Intraperitoneal Infusion of Recombinant Plasminogen Activator Inhibitor Type 2 Induced Apoptosis in Implanted Human Colon Cancer and Inhibited its Growth and Liver Metastasis

DAIHACHIRO SUWA[1], HIROYUKI KONNO[1], TATSUO TANAKA[1] and TETSUMEI URANO[2]

Departments of [1]Surgery and [2]Physiology, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu, Shizuoka 431-3192, Japan

Abstract. *Antitumor effects of plasminogen activator (PA) inhibitors (PAIs) were analyzed in a mouse model of human colon cancer xenografts. Either recombinant PA inhibitor-1 (rPAI-1) or inhibitor-2 (rPAI-2) was injected intraperitoneally to nude mice bearing human colon cancer xenografts for 6 weeks. Primary tumors in rPAI-2-treated group were smaller (0.45±0.13 g, n=16) than in the other two groups (control: 0.73±0.24 g, n=15; rPAI-1: 0.62±0.29 g, n=19). Primary tumors in the rPAI-2-treated group exhibited less mature ductal structures and were significantly smaller. The apoptotic index was higher in the rPAI-2-treated group (4.64±2.12%) than in the other groups (control: 1.94±0.82%; rPAI-1: 2.08±1.07%). Liver metastasis was less frequent in the rPAI-1(5/19) and rPAI-2-treated groups (1/16) than in the control group (14/15). PAI-2 more effectively suppressed tumor metastasis and progression, probably by inducing apoptosis; some different unknown mechanism may cause the difference in both antitumor effect and the histological findings. This may indicate the therapeutic potential of these PAIs in malignant patients.*

The plasminogen activator (PA)-plasmin system is considered to promote tumor growth, invasion and metastasis. Urokinase type PA (uPA) plays a key role together with its specific receptor (uPAR) on the cell surface (1). uPA catalyzes the conversion of plasminogen to active plasmin, which degrades a variety of extracellular matrix proteins either directly or indirectly through the activation of metalloproteases (2). Elevated uPA activity in tumor tissue is related to the proliferation and invasion of tumors, thus, its content in tumor tissues could be a prognostic marker in malignant tumors (3).

Correspondence to: Daihachiro Suwa, Department of Surgery, National Hospital Organization Tenryuu Hospital, 4201-2 Oro, Hamakita-ku, Hamamatsu, Shizuoka 434-8511, Japan. Tel: +81 53 5833111, Fax: +81 53 5833664, e-mail: dsuwa@apost.plala.or.jp

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uPA activity is regulated by two physiological inhibitors of PA, inhibitor type 1 (PAI-1) and type 2 (PAI-2), members of the serine protease inhibitor superfamily (4). These inhibitors have also been detected by immunohistochemical staining and their mRNAs have been detected by *in situ* hybridization in cancer cells (5), as well as in benign inflammatory cells in tumor tissues (2). Contrary to researchers' expectations that these inhibitors would suppress tumor growth *via* uPA inhibition, many clinical studies have demonstrated that high PAI-1 levels are related to a poor prognosis for the survival of patients suffering from a variety of cancers (6, 7). In contrast, PAI-2 was demonstrated to be related to good prognosis (8, 9) when it was present in large amounts in tumor tissues, at least under certain conditions. We have also reported that low PAI-2 expression was associated with higher potential of lymph node metastasis in cancers of the breast, stomach and lung (10-12).

To further examine the ability of PAI-2 to suppress both tumor growth and metastasis, the antitumor effects of intraperitoneal infusion of recombinant PAI-2 (rPAI-2) were assessed and compared to those of rPAI-1 in a human colon cancer orthotopic xenograft model.

Materials and Methods

Recombinant human PAI-1 (rPAI-1) and PAI-2 (rPAI-2). rPAI-1 was purified from *Escherichia coli* (*E. coli*) strain SG20043 containing plasmid pDL06 (13); rPAI-2 was also purified from *E. coli* strain SG20043 containing plasmid pPM7 (14). Both of these transformed *E. coli* strains were kindly provided by Dr. T. Ny, Umea University, Sweden. The purified proteins were filtered through a syringe filter and the amount containing endotoxin was shown to be less than 130 pg/ml. These PA inhibitors were titrated using two chain uPA (Mitsubishi Pharma Co., Osaka, Japan) and the specific activity was expressed in uPA units.

Human colon cancer xenografts. A human colon cancer xenograft model was used in this study (15). TK-4, an adenocarcinoma cell line, was established in our department from a metastatic liver lesion of a 50-year-old Japanese male with sigmoid colon cancer, and was maintained by passage in nude mice (*BALB/c-nu/nu*

males) (Clea Japan, Inc., Tokyo, Japan). Small pieces of tissue were resected aseptically during the exponential growth phase from TK-4 tumor which was maintained in nude mice. The colorectal parts of the intestines of male *BALB/c-nu/nu* mice at 5 weeks of age were carefully exteriorized. A tumor piece of 5 mm in diameter was then fixed on each serosa surface with a 6-0 Dexon (DavisGeck, Inc., Manati, Puerto Rico) transmural suture. The intestine was returned to the abdominal cavity, and the abdominal wall and skin were closed with 6-0 Dexon sutures. The animals were kept in a sterile environment (16).

Assay of tumor growth and liver metastasis. Animals of rPAI-1-treated group (n=19) or rPAI-2-treated group (n=19) were injected intraperitoneally with rPAI-1 or rPAI-2 at a dose of 300 IU from day 4 after tumor implantation, 6 days per week for 6 weeks. As a control, the same volume of phosphate-buffered saline (PBS) was given to the control mice. Mice were sacrificed 6 weeks after the implantation. Autopsy was performed immediately and the transplanted tumors growing on the caecal wall were removed. After the tumors were weighed, they were quickly frozen and stored in liquid nitrogen until use. Liver metastasis was evaluated macroscopically and then confirmed histologically.

Tissue extract samples. For extraction, tissue pieces of 300-500 mg wet weight were pulverized at 4˚C in 100 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 1% Triton X-100. After incubation at 4˚C for 3 hours, the extract was centrifuged at 12,000xg at 4˚C for 10 min. The supernatant was collected and stored in liquid nitrogen until use.

Assay of uPA, uPAR, PAI-1 and PAI-2. Antigen levels of uPA, uPAR, PAI-1 and PAI-2 were measured in the tissue extract samples using commercially available ELISA kits (Tint Elize uPA and Tint Elize PAI-1, Biopool, Umea, Sweden; IMUBIND uPAR and IMUBIND PAI-2, American Diagnostica, Greenwich, USA).

Evaluation of apoptosis. Xenografted tumor samples were fixed in 10% formalin and embedded in paraffin. Tissue sections of 3-μm thickness were stained with hematoxylin and eosin. The apoptotic index was quantified by counting the number of apoptotic cells seen under a light microscope at 400-fold magnification. The distinctive morphological features of apoptosis, as described by Kerr (17) and Walker (18) were used to recognize apoptotic cells, and any doubtful cells were disregarded. A minimum of 2,000 viable cancer cells were evaluated in the sections of the tumors.

Apoptosis was also evaluated by the TUNEL Assay. In formalin-fixed paraffin-embedded xenografted tumors, tissue sections of 3-μm thickness were deparaffinized, and apoptotic cells were visualized using an *In situ* Apoptosis Detection kit (Takara, Tokyo, Japan).

Statistical analysis. Student's *t*-test and the χ^2 test were used for statistical analysis.

Results

Tumor growth. All tumor pieces implanted on the caecum serosa showed local orthotopic growth. The tumor weight was 0.73±0.24 g in the control group, 0.62±0.29 g in the rPAI-1-treated group and 0.45±0.13 g in the rPAI-2-treated group. The tumor size in the rPAI-2-treated group was significantly smaller than the tumor sizes in the control ($p<0.01$) and the rPAI-1-treated ($p<0.05$) groups (Figure 1).

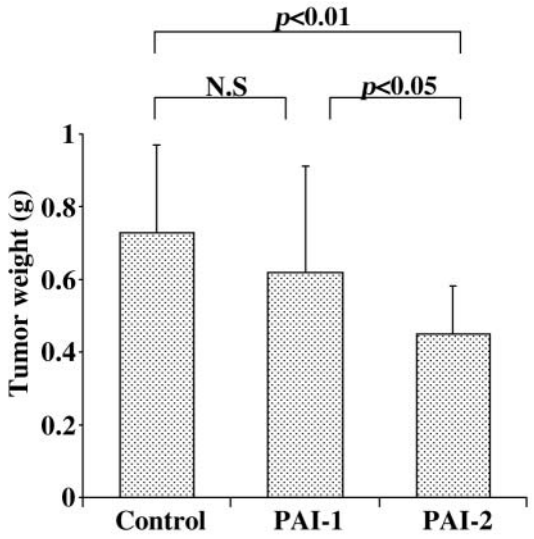

Figure 1. *Tumor weights of implanted human colon cancer growing in the nude mouse caecum in control, rPAI-1-treated and rPAI-2-treated mice. The data represent means±SD.*

Histological features. In the control and rPAI-1-treated groups, the histological findings of the implanted tumors were similar, whereas the findings in the rPAI-2-treated group differed from the others. In the rPAI-2-treated group, the ductal structure was less mature and smaller, and the interstitial space was more dominant than in the other groups (Figure 2).

Apoptotic index. The apoptotic index in the tumor specimens in the rPAI-2-treated group was significantly higher (4.64%±2.12%, $p<0.05$) than those in either the control group (1.94%±0.82%) or the rPAI-1-treated group (2.08%±1.07%) (Figure 3). Apoptosis seemed to be potently induced in the rPAI-2-treated group. The induction of apoptosis was also confirmed by the presence of apoptotic bodies in the TUNEL assay, and a significantly elevated rate of positive staining was observed in the rPAI-2-treated group.

Contents of uPA, uPAR, PAI-1 and PAI-2 in implanted primary tumor tissue. uPA antigen levels in both the rPAI-1-treated and rPAI-2-treated groups were significantly lower than that in the control group ($p<0.01$) (Figure 4). There was no significant difference in the uPAR level among these three groups (Figure 5). The PAI-2 level in the rPAI-2-treated group was significantly higher than that in the other two groups ($p<0.001$) (Figure 6), whereas PAI-1 could not be detected in any of the three groups.

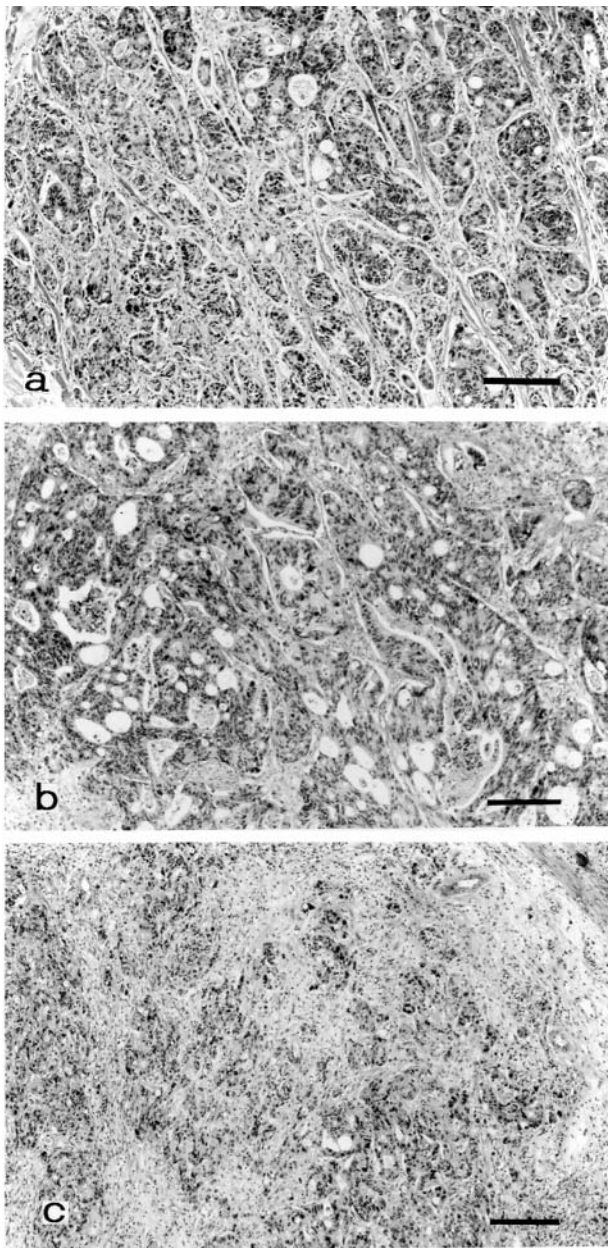

Figure 2. *Histological features of implanted primary tumor tissue in control group (a), rPAI-1-treated group (b) and rPAI-2-treated group (c). Paraffin-embedded sections were stained by hematoxylin and eosin. Bar indicates 150 μm.*

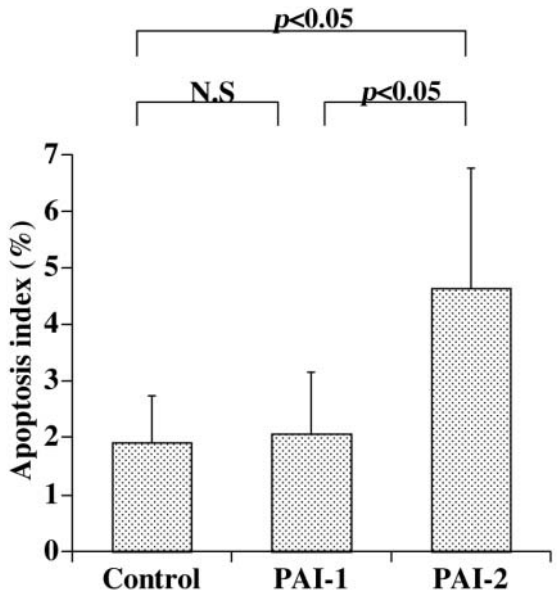

Figure 3. *Apoptotic indices of implanted primary tumor tissue in control, rPAI-1-treated and rPAI-2-treated mice. The data represent means±SD.*

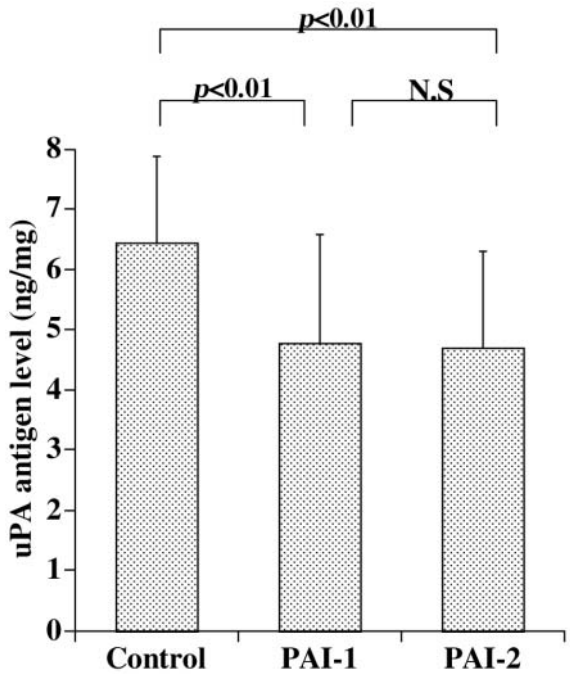

Figure 4. *Contents of uPA in implanted primary tumor tissue in control, rPAI-1-treated and rPAI-2-treated mice. The data represent means±SD.*

Liver metastasis. At 6 weeks after tumor implantation on the caecum, 14 out of 15 mice showed liver metastasis in the control group. The incidence of metastasis was suppressed significantly in the rPAI-1-treated group (5/19, $p<0.001$) and more dramatically in the rPAI-2-treated group (1/16, $p<0.001$). Multiple metastatic nodules numbering more than 5 were observed in some mice in the control group, but in no mice in either the rPAI-1- or the rPAI-2-treated groups (Table I).

Body weight. In the mice treated with rPAI-2, weight gain was significantly suppressed (20.81±1.34 g, $p<0.01$) compared to that in either the control group (22.16±1.52 g) or rPAI-1-treated group (22.12±1.93 g).

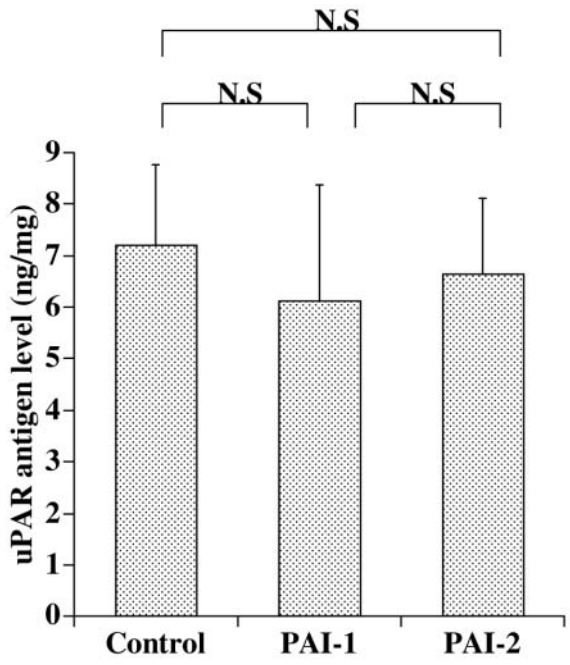

Figure 5. *Contents of uPAR in implanted primary tumor tissue in control, rPAI-1-treated and rPAI-2-treated mice. The data represent means±SD.*

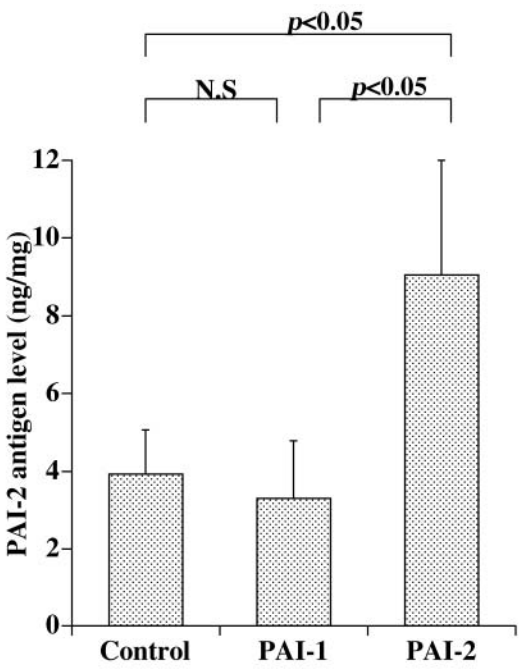

Figure 6. *Contents of PAI-2 in implanted primary tumor tissue in control, rPAI-1-treated and rPAI-2-treated mice. The data represent means±SD.*

Table I. *Inhibitory effect of rPAI-1 and rPAI-2 on liver metastasis.*

Group	Liver metastasis (no. of mice)
Control	14/15
PAI-1	5/19 *
PAI-2	1/16**

*$p<0.01$ *versus* control group; **$p<0.001$ *versus* control group.

Discussion

The present study revealed that the intraperitoneal infusion of rPAI-2 effectively inhibited tumor growth and liver metastasis in an orthotopic transplantation model. rPAI-1 also inhibited liver metastasis, although its efficacy was less than that of rPAI-2. A significant difference was observed after treatment between these two PAIs in the size and histology of primary implanted tumor. Tumor size was significantly smaller in the rPAI-2 treated group, in which development of cancer nests was limited and larger numbers of apoptotic cells were recognized. PAI-2 appears to suppress tumor growth more effectively than PAI-1 by mechanisms in which the induction of apoptosis is involved.

Several different mechanisms have been suggested for the roles of uPA and uPAR in pathological processes of malignant disease (19). Cleavage and degradation of matrix proteins after plasminogen activation indeed play important roles by which uPA promotes tumor growth, invasion and metastasis. The relevance of the activity of uPA bound to cell surface uPAR in the process was demonstrated by successful inhibition of metastasis by proteinase inhibitor (20), activity-neutralizing anti-uPA antibody (21) or amino-terminal fragment (ATF) peptide, which dissociates uPA from uPAR (22). In accordance with the possibility that uPA is involved mainly in the entry of tumor cells into the vascular circulation during the metastatic process, its inhibition by either rPAI-1 or rPAI-2 resulted in a lower incidence of liver metastasis as shown in the present study.

Another mechanism by which uPA plays a role in tumor growth is related to the mitogenic effect and/or growth-factor-like effect, which requires signal transduction. uPA is considered to promote cell growth after binding through its amino-terminal fragment to either uPAR (23) or other cell surface binding sites (24). Though the precise mechanism by which uPA transmits signals into the intracellular space has not been elucidated, many lines of evidence suggest that the enzyme activity of uPA is essential also for promoting cell proliferation (24,25) and is high during the growing phase (26). Most notably, the inhibition of uPA activity by PAI-2 has been reported to suppress proliferation of keratinocytes (26).

Another fact that transfection of THP-1 cells, non active PAI-2 producing monocyte-like cells, with plasmids expressing active PAI-2 suppressed cell proliferation which was attenuated by uPA also suggests that PAI-2 suppresses cell proliferation as a result of the inhibition of uPA activity (27). In the present study, significantly higher numbers of apoptotic cells were detected in transplanted tumors in the rPAI-2 treated group. Since depletion in growth factors is known to induce apoptosis, the inhibition of uPA activity by PAI-2 may be sufficient to quench its growth factor activity and to initiate apoptosis. This anti-proliferation effect of PAI-2 seems to have resulted in the suppression of growth of the implanted tumor as well as the formation of the typical large cancer nest, which was always seen in both the control and the rPAI-1-treated group. This is in accordance with the finding that PAI-2-expressing CHO cells grow more slowly than PAI-1-expressing CHO cells (14).

In contrast to the antiproliferative effect of exogenously supplied PAI-2, which was shown in the present study and previously (26), controversial results are reported for intracellular function of PAI-2 in apoptosis. During apoptosis, cleaved smaller molecular weight fragment of PAI-2 was generated in human myeloleukemia cells suggesting that PAI-2 is involved in apoptosis (28). A protective effect of PAI-2 from apoptosis was reported in TNF-α induced apoptosis model (29, 30). In contrast, suppression of cell proliferation was observed after expression of active PAI-2 in PAI-2 non-producing monocyte-like cells (27). Since PAI-2 is a non-secretory protein in certain cell types, its intracellular function may be different from its function on the cell surface, and may vary in different cell types.

rPAI-1 did not suppress the proliferation of the implanted primary tumor. Even though PAI-1 and PAI-2 possess similar inhibitory activities toward uPAR-bound uPA, higher levels of PAI-1 in tumor tissue are related to poorer prognosis of patients (31). In PAI-1 gene-knockout mice, less effective invasion (32) and proliferation (33) of an inoculated tumor was demonstrated, suggesting that PAI-1 promotes tumor progression and invasion *in vivo*. The promotion of tumor growth by PAI-1 seems to be caused by its protection from apoptosis besides its uPA inhibiting function (34) or by its potential to modify the cell adhesion capacity (19) and/or to promote angiogenesis (33), which are independent of uPA inhibitory activity. Biological function of endgeneouly added PAI-1 and exogeneously added PAI-1 may differ.

The uPA antigen levels in implanted tumors in the rPAI-1-and rPAI-2-treated groups were significantly reduced. The internalization and degradation of uPAR-bound uPA/PAI-1 complexes *via* the function of a LDL receptor-related protein (35) may be the reason for this reduction. The degradation of the uPA-PAI-2 complex into 70 and 20 kDa fragments bound to the cell surface uPAR has also been reported, although its internalization is questionable (36). Such accelerated degradation of uPA either on the cell surface or in the cytosol after complex formation with PAIs seems to have resulted in the depression of the uPA level after intraperitoneal infusion of these inhibitors. The depression of uPA may indeed have contributed to the lower frequency of liver metastasis.

In the present study we demonstrated that both rPAI-1 and rPAI-2 suppressed liver metastasis in a human colon cancer orthotopic xenograft model. This may indicate the therapeutic potential of these PAIs in malignancies. The significant difference in suppression of primary tumor growth, as well as the completely different histological findings resulting from rPAI-1 and rPAI-2 treatment, also provides clues to understanding the mechanism by which these inhibitors regulate tumor growth.

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