	48	IIb	NKL	PR	6.4 ± 1.3		20.0 ± 7.2		−
7	59	IIIb	NKL	PR	8.6 ± 1.5		27.2 ± 10.3		1+
8	57	IIIb	NKL	PR	8.0 ± 1.6		24.4 ± 4.2		−
9	53	IIb	NKL	SBD	2.8 ± 0.8	3.4 ± 1.3	89.8 ± 9.5	69.1 ± 16.9	1+
10	36	IIIb	K	SBD	3.0 ± 0.7		59.6 ± 4.7		2+
11	56	IIIb	K	SBD	4.2 ± 1.5		60.2 ± 18.8		2+
12	54	IIIb	NKL	SBD	3.4 ± 1.7		66.6 ± 11.3		1+
13	35	Ib	K		2.8 ± 1.3	2.2 ± 1.8	63.2 ± 13.0	69.9 ± 24.3	2+
14	71	Ib	NKL		1.4 ± 0.9		86.8 ± 10.7		−
15	44	Ib	AD		1.8 ± 1.5		76.6 ± 10.5		2+
16	45	IIb	K		1.4 ± 1.1		79.6 ± 19.3		1+
17	61	IIb	K		4.4 ± 1.5		42.2 ± 5.4		2+
18	52	IIb	NKL		5.4 ± 1.3		48.4 ± 8.0		2+
19	34	IIb	NKL		1.8 ± 1.3		55.8 ± 13.0		1+
20	51	IIb	NKL		0.8 ± 0.8		118.6 ± 18.9		2+
21	47	IIb	NKS		1.2 ± 0.8		70.0 ± 14.3		1+
22	32	IIb	ADSQ		1.4 ± 1.1		57.8 ± 10.6		1+

[a] Stages are presented according to the classification of the International Federation of Gynecology and Obstetrics.

[b] K, keratinizing-type squamous cell carcinoma; NKL, nonkeratinizing large cell-type squamous cell carcinoma; NKS, nonkeratinizing small cell-type squamous cell carcinoma; AD, adenocarcinoma; ADSQ, adenosquamous carcinoma.

[c] Occurrence of TUNEL-positive nuclei within five fields at ×200.

[d] Nonparametric test showed that the mean AI or IMVD was significantly higher or lower in the 2 CR patients (cases 1 and 2) and the 6 PR patients (cases 3–8) with IAC than it was in the 4 SBD patients (cases 9–12) and the 10 patients (cases 13–22) who received no chemotherapy ($P < 0.01$), respectively.

[e] Number of intratumoral microvessels within five fields at ×200.

[f] The immunoreactivity of dThdPase is expressed as a percentage and assigned to one of three subgroups: −, <10%; 1+, 10–50%; and 2+, >50%.

[g] Mean ± SD.

peroxidase complex for 30 min at room temperature and stained with a solution of 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 in Tris-HCl buffer (DAB solution) at pH 7.6 for 3–6 min at room temperature. The sections were counterstained with 1% hematoxylin. Negative controls were obtained by omitting TdT from the buffer solution. The AI was calculated as the ratio of TUNEL-positive cancer cells:the total number of cancer cells and obtained in more than five 10 × 20 microscopic fields.

Determination of IMVD. Immunohistochemical staining for CD34 to highlight endothelial cells (10) was performed using the avidin-biotin-peroxidase complex method. Briefly, de-waxed and rehydrated tissue sections were incubated overnight at 4°C with mouse monoclonal anti-CD34 antibody (QB-END/10; Novocastra Laboratory, Newcastle, United Kingdom) at a 1:50 dilution and then washed with PBS. Biotinylated horse antimouse immunoglobulin (DAKO, Kyoto, Japan) was then added to the sections for 30 min at room temperature. Peroxidase-conjugated avidin (DAKO) was applied after the sections were washed with PBS. Peroxidase activity was detected by exposure of the sections to the DAB solution as described above. The sections were counterstained with hematoxylin. Normal mouse IgG was used as a substitute for the primary antibody for the negative controls. The sections showed a frequently heterogeneous staining pattern for anti-CD34 antibody. For the determination of IMVD, the five most vascular areas within a section were selected and counted under a light microscope with a 200-fold magnification (*i.e.*, ×20 objective lens and ×10 ocular lens; 0.7386 mm^2/field) as described by Weidner *et al.* (11). The average numbers were recorded as the IMVD for each case.

Immunohistochemical Staining for dThdPase. To evaluate the effect of preoperative chemotherapy on the angiogenic activity of the tumor cells, we performed an immunohistochemical study for dThdPase, also known as PD-ECGF, which is an enzyme implicated in tumor-associated angiogenesis (12, 13). A mouse monoclonal anti-dThdPase antibody was kindly provided by the Nippon Roche Research Center (Kamakura, Japan). Tumor sections were heated twice in an oven at 70°C for 5 min and then incubated overnight at 4°C with anti-dThdPase antibody at a 1:100 dilution and washed with PBS. The following steps were the same as those used for the anti-CD34 protocol. The immunoreactivity of dThdPase is expressed as a percentage of the number of dThdPase-positive cancer cells:the total number of cancer cells and assigned to one of three subgroups: (*a*) −, <10%; (*b*) 1+, 10–50%; and (*c*) 2+, >50%.

Statistical Analysis. A nonparametric test was used to determine the statistical correlation among AI, IMVD, and dThdPase expression before and after IAC. The Spearman rank correlation coefficient was also used to analyze the relationship between the AI and IMVD.

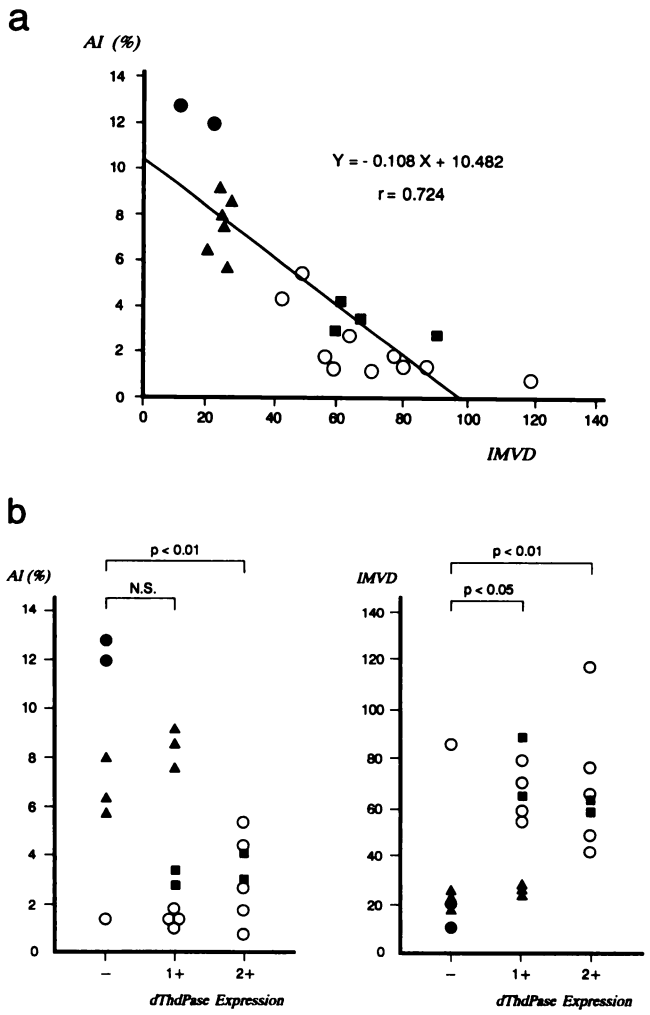

Fig. 2. The correlation among AI, IMVD, and dThdPase expression (●, CR; ▲, PR; ■, SBD; ○, nontreated). *a*, regression analysis with the Spearman rank correlation coefficient on plots of AI *versus* IMVD on a per case basis ($r = 0.724$; $P < 0.01$). *b*, the relationship of AI and IMVD to the expression of dThdPase.

Results

The evaluation of clinical response after IAC revealed a CR in 2 of the 12 patients, a PR in 6 patients, and SBD in 4 patients. Tissue samples from these 12 patients before and after IAC and from an additional 10 patients who received no preoperative chemotherapy were examined in the TUNEL and immunohistochemical studies.

Intense TUNEL signals were observed in the nuclei of some tumor cells before and after IAC (Fig. 1, *a* and *b*). The immunoreactivity of anti-CD34 antibody was located only on the cytoplasm of endothelial cells, and not on tumor cells or interstitial cells (Fig. 1, *c* and *d*). In contrast to the immunoreactivity of anti-CD34 antibody, that of dThdPase was observed only in the cytoplasm of tumor cells (Fig. 1, *e* and *f*). The negative control slides for TUNEL, CD34, and dThdPase exhibited no specific staining.

Table 1 shows the overall results including clinical data, histological type, AI, IMVD, and dThdPase expression. The mean AI was significantly higher in the 8 patients (cases 1–8) with CR or PR to IAC than it was in the 4 patients with SBD (cases 9–12) and in the 10 patients who received no chemotherapy (cases 13–22; $P < 0.01$). In contrast to the AI, the mean IMVD was significantly lower in the chemoresponsive group (cases 1–8) than it was in the nonchemoresponsive (cases 9–12) and control (cases 13–22) groups ($P < 0.01$). Regression analysis with the Spearman rank correlation coefficient on plots of AI *versus* IMVD on a per case basis revealed a significant inverse correlation between them ($r = 0.724$; $P < 0.01$), as shown in Fig. 2*a*. The relationship of AI and IMVD to the expression of dThdPase was also evaluated. As shown in Fig. 2*b*, the AIs in the tumors with 2+ dThdPase-positive staining were significantly lower than those in the tumors that were negative for dThdPase staining. However, the IMVDs in tumors with dThdPase-positive staining from 1+ to 2+ were significantly higher than those in the tumors that were negative for dThdPase staining. The results of AI, IMVD, and dThdPase expression after IAC were compared with those before IAC using the biopsy specimens. There was a significant increase in the AI and a significant decrease in the IMVD after IAC ($P < 0.01$). In addition, dThdPase immunoreactivity was markedly decreased in the chemoresponsive patients after IAC, as shown in Fig. 3.

Discussion

Preoperative IAC has recently been investigated as a new therapeutic approach to locally advanced cervical cancer because of the disappointing results with conventional treatments, and data from different studies suggest that cervical cancer is a chemosensitive tumor (14, 15). The use of IAC may shrink bulky tumors before surgical and radiation treatment and may also reduce the incidence of lymph node metastasis. After IAC, radical surgery can remove residual central disease, enable the evaluation of lymph node status, and

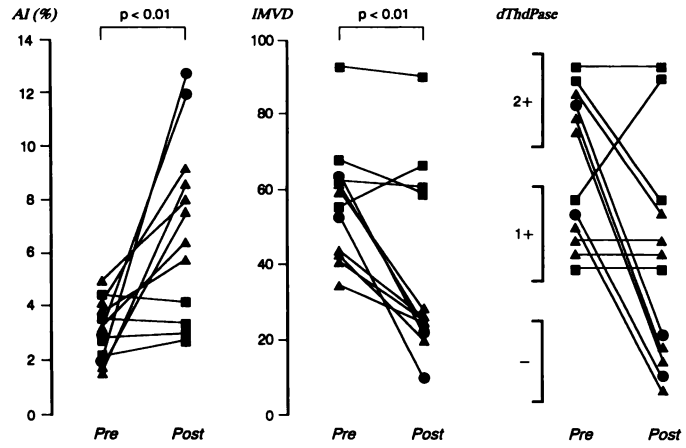

Fig. 3. Changes in AI, IMVD, and dThdPase expression before and after IAC (●, CR; ▲, PR; ■, SBD). There was a significant increase in the AI and a significant decrease in the IMVD after IAC ($P < 0.01$). The dThdPase immunoreactivity was markedly decreased in chemoresponsive patients after IAC.

presumably improve the cure rate in a high percentage of cases that had previously been considered to be inoperable (15). Various combinations of drugs have been used in such studies; however, the mechanism that leads to cell death and reduces the micrometastases induced by preoperative chemotherapy remains to be elucidated.

The present results demonstrated that apoptosis occurred more frequently in the tissues from the 8 CR and PR patients than in those from the 4 SBD patients and the 10 who received no preoperative chemotherapy. The AIs of the chemoresponsive cases after IAC were also significantly higher than those before IAC. These results suggest that preoperative IAC using a combination of mitomycin-C, therarubicin, and cisplatin may induce apoptotic cell death in locally advanced cervical cancer. A marked decrease in the IMVD was observed in the tissues from the chemoresponsive patients after IAC, and there was a significant inverse correlation between the AI and the IMVD. The inhibition of angiogenesis limits tumor growth by elevating the incidence of apoptosis (5, 6). In other words, the ability of tumor cells to undergo apoptosis can be enhanced by the inhibition of neovascularization. The present finding that the AIs in the hypovascular condition were significantly higher than those in the hypervascular condition indicates that apoptotic cells concomitantly increase when intratumoral microvessels decrease. The antitumor effects of preoperative IAC may be associated not only with the chemosensitivity of individual tumor cells themselves, but also with the extent of tumor angiogenesis inhibition.

A recent study has demonstrated that dThdPase is almost identical to PD-ECGF (12). dThdPase, an enzyme involved in pyrimidine nucleotide metabolism, has been shown to have angiogenic activity (12, 13). Haraguchi *et al.* (16) reported that dThdPase, through the degradation products of thymidine, stimulates the chemotaxis of endothelial cells and possibly other cells and thus indirectly stimulates angiogenesis. Our present study of human cervical cancer indicated that dThdPase immunoreactivity in tumor cells was well correlated with IMVD, and its expression was markedly decreased in chemoresponsive patients after IAC. The inhibitory effects of preoperative IAC on tumor angiogenesis may be mediated by a decreased level of dThdPase produced by tumor tissues. Various other peptide growth factors such as vascular endothelial growth factor (17), basic fibroblast growth factor (18), and transforming growth factor-α (19) have been found to stimulate the proliferation and motility of endothelial cells, thus inducing new blood vessel formation. Very recently, we also reported that epidermal growth factor and transforming growth factor-α stimulated the production of dThdPase in human cervical cancer cells, which may be associated with their invasive activity (20). Because tumor angiogenesis is a complex multistep process controlled by such various growth factors, it is unlikely that the inhibitory effects of preoperative IAC on the angiogenic phenotype of cervical cancer can be explained by the expression of dThdPase alone. Additional studies are needed to clarify the molecular events leading to the tumor angiogenesis inhibition induced by IAC.

To our knowledge, this is the first report to highlight the relationship between apoptosis and angiogenesis in human solid tumors after preoperative chemotherapy. Although preliminary, these results are potentially important in translating proven laboratory data into clinically relevant findings that may influence the treatment of human malignant tumors.

Acknowledgments

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