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Localization of Urokinase-type Plasminogen Activator, Plasminogen Activator Inhibitor-1, 2 and Plasminogen in Colon Cancer

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We examined the localization of urokinase-type plasminogen activator (u-PA), plasminogen activator inhibitors (PAI-1 and PAI-2) and plasminogen (plg) in 26 cases of colon cancer by immunohistochemical staining. The u-PA antigen was detected in the cytoplasm of cancer cells (18/26) and stromal cells adjacent to cancer tissues (9/26). The localization of u-PA mRNA examined by *in situ* hybridization was consistent with that of u-PA antigen. The PAI-1 antigen was detected in fibroblasts and endothelial cells (22/26), while PAI-2 antigen was found in cancer cells (20/26). The plg antigen was seen in the extracellular matrix of the cancer stroma. The u-PA expression in cancer cells was significantly more frequently detected in cases with lymph node metastasis than in cases without metastasis. In either PAI-1- or PAI-2-expressing cases, lymph node metastasis seemed to be restrained. These findings indicate that cancer cells themselves produce u-PA, and suggest that u-PA converts plg into plasmin, which dissolves the extracellular matrix surrounding cancer cells, resulting in cancer invasion and metastasis. PAI-1 and PAI-2 may have inhibitory actions on cancer invasion and metastasis mediated by u-PA.

Key words: Plasminogen activator — Plasminogen activator inhibitor — *In situ* hybridization — Colon cancer — Immunohistochemistry

Cancer cells are characterized by disordered proliferation, invasion and metastasis. The latter two features are not simply a reflection of their disordered proliferation, but involve degradation of the extracellular matrix surrounding them. In this process, proteases have been considered to play an important role,[1–3] and among various proteases, the plasminogen-activating system has recently been the subject of much attention.

Urokinase-type plasminogen activator (u-PA) converts plasminogen (plg) into its active proteolytic form, plasmin, which degrades the extracellular matrix by itself or through activation of collagenase, thereby contributing to tumor invasion and metastasis.[4, 5] In colon cancers, recent findings have demonstrated that u-PA levels are higher in adenomas than in normal tissues, and higher in cancer tissues than in adenomas.[6, 7] In addition, u-PA levels in homogenized colon cancer tissues increase as the clinical stage (Dukes' staging system) advances.[8]

In the fibrinolytic system, there are two specific plasminogen activator inhibitors. plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). It has been suggested that they might inhibit invasion and metastasis. For instance, the matrix degradation by HT-1080 human fibrosarcoma cells, which express a high level of u-PA, was decreased by PAI-1[9] or PAI-2[10] and was restored by the further addition of monoclonal antibody to PAI-1.[9] Moreover, Laug *et al.*[11] transfected PAI-2 cDNA into HT-1080 cells, and observed a decrease in matrix degradation and invasion. In this study, we examined the localization of u-PA, PAI-1, PAI-2 and plg in colon cancer immunohistochemically and that of u-PA mRNA using *in situ* hybridization.

MATERIALS AND METHODS

Tissue preparation Twenty-six cases of surgically resected colon cancers, all of which were confined to the subserosal layer, and 10 cases of normal tissues resected at sites at least 10 cm away from a tumor were evaluated. The specimens were immediately fixed in 4% (w/v) paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) at 4°C for 4 h and embedded in paraffin. The specimens were then sectioned at 4 μm thickness. The sections were mounted on poly-L-lysine-coated slides and subjected to immunohistochemical staining and *in situ* hybridization.

Immunohistochemical staining Monoclonal antibodies against u-PA and PAI-1, and antisera against plg were provided by Dr. Y. Sakata (Division of Hemostasis and Thrombosis Research, Jichi Medical School). Monoclonal antibody against PAI-2 was purchased from Biopool Co. (Umeå, Sweden). Immunohistochemical staining was performed using the DAKO-SAB kit (Dakopatts, Copenhagen, Denmark) according to the

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manufacturer's instructions. To unmask tissue antigens, the sections were digested with 0.3% (w/v) pepsin (Sigma, St. Louis, MO) in 0.01 *N* HCl for 15 min at room temperature. The sections were incubated overnight with primary antibody, which was diluted with PBS containing 0.1% goat normal serum to 10 μg/ml (u-PA, PAI-1 and plg) or to 2 μg/ml (PAI-2), at 4°C. Visualization was done with H_2O_2-supplemented aminoethylcarbazole chromogen. The sections were counterstained with hematoxylin and mounted. In the control experiments, the sections were incubated with non-immune IgG in place of the primary antibody.

Riboprobe preparation Human u-PA cDNA,[12] subcloned into the vector pBR322, was provided by the Japanese Cancer Research Resources Bank. The *Pst* I fragment of u-PA cDNA (1.58 kb) was subcloned into the vector pBluescript KS+ (Stratagene, La Jolla, CA). Sense and anti-sense RNA probes were prepared through transcription of the linearized plasmid with T7 or T3 polymerase using digoxigenin (Dig)-labeled UTP[13] (Fig. 1A). The transcription was carried out with the Dig RNA labeling kit (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The RNA probe was hydrolyzed in a solution of 40 m*M* $NaHCO_3$ and 60 m*M* Na_2CO_3 to an average size of 100 nucleotides.

Sensitivity of detection of the hybridized Dig-labeled probe Target human u-PA cDNA was spotted onto Hybond-N+ membrane (Amersham, Buckinghamshire, England) in the range of 3.3 ng–0.3 pg, and hybridized with the Dig-labeled riboprobe essentially as described by Lion and Haas.[14] The hybridization signals were detected at 3.3 ng down to 0.3 pg (Fig. 1B), showing that the sensitivity of the u-PA riboprobes labeled with Dig-UTP was comparable to that of the radiolabeled system.

***In situ* hybridization** In six cases which were u-PA-positive immunohistochemically, the localization of u-PA mRNA was examined by using *in situ* hybridization. All solutions for *in situ* hybridization were prepared in RNase-free water which had been supplemented with diethyl pyrocarbonate (DEPC) (Nacalai, Kyoto), incubated overnight and autoclaved. All glassware was heat-sterilized (220°C, 2 h) or autoclaved after DEPC-treatment. *In situ* hybridization was performed essentially as described by Springer *et al.*[15] In brief, the deparaffinized sections were followed by incubation with 0.2 *N* HCl for 10 min, and proteinase K (Merck, Darmstadt, Germany, 10 μg in 20 m*M* Tris-HCl pH 7.5 and 2 m*M* $CaCl_2$) for 10 min. Then post-fixation was done in 4% (w/v) paraformaldehyde in PBS for 10 min. The sections were immersed in 0.25% (w/v) acetic anhydride in 0.1 *M* triethanolamine for 10 min, and rinsed with glycine in PBS (2 mg/ml) for 10 min. The sections were prehybridized in 50 μl of prehybridization buffer [10 m*M* Tris-HCl (pH 7.5), 0.6 *M* NaCl, 1 m*M* EDTA, 50% (v/v) formamide, 1×Denhardt's solution, 0.5 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA] at 37°C for 1 h. Hybridization was performed in a mixture (20 μl) of prehybridization buffer, 10% dextran sulfate, and Dig-labeled RNA probe (1 μg/ml) at 37°C overnight. Following hybridization, the sections were washed with 2×SSC/50% formamide (20 min×3 at 37°C), 2×SSC (20 min×3 at 37°C) and 1×SSC (20 min×3 at 37°C), and then treated with RNase A (Sigma, St. Louis, MO, 10 μg/ml in 2×SSC, 20 min). The sections were rinsed in Buffer I (0.1 *M* Tris-HCl, pH 7.5, 0.15 *M* NaCl, 2 m*M* $MgCl_2$) for 10 min, incubated with 5% bovine serum albumin in Buffer I for 30 min, then incubated with

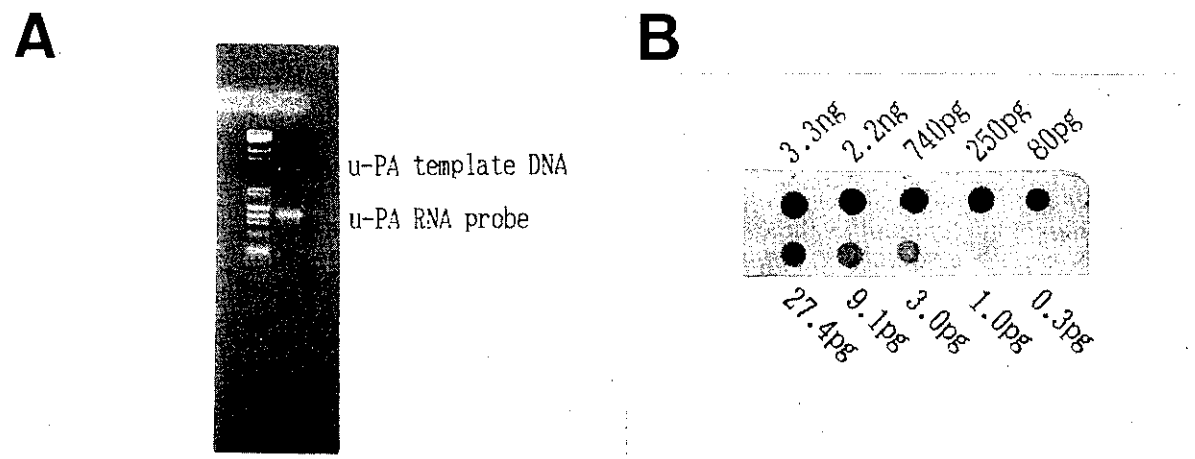

Fig. 1. Riboprobe preparation for *in situ* hybridization. (A) Sense and anti-sense RNA probes were synthesized by linearizing the plasmid and transcribed with T7 or T3 polymerase using digoxigenin (Dig)-labeled UTP. (B) The signals representing 3.3 ng down to 0.3 pg were revealed with dot blot hybridization.

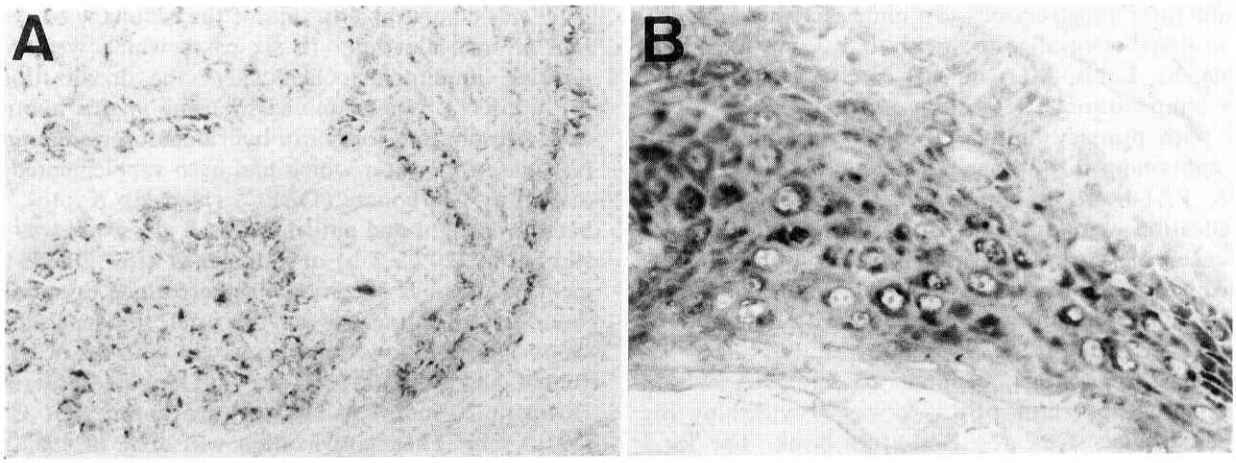

Fig. 2. Localization of u-PA antigen and mRNA in A431 cells transplanted in nude rats. u-PA antigen (A) and u-PA mRNA (B) were positive.

anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer) diluted 1:500 in Buffer I for 10 min. This was followed by washing with Buffer I (5 min×3), and washing with Buffer II (0.1 *M* Tris-HCl, pH 9.5, 0.15 *M* NaCl, 50 m*M* $MgCl_2$) for 5 min. For detection of signals, the sections were incubated with chromogen solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies, Gaithersburg, MD) for about 60 min. The A431 vulvar epidermoid carcinoma cell line was provided by the Japanese Cancer Research Resources Bank (JCRB). Cells (1×10^7) were injected under the skin of a nude rat, and a solid tumor was obtained after two weeks. We used this tumor as a positive control for *in situ* hybridization of u-PA mRNA[16] (Fig. 2). Sense probes were used as a negative control.

The chi-square test was used to evaluate the significance of differences between the groups, and a P value of less than 0.05 was taken as the criterion of significance.

RESULTS

Immunohistochemistry for u-PA The u-PA antigen was detected in a fine granular pattern in the cytoplasm of cancer cells in 18 of the 26 cases (69.2%) examined (Fig. 3A, B). The positive staining was mostly localized at the periphery, not in the central portion, of the tumors. Lymph node metastasis was more frequently found in the cases with u-PA-positive cancer cells (13/18, 72.2%) than in the negative cases (2/8, 25.0%) ($P<0.05$) (Table I).

In 9 cases, u-PA antigen was found in the stromal cells adjacent to cancer tissues. Higher magnifications revealed that many of the u-PA-positive stromal cells had the morphologic appearance of macrophages (Fig. 3C, D). The u-PA antigen was not detected either in the normal mucosa or in both epithelial and stromal cells of normal colonic tissues. Among the 18 cases exhibiting u-PA-positive cancer cells, 6 cases (33.3%) also showed positive in the stromal cells. Of the 8 cases in which u-PA antigen was negative in cancer cells, 3 cases (37.5%) were u-PA-positive in the stromal cells. There was no significant difference in the degree of u-PA stainings in the stromal cells between these two groups.

Immunohistochemistry for PAI-1 and PAI-2 PAI-1 was detected in the cytoplasm of fibroblasts and endothelial cells adjacent to cancer tissues in 22 of 26 cases (84.6%) (Fig. 4A, B). On the other hand, PAI-2 was stained clearly in the cytoplasm of cancer cells in 20 of 26 cases (76.9%) (Fig. 4C, D). Thus, the localization of PAI-2 was completely different from that of PAI-1. Different from the distribution of u-PA staining, the intensity of PAI-2 staining appeared similar at the periphery and at the central portion of the tumor. In u-PA- and PAI-1-positive cases, lymph node metastasis was seen in 68.8% (11/16). In u-PA-positive and PAI-1-negative cases, both had lymph node metastasis (2/2); on the other hand, lymph node metastasis was only seen in 16.7% (1/6) in u-PA-negative and PAI-1-positive cases. With regard to the relationship between u-PA and PAI-2 expressions, u-PA- and PAI-2-positive cases had lymph node metastasis in 70.6% (12/17). The u-PA-positive and PAI-2-negative case had lymph node metastasis, but in u-PA-negative and PAI-2-positive cases, no lymph node metastasis was seen (0/3) (Table I).

Immunohistochemistry for plg Plasminogen was strongly stained in the cytoplasm of normal hepatocytes (Fig. 4E). In the cancer lesion, plg was positive at the extra-

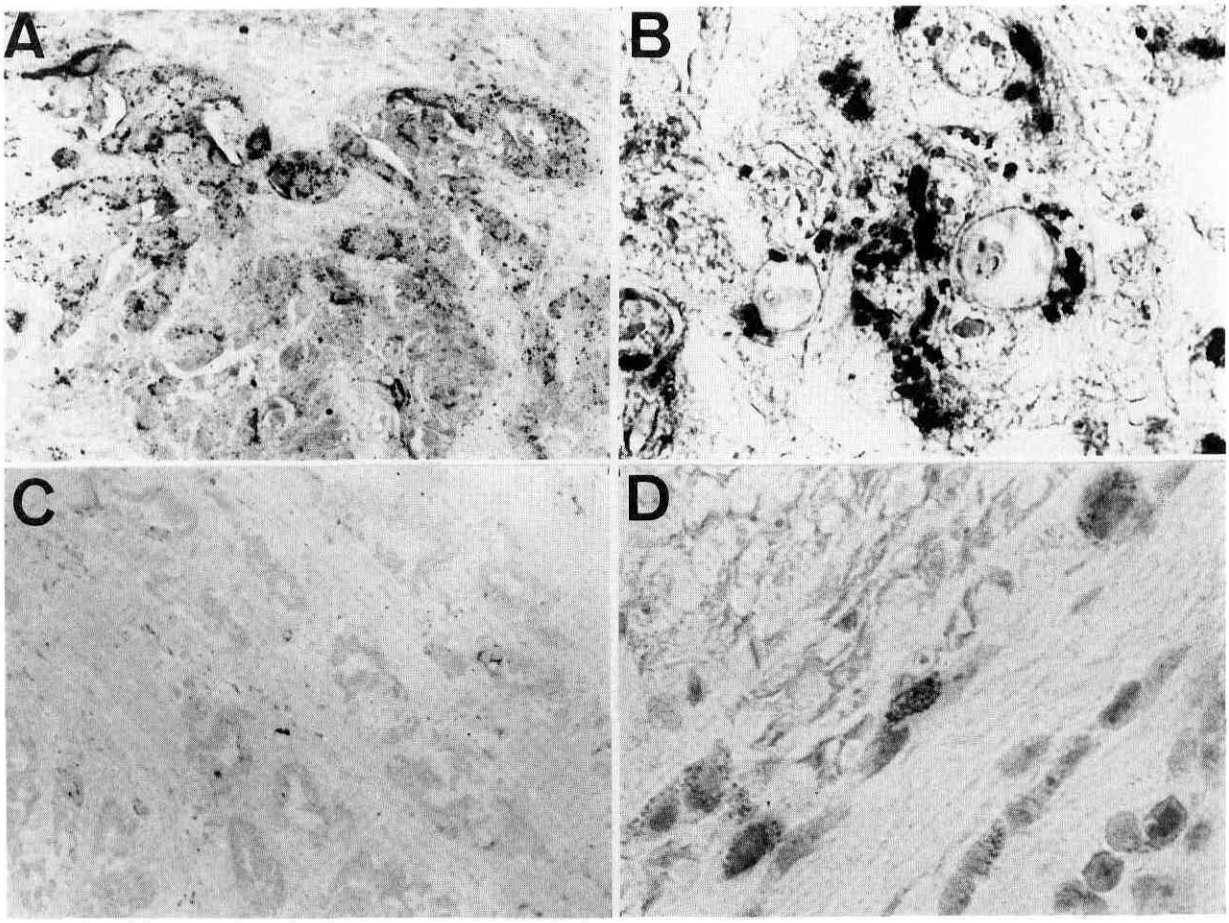

Fig. 3. Immunolocalization of u-PA antigen in colon cancer. The u-PA antigen was detected in a fine granular pattern in the cytoplasm in cancer cells (A). Higher magnification revealed that u-PA antigen was mainly located around the nucleus (B). The u-PA antigen was also found in stromal cells adjacent to the tumor (C). Higher magnification revealed that many of these cells had the morphologic appearance of macrophages (D).

Table I. Immunohistochemical Expression of u-PA, PAI-1 and PAI-2 in Relation to Lymph Node Metastasis

	Lymph node metastasis		Total	
	(+)	(−)		
u-PA(+)	13	5	18	
u-PA(−)	2	6	8	$P<0.05$
u-PA(+) PAI-1(+)	11	5	16	
u-PA(+) PAI-1(−)	2	0	2	
u-PA(−) PAI-1(+)	1	5	6	
u-PA(−) PAI-1(−)	1	1	2	
u-PA(+) PAI-2(+)	12	5	17	
u-PA(+) PAI-2(−)	1	0	1	
u-PA(−) PAI-2(+)	0	3	3	
u-PA(−) PAI-2(−)	2	3	5	

cellular matrix of the tumor stroma (Fig. 4F). No staining was detected in cancer or stromal cells.

***In situ* hybridization for u-PA** The u-PA mRNA was confined to cancer cells and stromal cells in the tumor (Fig. 5A, C). Higher magnification revealed that u-PA mRNA was mainly localized in the cytoplasm around the nucleus (Fig. 5B, D). The u-PA mRNA was not detected in normal tissues. These findings were consistent with the results of the above immunohistochemical studies. As a negative control, sense probes were applied to the sections, and no specific signals were detected (data not shown).

DISCUSSION

In this study, we have demonstrated that u-PA was expressed in the cytoplasm of both cancer cells and stromal cells near the tumor. In previous studies, u-PA has been reported to be present in cancer cells by Markus *et al.*[17] Kohga *et al.*[18] and Sier *et al.*[19] detected u-PA not

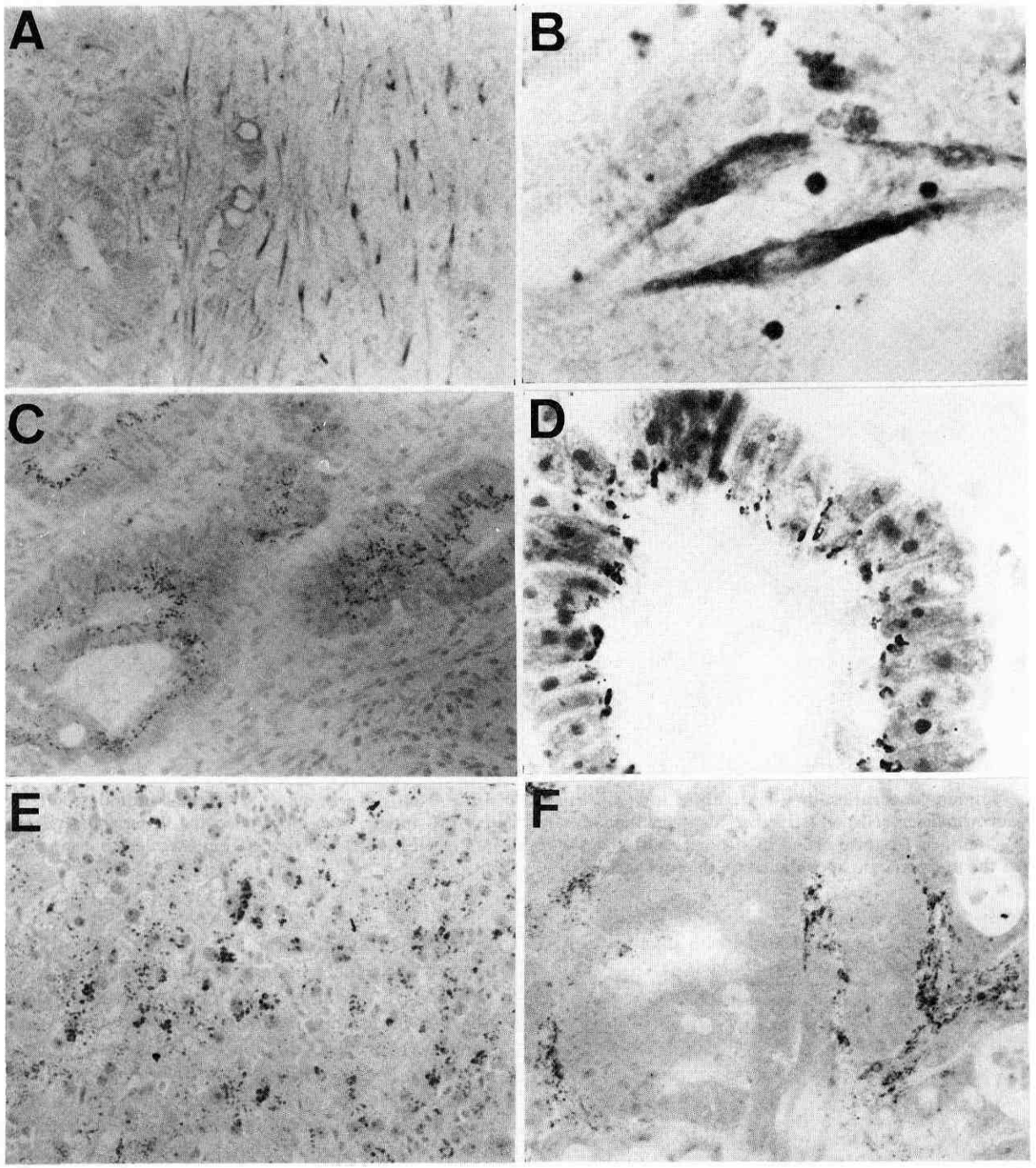

Fig. 4. Immunolocalization of PAI-1, PAI-2 and plg. PAI-1 was detected in the cytoplasm of fibroblasts and endothelial cells (A). (B) is higher magnification. PAI-2 was expressed in the cytoplasm of cancer cells (C). (D) is higher magnification. Plg was expressed in the cytoplasm of normal hepatocytes (E). In the tumor lesion, plg was positive at the extracellular matrix of the stroma (F).

only in cancer cells but also in the stromal cells. In contrast, Grφndahl-Hansen *et al.*[20] detected u-PA antigen in the fibroblast-like cells of the tumor stroma but not in cancer cells. The reason for the apparent discrepancies between these studies and our report is unknown. There is a possibility that the different results are related to the

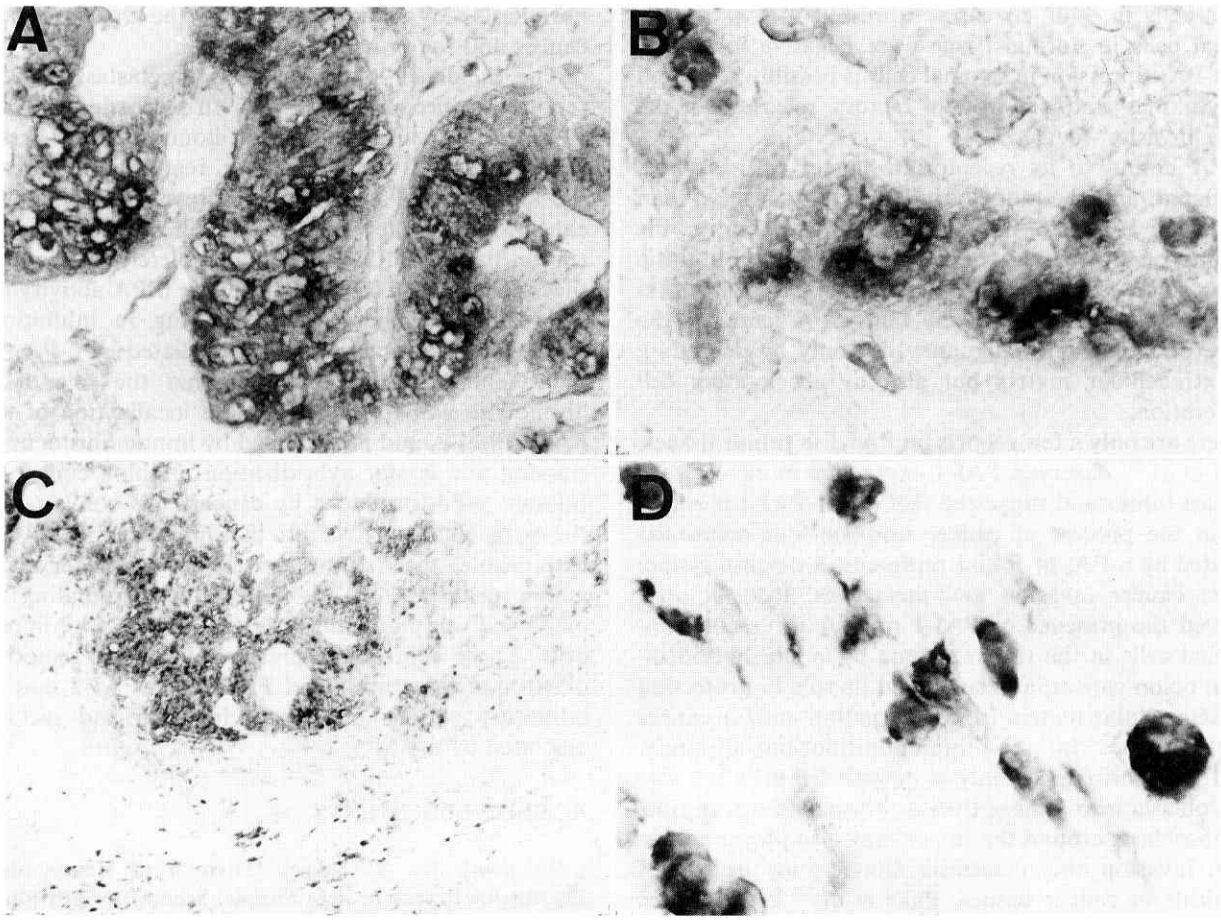

Fig. 5. *In situ* hybridization of u-PA mRNA with Dig-labeled anti-sense probe. U-PA mRNA was localized in cancer cells (A). Higher magnification revealed that u-PA mRNA was localized around the nucleus (B). The u-PA mRNA was also detected in stromal cells adjacent to the tumor (C). (D) is higher magnification. These findings were consistent with the results of immunohistochemical studies.

specificity of the antibodies used. u-PA exists in different forms in human tissues, such as single-chain u-PA (scu-PA), high-molecular-weight u-PA (HMWu-PA), low-molecular-weight u-PA (LMWu-PA), u-PA/plasminogen activator inhibitor (PAI) complex, u-PA/u-PA receptor complex and so on. In our study, the immunohistochemical intensity of u-PA using monoclonal antibody in stromal cells was much less than that in cancer cells (see Fig. 3A, C) in spite of the same intensity of u-PA mRNA (see Fig. 5C) and u-PA immunohistochemical staining using polyclonal antibody (data not shown). These findings raise the possibility that the u-PA in cancer cells and in stromal cells might be in different forms.

u-PA is present in the circulation in minute quantities.[21] When u-PA binds to the u-PA inhibitor or u-PA receptor, it is incorporated into the cellular cytoplasm.[22, 23] Therefore, even if u-PA antigen is detected, it does not necessarily mean that u-PA was produced there. For this reason, identification of u-PA mRNA is required, and in the present *in situ* hybridization study, we were successful in demonstrating the presence of u-PA mRNA in cancer and stromal cells.

The expression of u-PA was more marked at the periphery of the tumor rather than in the central area. In addition, lymph node metastasis was frequently observed in the patients positive for u-PA in cancer cells. These findings suggested the involvement of u-PA in cancer invasion and metastasis, and u-PA might be useful as an index of biological malignancy of cancer cells.

Previous *in vitro* studies have shown that many kinds of cells, including malignant cells, produce u-PA, which is regulated by various cytokines and growth factors.[4, 24–26] In this study, the stromal cells adjacent to

tumor cells in 9 of 26 cases expressed u-PA, but the stromal cells in normal tissue were negative for u-PA. The u-PA expression in stromal cells is possibly regulated by cytokines and/or growth factors released at the cancer invasion focus.

u-PA bound to its receptor in cancer cells is phosphorylated, and the modified u-PA is less sensitive than unmodified u-PA to inhibitors.[27,28] Furthermore, the amino-terminal fragment of u-PA (this region contains a similar structure to epidermal growth factor) activates cell proliferation.[29] It is likely that u-PA increases the degree of biological malignancy not only by degrading the extracellular matrix but also by accelerating cell proliferation.

There are only a few reports on PAI-1 in tumor tissues. Reilly *et al.*[30] observed PAI-1 expression in cancer cells in breast tumor and suggested that either PAI-1 is necessary in the process of cancer invasion and metastasis mediated by u-PA, or PAI-1 represents a defense system against cancer invasion and metastasis. Pyke *et al.*[31] observed the presence of PAI-1 mRNA in vascular endothelial cells in the tumor stroma by *in situ* hybridization in colon cancer, and suggested its role in protecting the extracellular matrix from destruction and/or cancer vascularization. In this immunohistochemical study, PAI-1 was positive not only in endothelial cells but also in fibroblasts near cancer tissues. These findings suggest that fibroblasts around the tumor may also play a part in cancer invasion and metastasis. Concerning the role of fibroblasts in cancer tissues, Pyke *et al.*[32] reported the presence of mRNA of type IV collagenase (molecular weight, 72,000) in fibroblasts around skin cancer, and Basset *et al.*[33] also reported the expression of stromalysin 3 in fibroblasts near breast cancer, both suggesting their participation in remodeling of cancer stroma. The role of fibroblasts may differ depending on the type and grade of cancer and the type of proteases.

The incidence of lymph node metastasis has been reported to become lower with an increasing amount of PAI-2 in the cancer tissues of stomach[34] and breast.[35] Gleeson *et al.*,[36] to the contrary, reported an increase in the amount of PAI-2 with progression of endometrial cancer. We detected PAI-2 in cancer cells, and the localization pattern of PAI-2 was completely different from that of PAI-1. PAI-2 might regulate u-PA activity in the cytoplasm of cancer cells, resulting in inhibition of cancer invasion and metastasis mediated by u-PA.

In conclusion, we speculate that the role of local fibrinolytic activity, based on the localization of u-PA, PAI-1, PAI-2, and plg revealed by immunohistochemical staining and *in situ* hybridization in colon cancer, is as follows: u-PA produced by cancer cells converts plg in the extracellular matrix into plasmin, which dissolves the extracellular matrix, and contributes to cancer invasion and metastasis. PAI-1 is located in surrounding fibroblasts and vascular endothelial cells, and PAI-2 in cancer cells. These expressions presumably reflect remodelling of stromal structures, and PAI-1 and PAI-2 may have inhibitory actions on cancer invasion and metastasis mediated by u-PA.

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