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Expression of Trypsin in Human Cancer Cell Lines and Cancer Tissues and Its Tight Binding to Soluble Form of Alzheimer Amyloid Precursor Protein in Culture[1]

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It was recently found that overexpression of the trypsin gene in tumor cells stimulates their growth in culture and in nude mice. In the present study, expression of trypsin in various human cancer cell lines and tissues was studied by gelatin zymography and immunoblotting before and after enterokinase treatment and by immunohistochemistry. The analyses showed that many stomach, colon, and breast cancer cell lines secreted trypsinogens-1 and/or -2, as well as an unidentified serine proteinase of about 70 kDa, into culture medium. Lung cancer cell lines secreted 18- and 19-kDa unidentified trypsin-like proteins. Stomach cancer cell lines frequently secreted active trypsin, suggesting that they produced an endogenous activator of trypsinogen, most likely enterokinase. Active trypsin formed a complex with a soluble form of Alzheimer amyloid precursor protein (sAPP), a Kunitz-type trypsin inhibitor, which was secreted by all cell lines tested. This indicated that sAPP is a primary inhibitor of secreted trypsin. Immunohistochemical analysis showed that trypsin(ogen) was frequently expressed at high levels in stomach and colon cancers, but scarcely in breast cancers. In the stomach cancers, the trypsin immunoreactivity was higher in the malignant, non-cohesive type than in the cohesive type. These results support the hypothesis that tumor-derived trypsin is involved in the malignant growth of tumor cells, especially stomach cancer cells.

Key words: APP, cancer, trypsin, tumor invasion, zymography.

It has long been known that trypsin is produced as a zymogen (trypsinogen) in the acinar cells of pancreas, secreted into the duodenum, activated into the mature form of trypsin by enterokinase, and functions as an essential food-digestive enzyme (*1*). Previous studies have shown that trypsin or trypsin-like enzymes are produced by non-pancreatic tissues and cells. Koivunen *et al.* purified two trypsinogen-like proteins from cyst fluid of ovarian mucinous adenocarcinoma (*2*) and showed their production by human colon carcinoma, fibrosarcoma, and leukemia cell lines (*3*). We found that a few human stomach and ovarian cancer cell lines secrete active forms of trypsins-1 and -2 and their zymogens (*4, 5*). Immunohistochemical studies suggested that trypsin(ogen) is synthesized in human ovary (*6*) and lung (*7*) cancer tissues. More recently, we found that the trypsin gene is widely expressed in normal human tissues including various epithelial tissues, brain, and spleen (*8*) and in vascular endothelial cells (*9*).

It has been established that extracellular matrix-degrading proteinases play a critical role in tumor invasion and metastasis (*10*). Trypsin has potent proteolytic activity toward a wide variety of extracellular matrix proteins such as laminin and fibronectin (*4*). Trypsin is also known as a potent activator of latent form of various matrix metalloproteinases (MMPs) and serine proteinases (*11-13*). Overexpression of an exogenous trypsinogen gene in a human stomach adenocarcinoma cell line increases its tumorigenicity in the abdominal cavity of nude mice (*14*). In another human stomach adenocarcinoma cell line, a clone secreting trypsin and its zymogen at high levels is more malignant in nude mice than another clone secreting them at low levels (*15*). These facts suggest that tumor-derived trypsin might

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Abbreviations: sAPP, soluble form of Alzheimer amyloid protein precursor; MMPs, matrix metalloproteinases; EK, enterokinase; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

contribute to growth and invasion of tumor cells *in vivo*. In addition, recent studies showed that trypsinogens secreted by cultured cells can be detected with high sensitivity by treatment of culture medium with the trypsinogen activator enterokinase and subsequent gelatin zymography (*15*).

In the present study, we examined secretion of trypsin(ogen) from human colon, stomach, breast, and lung cancer cell lines and its expression in human stomach, colon, and breast cancer tissues.

MATERIALS AND METHODS

Cells and Culture Conditions—Human colon adenocarcinoma cell lines used were: CaR-1, Colo-201, RCM-1, SW-480, WiDr, and SW-620 (established from lymph node metastasis). Human stomach cancer cell lines used were: adenocarcinoma (STKM-1, STKM-2, MKN-28, MKN-45, MKN-74, and NUGC-3); adenosquamous carcinoma (MKN-1); unspecified (AZ-521); signet-ring cell carcinoma (KATO-III); and choriocarcinoma (SCH). Human breast carcinoma cell lines used were: BT-20, MMK-29, MCF-7, and MDA-MB-157. Human lung cancer cell lines used were: adenocarcinoma (A-549, RERF-LC-MS, and PC-3); small cell carcinoma (Lu-65, Lu-134-A-H, and YLC-KKI); giant cell carcinoma (Lu-99); and squamous cell carcinoma (VMRC-LCP). STKM-1 and STKM-2 were established by Dr. S. Yanoma, Kanagawa Cancer Center, Japan. MMK-29 was a kind gift from Dr. T. Chishima, Yokohama City University School of Medicine. Three human breast cancer cell lines (BT-20, MCF-7, and MDA-MB-157) were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA). The other cell lines were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo). All these cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2–95% air. RPMI 1640 supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid, 1.2 mg/ml $NaHCO_3$, and 2 mM glutamine was used as the basal medium. Cultures were maintained in the basal medium supplemented with 10% fetal calf serum (FCS).

Preparation of Serum-Free Conditioned Media of Human Cancer Cell Lines—Serum-free culture media conditioned by human colon, stomach, breast and lung cancer cell lines were prepared as reported previously (*4*). Briefly, cells were grown to confluence in the serum-containing medium, rinsed twice with Ca^{2+}- and Mg^{2+}-free PBS, then cultured in serum-free RPMI 1640 for 2 days. The serum-free conditioned media were clarified by centrifugation, and proteins were precipitated with ammonium sulfate of 80% saturation. The resultant protein precipitates were collected by centrifugation and dissolved in and dialyzed against 20 mM Tris-HCl (pH 7.5). The final volume was adjusted to make a 50-fold concentrated conditioned medium.

Gelatin Zymography—Zymographic analysis of gelatinolytic activities was carried out on 12.5% polyacrylamide gels containing 1 mg/ml gelatin as described previously (*4*). To activate trypsinogen to trypsin, 1.5 μl of concentrated conditioned medium of MKN-28 and 15 μl of concentrated conditioned medium of other cell lines were pretreated at 37°C for 1 h with a final concentration of 50 μg/ml enterokinase (EK) (Biozyme Laboratories, Blaenavon, Great Britain). Proteins separated on the gels were renatured in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 2.5% (v/v) Triton X-100, and incubated in 50 mM Tris-HCl (pH 7.5) in the presence of 5 mM EDTA to inhibit matrix metalloproteinase activities. The resultant gels were stained with Coomassie Brilliant Blue R-250. Molecular weight (M_r) markers used were rabbit skeletal muscle myosin (M_r 200,000), *Escherichia coli* β-galactosidase (M_r 116,250), rabbit muscle phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 66,200), hen egg albumin (M_r 45,000), bovine carbonic anhydrase (M_r 31,000), and soybean trypsin inhibitor (M_r 21,500).

Immunoblotting—Immunoblotting was carried out as described previously (*4*). The conditioned media of human cancer cell lines treated or not treated with enterokinase were analyzed by immunoblotting with a rabbit polyclonal antibody against human trypsin (Athens Research and Technology, Athens, GA, USA) under reducing conditions for detection of trypsin-1 and trypsin-2. The anti-trypsin antibody did not react with bovine trypsin. To detect a SDS-stable complex between trypsin and a soluble form of human Alzheimer amyloid precursor protein (sAPP) and its partially degraded products, the concentrated conditioned media treated or not treated with enterokinase were analyzed by immunoblotting under non-reducing conditions with the anti-trypsin antibody and a mouse monoclonal antibody against sAPP (clone 22C11; Boehringer Mannheim GmbH, Mannheim, Germany), which recognizes an N-terminal portion of sAPP. In some experiments, mouse anti-sAPP monoclonal antibody OM84 capable of recognizing a C-terminal portion of human sAPP, which was prepared by Y. Okabe in our laboratory, was used. In one experiment, the complex between human pancreatic trypsin (Athens Research and Technology) and purified sAPP (*16*) was produced by incubating 0.3 μg trypsin with 5 or 50 μg sAPP in 50 μl of Tris-HCl (pH 7.5) at 37°C for 5 min. The resultant reaction mixtures containing 0.3 μg trypsin or 1 μg sAPP were subjected to immunoblotting with the anti-trypsin polyclonal and the anti-sAPP monoclonal antibodies.

Immunohistochemistry—All human stomach, colon, and breast cancer tissues were sampled by autopsy with informed consent of patient's families, and immediately fixed with 10% formalin. The paraffin-embedded sections were mounted on aminoacyl silane-coated glass slides and used for immunohistochemistry. The histologic types of the tumors were determined according to the general rules of clinicopathologic study of colonic, stomach, and breast cancers. The stomach cancers were histologically classified according to the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer (Japanese Reseach Society Committee on Histological Classification of Gastric Cancer). In addition, we divided gastric cancers into two types according to Uchino *et al.* (*17*), namely, cohesive and non-cohesive types. Immunohistochemical staining of human trypsin(ogen) was carried out as reported previously (*6*). Briefly, 4-μm thick paraffin sections were dewaxed, dehydrated, and immersed in methanol containing 0.3% hydrogen peroxide to inactivate intrinsic peroxidase. All sections were treated with Protease XXIV (Sigma, St. Louis, MO, USA) at room temperature for 15 min. After incubation with 10% normal rabbit serum/PBS for 1 h at room temperature, sections were incubated with a mouse monoclonal antibody against hu-

man pancreatic trypsin (Chemicon, Temecula, CA, USA) (diluted to 100-fold with 3% normal rabbit serum/PBS) at 4 C overnight, which was specifically reactive to trypsinogen-1 and trypsin-1. The labeled antigen was detected with a HistoFine Kit (Nichirei Pharmaceutical, Tokyo), and visualized by the 3,3-diaminobenzidine (DAB) reaction. As negative controls, sections were similarly processed with mouse normal IgG (Sigma) in place of the primary antibody.

Northern Blotting--All fresh tissues were snap-frozen in liquid nitrogen immediately and stored at −80°C until use. Total RNAs were prepared from tissues with TRIZOL reagent (GIBCO BRL, Grand Island, NY, USA) according to the manufacturer's protocol. Twenty micrograms each of total RNAs was separated on 1% agarose gels, blotted onto nylon membranes and hybridized with a ^{32}P-labeled human trypsinogen-1 cDNA probe at 42°C for 24 h. Membranes were washed twice in 2 × SSC, 0.1% SDS at 65°C for 30 min and once in 0.1 × SSC, 0.1% SDS at 65°C for 30 min. Thereafter, membranes were exposed to Kodak XAR-5 films with intensifying screens at −80°C for 3 days. To normalize the amount of RNAs blotted, the same membranes were rehybridized with a human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA probe after deprobing the trypsinogen probe.

RESULTS

Analysis of Trypsin and Trypsinogen Secreted by Human Colon Cancer Cell Lines--Our previous study using gelatin zymography showed that 2 of 10 stomach adenocarcinoma cell lines tested, STKM-1 and MKN-28, secreted trypsin activities (*4*). Later studies revealed that unlike the zymogens of matrix metalloproteinases (MMPs), the zymogen of trypsin, trypsinogen, cannot be detected by gelatin zymography without previous activation (*9, 15*). In order to detect both trypsin and trypsinogen, culture media conditioned by various cancer cell lines (conditioned media) were treated with the trypsinogen activator enterokinase (EK) before gelatin zymography in this study.

Gelatinolytic activities in the conditioned media of six kinds of human colon cancer cell lines were analyzed by gelatin zymography (Fig. 1A). The activities of MMPs were eliminated by the addition of EDTA, an inhibitor of MMPs. Gelatinolytic activities of 23 and 25 kDa were detected in four cell lines (Colo-201, SW-480, SW-620, and WiDr) after EK treatment. Colo-201 showed the 25-kDa activity without EK treatment, suggesting that the cells produced an endogenous activator of trypsinogen, probably EK. CaR-1 and RCM-1 secreted neither the 23- nor 25-kDa trypsin even after EK treatment.

Some cultured tumor cell lines secrete two trypsin(ogen)

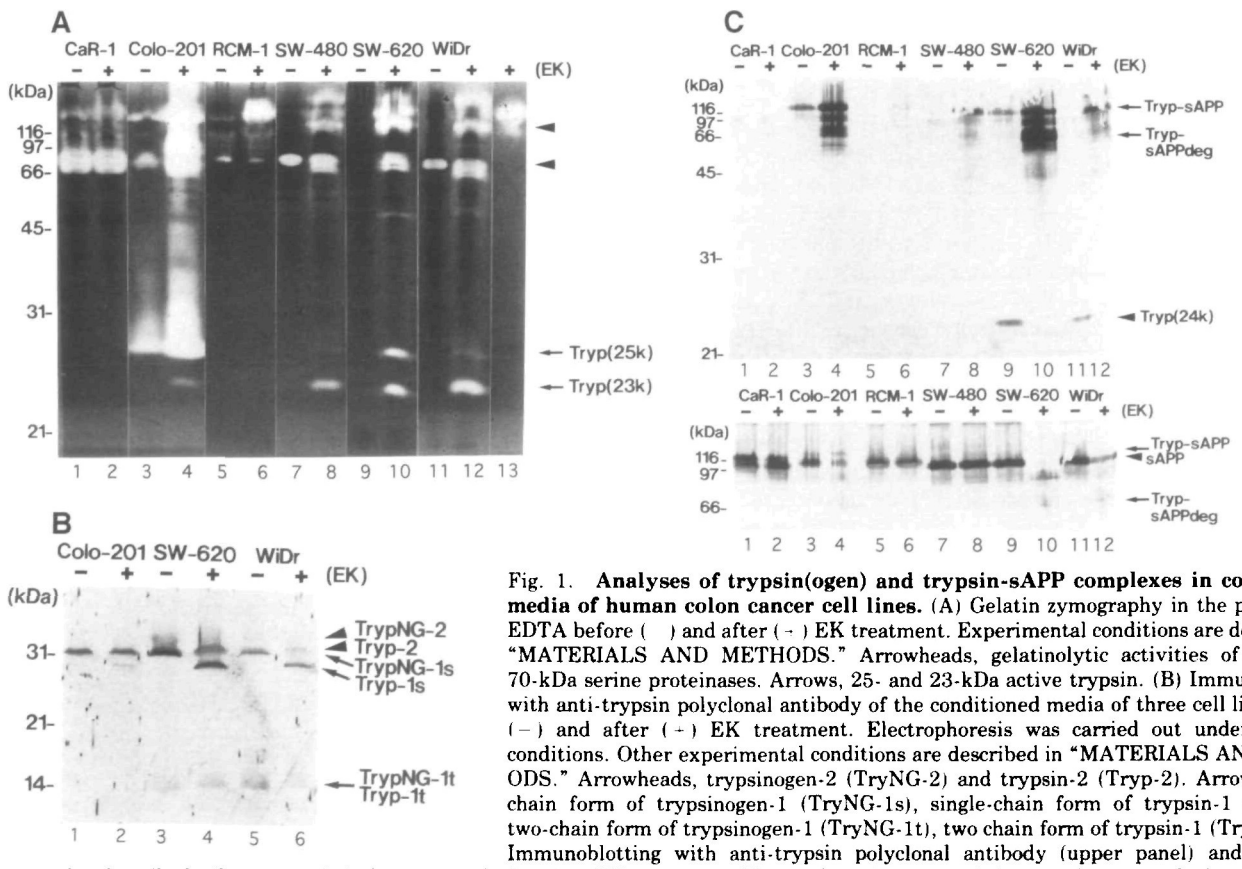


Fig. 1. **Analyses of trypsin(ogen) and trypsin-sAPP complexes in conditioned media of human colon cancer cell lines.** (A) Gelatin zymography in the presence of EDTA before (−) and after (+) EK treatment. Experimental conditions are described in "MATERIALS AND METHODS." Arrowheads, gelatinolytic activities of 130- and 70-kDa serine proteinases. Arrows, 25- and 23-kDa active trypsin. (B) Immunoblotting with anti-trypsin polyclonal antibody of the conditioned media of three cell lines before (−) and after (+) EK treatment. Electrophoresis was carried out under reducing conditions. Other experimental conditions are described in "MATERIALS AND METHODS." Arrowheads, trypsinogen-2 (TryNG-2) and trypsin-2 (Tryp-2). Arrows, single-chain form of trypsinogen-1 (TryNG-1s), single-chain form of trypsin-1 (Tryp-1s), two-chain form of trypsinogen-1 (TryNG-1t), two chain form of trypsin-1 (Tryp-1t). (C) Immunoblotting with anti-trypsin polyclonal antibody (upper panel) and anti-APP monoclonal antibody (lower panel) before (−) and after (+) EK treatment. Electrophoresis was carried out under non-reducing conditions. Other experimental conditions are described in "MATERIALS AND METHODS." Arrows, 130-kDa trypsin-sAPP complex and its partially degraded product of 70 kDa. Arrowheads, 24-kDa trypsinogen and 110-kDa and 120-kDa sAPPs. Ordinate, molecular size in kDa.

isoforms, trypsin(ogen)-1 and trypsin(ogen)-2, which have molecular sizes of 23 and 25 kDa in the mature form, respectively (*6, 15*). In addition, a 25-kDa two-chain form of trypsin which had undergone proteolytic cleavage at the Arg^{107}-Val^{108} bond was found in culture medium of the stomach carcinoma cell line STKM-1 (*18*). To identify trypsin forms secreted by colon cancer cell lines, their conditioned media were analyzed by immunoblotting under reducing conditions with an anti-trypsin polyclonal antibody which was reactive to both trypsin(ogen)-1 and trypsin(ogen)-2 (Fig. 1B). Under reducing conditions, the 23-kDa trypsin-1 and the 25-kDa trypsin-2 in Fig. 1A were separated at apparent molecular sizes of 30 kDa and 32 kDa, respectively. The conditioned medium of Colo-201 showed only trypsin-2 regardless of EK treatment. The conditioned media of SW-620 and WiDr showed trypsinogen-1 at 32 kDa as a major isoform and trypsinogen-2 at 33 kDa as a minor isoform before EK treatment. They were converted to 30- and 32-kDa forms, respectively, after EK treatment. Both conditioned media showed an additional broad band of approximately 14 kDa which seemed to correspond to two-chain forms of trypsinogen-1 and trypsin-1 before and after EK treatment, respectively.

Identification of Trypsin-sAPP Complexes in Conditioned Media—Our previous study on human stomach carcinoma cell line MKN-1 transfected with trypsinogen-1 cDNA suggested that the 130- and 70-kDa gelatinolytic activities in the zymograms might be due to SDS-stable complexes between trypsin and sAPP (*14*). There are three forms of APP, APP770, APP751, and APP695 (*19*). Of these, APP751 and APP770 contain a Kunitz-type proteinase inhibitor (KPI) domain. These membrane-bound APPs are proteolytically processed by α-secretase to release sAPPs.

In the conditioned media of all of the six colon cancer cell lines, gelatinolytic activity was detected at approximately 70 kDa, in addition to the 23- and 25-kDa trypsin activities and the EK activity of about 130 kDa (Fig. 1A). Colo-201, SW-480, SW-620, and WiDr showed additional bands at 120-130 kDa. To examine whether the 130- and 70-kDa gelatinolytic activities in Fig. 1A are trypsin-sAPP complexes, the conditioned media of colon cancer cell lines were analyzed by immunoblotting with an anti-trypsin antibody and an anti-sAPP antibody under non-reducing conditions (Fig. 1C). In the analysis with the anti-trypsin antibody, the EK-treated conditioned media of Colo-201, SW-620, and WiDr showed intensely stained bands between 70 and 130 kDa, possibly due to trypsin-sAPP complexes (Fig. 1C, upper panel).

Analysis with the anti-sAPP antibody 22C11, which recognizes an N-terminal portion of APP, showed a strong band of 110 or 120 kDa in the conditioned media of all six cell lines (Fig. 1C, lower panel). The EK treatment of the conditioned media of Colo-201, SW-620, and WiDr decreased the intensity of the main band and formed several faint bands at lower molecular weight regions. In the conditioned media of Colo-201 and SW-620, a weak immunostained band was seen at the position corresponding to the 130-kDa immunostained band for trypsin. These immunoblotting patterns for sAPP were well correlated with those for trypsin. Essentially the same immunoblot was obtained when another monoclonal antibody (OM84) to sAPP, which recognizes a C-terminal portion of sAPP, was used (data not shown). These results suggested that EK-activated trypsin digested trypsin-sAPP complex and probably KPI-less sAPP (sAPP695). The 130-kDa band detected by the anti-sAPP antibody appeared to be the complex between trypsin and undigested sAPP.

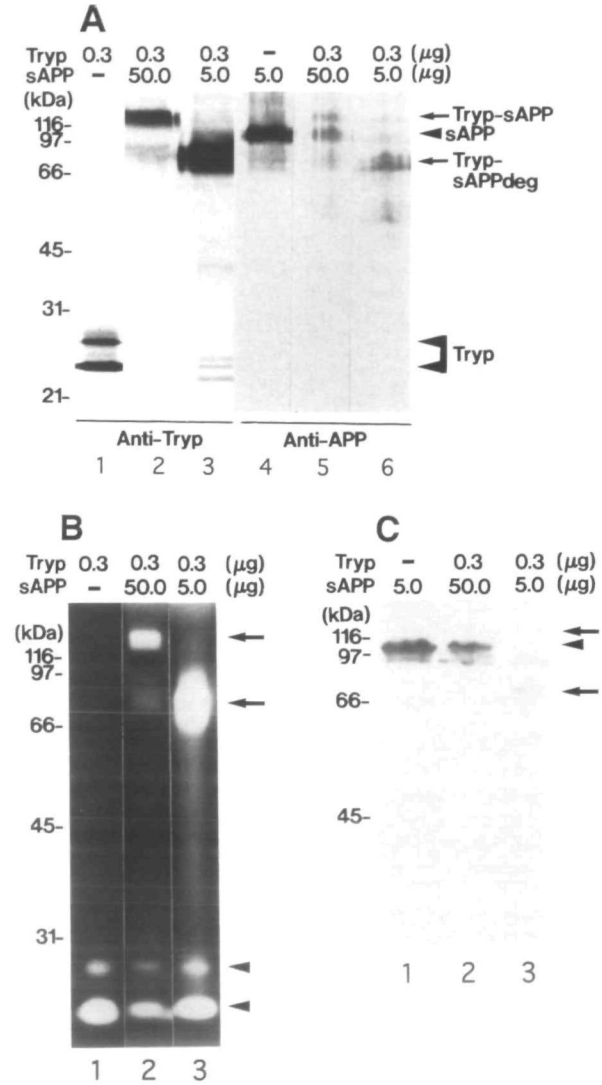


Fig. 2. **Formation of trypsin-sAPP complex.** Human pancreatic trypsin (0.3 μg) and 5 or 50 μg of purified human sAPP were mixed and incubated at 37°C for 5 min. (A) Immunoblotting under non-reducing conditions with anti-trypsin antibody (lanes 1-3) and with anti-sAPP antibody (lanes 4-6). The resultant reaction mixtures containing 0.3 μg trypsin (lanes 1-3) or 1 μg of sAPP (lanes 4-6) were subjected to immunoblotting with anti-trypsin antibody and with anti-sAPP antibody, respectively. (B) Gelatin zymography in presence of EDTA under non-reducing conditions. The resultant reaction mixtures containing 0.03 μg of trypsin were subjected to gelatin zymography. (C) Protein staining after SDS-PAGE under non-reducing conditions. The resultant reaction mixtures containing 5 μg of sAPP were subjected to CBB-staining. Other experimental conditions are described in "MATERIALS AND METHODS." Ordinate, molecular size in kDa. Arrows, 130-kDa trypsin-sAPP complex and its partially degraded product of 70 kDa. Arrowheads, 100-kDa sAPP and 25- and 23-kDa active trypsin. Experiments (A) and experiments (B) (C) were carried out separately. Small differences in band patterns between (A), lanes 5 and 6, and (C), lanes 2 and 3, are due to the difference in samples.

Some inconsistencies were noted in the results of gelatin zymography (Fig. 1A) and immunoblotting under reducing conditions (Fig. 1B) and non-reducing conditions (Fig. 1C). For example, in Fig. 1C, the 23- and 25-kDa trypsins were scarcely detected. In addition, the intensity of the immunoreactive bands for trypsin(ogen)s was much stronger in the EK-treated conditioned media than in the non-treated ones (Fig. 1C). These apparent discrepancies seem to derive at least partly from the differences in sensitivity between zymography and immunoblotting, in specific gelatinolytic activity between free and sAPP-bound trypsins, in immunoreactivity of the antibody between trypsinogen and trypsin, and in electrophoretic mobility and protein-protein association between non-reducing and reducing SDS-PAGEs. Gelatin zymography had a much higher sensitivity than immunoblotting in detection of trypsin and gelatinases (data not shown).

Although the conditioned media of CaR-1 and most other cell lines showed a 70-kDa gelatinolytic activity even before EK treatment, the corresponding immunostained band was not detected with the anti-trypsin antibody (Fig. 1A; 1C, upper panel). This activity was due to a serine

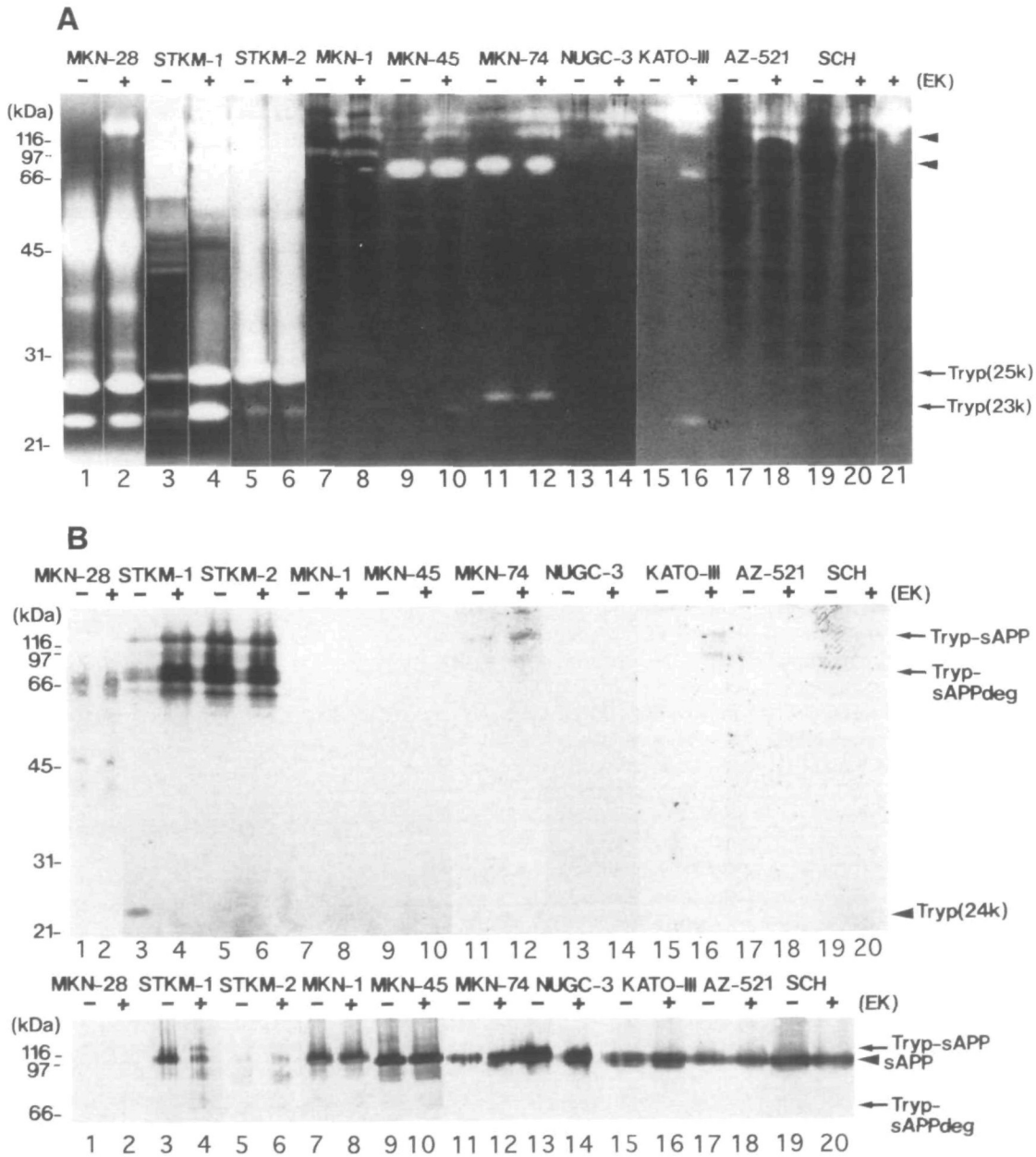


Fig. 3. **Analyses of trypsin(ogen) and trypsin-sAPP complexes in conditioned media of human stomach cancer cell lines.** (A) Gelatin zymography in the presence of EDTA before (−) and after (+) EK treatment. Experimental conditions are described in "MATERIALS AND METHODS." Arrowheads, gelatinolytic activities of 130- and 70-kDa serine proteinases. Arrows, 25- and 23-kDa active trypsin. (B) Immunoblotting with anti-trypsin polyclonal antibody (upper panel) and anti-APP monoclonal antibody (lower panel) before (−) and after (+) EK treatment. Electrophoresis was carried out under non-reducing conditions. Other experimental conditions are described in "MATERIALS AND METHODS." Arrows, 130-kDa trypsin-sAPP complex and its partially degraded product of 70 kDa. Arrowheads, 24-kDa trypsinogen (upper panel) and 110- and 120-kDa sAPPs (lower panel). Ordinate, molecular size in kDa.

proteinase, because it was blocked by diisopropylfluorophosphate (DFP) (data not shown). This 70-kDa band was not detectable by immunoblotting with antibodies against human plasminogen and tissue plasminogen activator. This serine proteinase was not further characterized in this study.

To confirm the formation of trypsin-sAPP complexes, two different amounts of purified sAPP were incubated with a constant amount of trypsin, then analyzed by SDS-PAGE under non-reducing conditions, immunoblotting with anti-trypsin and anti-APP antibodies, and gelatin zymography (Fig. 2). Less than 10% of the purified sAPP was the KPI-containing sAPPs (sAPP770 and sAPP751), and the rest was sAPP695. When 50 μg of sAPP was treated with 0.3 μg of trypsin, the 23- and 25-kDa trypsin bands were almost completely converted to a single 130-kDa band of the trypsin-sAPP complex in the immunoblotting with anti-trypsin antibody (Fig. 2A, lane 2). The 130-kDa band was also detected by the anti-sAPP antibody (Fig. 2A, lane 5). When 5 μg of sAPP was treated with 0.3 μg of trypsin, a 70-kDa major band was detected by both antibodies, indicating that the 130-kDa trypsin-sAPP complex was converted to the 70-kDa complex by limited proteolysis (Fig. 2A, lanes 3 and 6). Gelatin zymography also showed the formation of the trypsin-sAPP complexes of 130 and 70 kDa (Fig. 2B). With 5 μg of sAPP, a smear of gelatinolytic activity was faintly observed between the 70- and 25-kDa activities (Fig. 2B, lane 3). The protein staining after SDS-PAGE showed that sAPP was quickly degraded by excess trypsin at a high trypsin/sAPP ratio (Fig. 2C, lane 3).

Stomach and Breast Cancer Cell Lines—Gelatinolytic activities in the conditioned media of 10 kinds of human stomach cancer cell lines were analyzed by gelatin zymography (Fig. 3A). It has previously been reported that MKN-28 and STKM-1 cell lines secrete trypsin (*4*). STKM-2, as well as MKN-28 and STKM-1, showed a 25-kDa trypsin activity even in the absence of EK treatment. Regardless of EK treatment, MKN-28 secreted a strong 50-kDa gelatinolytic activity, whereas STKM-1 and STKM-2 showed strong activity in a range of 55-130 kDa. The gelatinolytic activity at 23 or 25 kDa was slightly detected in MKN-45 and KATO-III, and the activity of 70 kDa was moderately or slightly detected in MKN-45, MKN-74, and KATO-III.

Immunoblotting analysis with the anti-trypsin antibody showed strong signals at 130, 70, and 60 kDa in STKM-1 and STKM-2 and three weak signals between 66 and 40 kDa before and/or after EK treatment in MKN-28 (Fig. 3B, upper panel). Immunoblotting analysis with the anti-sAPP antibody intensely stained the sAPP of 110-120 kDa in most of the cell lines tested, except for MKN-28 and STKM-2, in which the main band was absent or faintly detected due to its degradation by active trypsin (Fig. 3B, lower panel). The conditioned medium of MKN-28 showed no immunoreactive signal for sAPP. However, when MKN-28 cells were incubated in the presence of soybean trypsin inhibitor, the 110-kDa sAPP was clearly detected on the immunoblotting (data not shown). The 130-kDa trypsin-sAPP complex was also detected by anti-trypsin and anti-sAPP antibodies in the MKN-28 conditioned medium. These results indicate that in the absence of the inhibitor the active trypsin secreted from the cells quickly degrades sAPP into fragments which cannot be detected with the anti-sAPP antibody used. Therefore, it seems likely that the gelatinolytic activities found between 66 and 25 kDa in MKN-28 and STKM-2 are at least in part due to degradation products of trypsin-sAPP complexes (Fig. 3A).

The 70-kDa gelatinolytic activity found in MKN-45 and

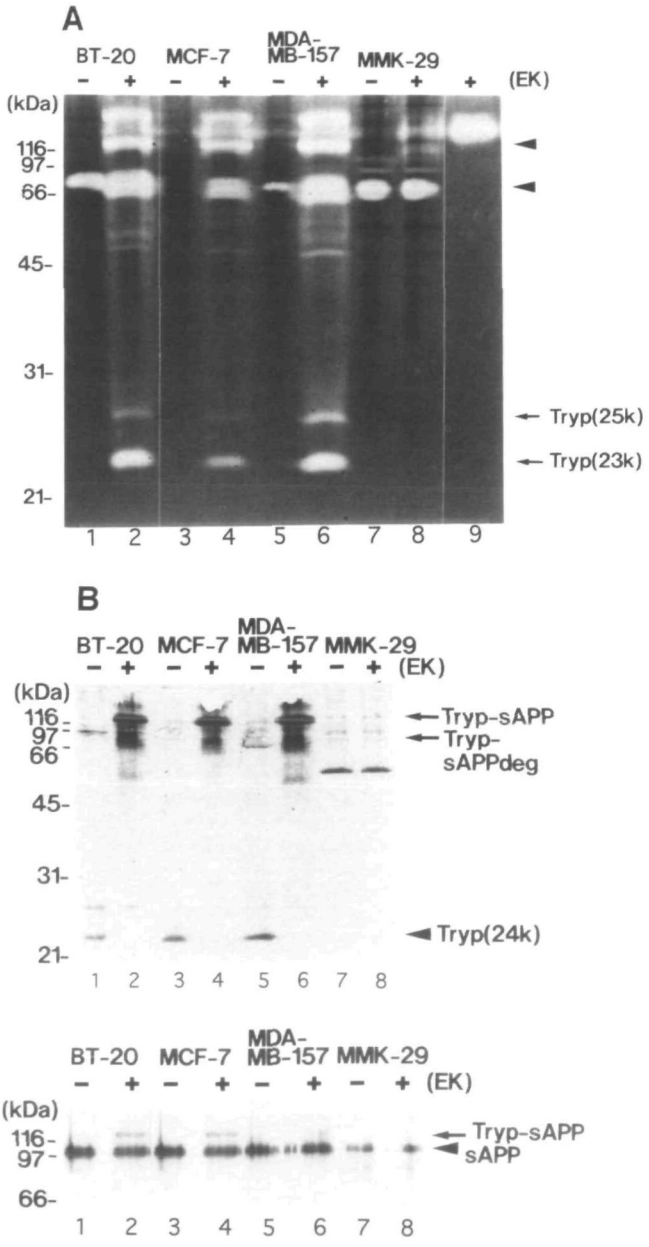
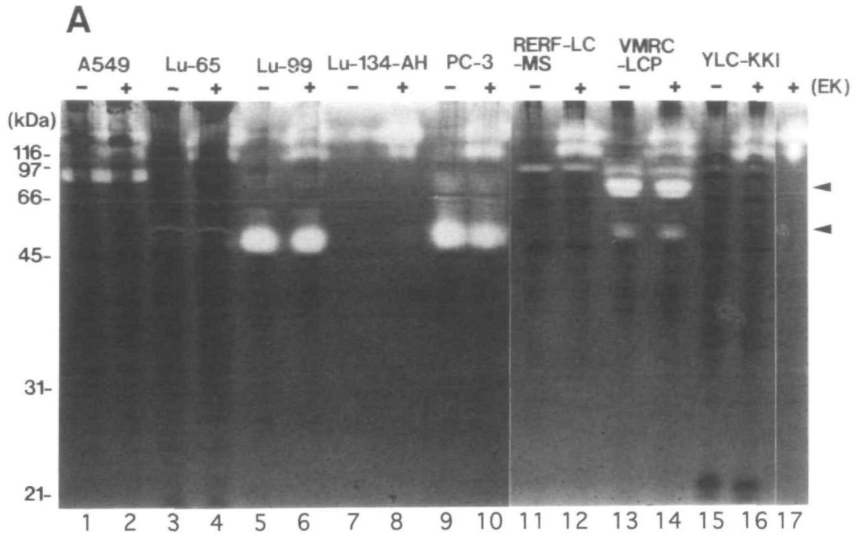


Fig. 4. **Analyses of trypsin(ogen) and trypsin-sAPP complexes in conditioned media of human breast cancer cell lines.** (A) Gelatin zymography in the presence of EDTA before (−) and after (+) EK treatment. Experimental conditions are described in "MATERIALS AND METHODS." Arrowheads, gelatinolytic activities of 130- and 70-kDa serine proteinases. Arrows, 25- and 23-kDa active trypsin. (B) Immunoblotting with anti-trypsin polyclonal antibody (upper panel) and anti-APP monoclonal (lower panel) antibody before (−) and after (+) EK treatment. Electrophoresis was carried out under reducing conditions. Other experimental conditions are described in "MATERIALS AND METHODS." Arrows, 130-kDa trypsin-sAPP complex and its partially degraded product of 70 kDa. Arrowheads, 24-kDa trypsinogen (upper panel) and 110-kDa sAPP (lower panel). Ordinate, molecular size in kDa.



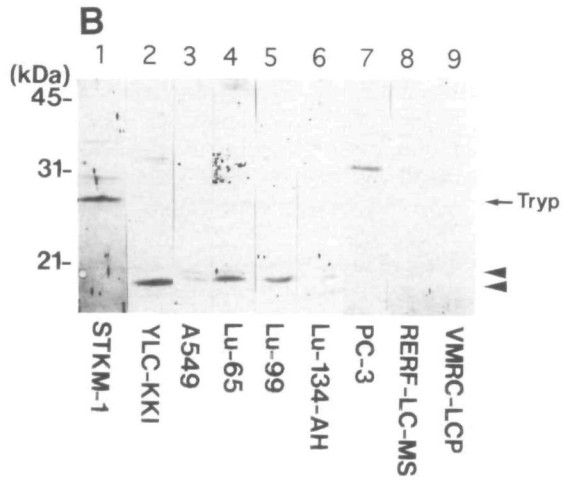


Fig. 5. **Analyses of serine proteinases and trypsin-like proteins in conditioned media of eight human lung cancer cell lines.** (A) Gelatin zymography in the presence of EDTA before (−) and after (+) EK treatment. Experimental conditions are described in "MATERIALS AND METHODS." Arrowheads, gelatinolytic activities of 50- and 70-kDa serine proteinases. (B) Immunoblotting under reducing conditions with anti-trypsin polyclonal antibody after EK treatment. The conditioned medium of stomach cancer cell line STKM-1 was run as a positive control secreting trypsinogen. Other experimental conditions are described in "MATERIALS AND METHODS." Arrow, 23-kDa active trypsin. Arrowheads, 18- and 19-kDa proteins immunoreactive to anti-trypsin antibody. Ordinate, molecular size in kDa.

TABLE I. **Analyses of trypsin and trypsin-sAPP complexes in conditioned media of human colon, stomach, and breast cancer cell lines.**

Cells	EK(−)		EK(+)	
	Trypsin (23 and 25 kDa)	Trypsin-sAPP complexes (130, 70, and 50 kDa)	Trypsin (23 and 25 kDa)	Trypsin-sAPP complexes (130, 70, and 50 kDa)
Colon	1/6	1/6	4/6	4/6
Stomach	4/10	4/10	6/10	5/10
Breast	0/4	0/4	3/4	3/4

The results obtained by gelatin zymography and immunoblotting with anti-trypsin and anti-APP antibodies are summarized (see Figs. 1, 3, and 4). Colon cancer cell lines positive for trypsin and trypsin-sAPP complexes are as follows: EK(−): Colo-201; EK(+): Colo-201, SW-480, SW-620, and WiDr. Stomach cancer cell lines positive for trypsin and/or trypsin-sAPP complexes are as follows: EK(−): MKN-28, STKM-1, STKM-2, and MKN-74; EK(+): MKN-28, STKM-1, STKM-2, MKN-45, MKN-74, and KATO-III. Breast cancer cell lines positive for trypsin and trypsin-sAPP complexes are as follows: EK(+): BT-20, MCF-7, and MDA-MB-157.

MKN-74 before and after EK treatment seemed to be the same unidentified serine proteinase as found in the conditioned media of CaR-1 and other colon cancer cell lines (Fig. 1A).

Trypsin(ogen) secretion was also analyzed in four breast cancer cell lines by gelatin zymography and immunoblotting. BT-20, MCF-7, and MDA-MB-157 showed the 23- and 25-kDa trypsin activities, as well as the 70- and 130-kDa trypsin activities, after EK treatment (Fig. 4A). The 130- and/or 70-kDa trypsin-sAPP complexes were detected by immunoblotting with both anti-trypsin and anti-sAPP antibodies (Fig. 4B, upper and lower panels). In addition, the immunoblotting with the anti-trypsin antibody of the EK-untreated conditioned media of the three cell lines showed a 24-kDa trypsinogen band. The conditioned medium of the other cell line, MMK-29, showed the unidentified 70-kDa activity before and after EK treatment in gelatin zymography (Fig. 4A). Immunoblotting of MMK-29 with the anti-trypsin antibody showed an unidentified 55-kDa band before and after EK treatment (Fig. 4B).

The results obtained with human colon, stomach, and breast cell lines are summarized in Table I. The data indicate that a majority of colon, stomach, and breast cancer cell lines secrete trypsinogen, and that stomach cancer cell lines frequently produce an endogenous trypsinogen activator, thus releasing active trypsin.

Secretion of Trypsin-Like Protein from Human Lung Cancer Cell Lines—In addition to the three types of cancer cell lines, eight lung cancer cell lines were tested for trypsin(ogen) secretion by gelatin zymography and immunoblotting. In the gelatin zymography, no tryptic activities at 23 and 25 kDa were detected in any cell lines regardless of EK treatment, though 50- and 70-kDa gelatinolytic activities were detected in some cell lines (Fig. 5A). Trypsin-sAPP complexes were not detected by immunoblotting with the anti-trypsin polyclonal antibody and the anti-sAPP monoclonal antibody, though the 110- or 120-kDa band was detected in all cell lines (data not shown). However, the immunoblotting with the anti-trypsin antibody showed 18- and 19-kDa immunostained bands in five cell lines (YLC-KKI, A-549, Lu-65, Lu-99, and Lu-134-AH) before and after EK treatment (Fig. 5B). These bands were not detected by immunoblotting with an anti-trypsin monoclonal antibody which specifically reacted to trypsinogen-1 (data not shown). When RT-PCR analysis

was carried out using PCR primers for human pancreatic trypsinogen-1 cDNA, which amplified trypsinogen-1, -2, -3, and -4 mRNAs, none of the cell lines produced any specific fragments (data not shown). These proteins showed no gelatinolytic and caseinolytic activities on zymographic analysis (data not shown). Other cell lines including colon, stomach, and breast cancers did not secrete these low molecular weight immunoreactive proteins. These results suggest that human lung cancer cells secrete unique trypsin-related proteins.

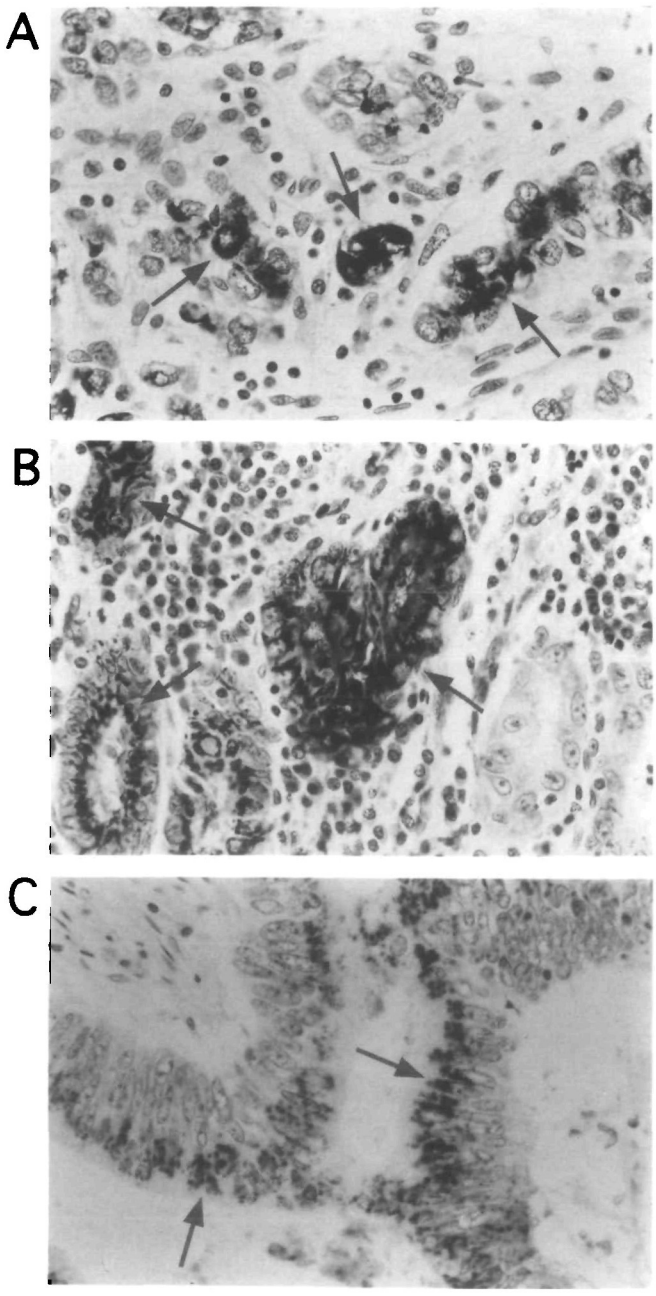


Fig. 6. **Typical examples of immunohistochemical staining for trypsin in stomach and colon cancer tissues.** Trypsin immunoreactivities were noted in signet-ring cell carcinoma of the stomach (A), and well-differentiated tubular adenocarcinoma of the stomach (B) and of the colon (C). Immunohistochemical staining with mouse normal IgG did not show any significant staining (data not shown). Arrows, positive staining of tumor cells for trypsin(ogen).

Expression of Trypsin(ogen) in Human Cancer Tissues—It was previously reported that trypsin(ogen) is immunohistochemically detected in human ovary (*6*) and lung (*7*) cancer tissues. In this study, the expression of trypsin in human stomach, colon, and breast cancer tissues was immunohistochemically analyzed with an anti-trypsin monoclonal antibody. In human stomach cancer tissues, trypsin immunoreactivity was highly detected in tumor cells of signet-ring cell carcinoma (Fig. 6A), well- and moderately-differentiated tubular adenocarcinoma (Fig. 6B), and poorly-differentiated adenocarcinoma (data not shown). Trypsin immunoreactivity was detected in 17 cases (65%) out of 26 stomach cancer tissues examined (Table II). In terms of the non-cohesive type (poorly differentiated adenocarcinoma with scirrhous growth and signet-ring cell carcinoma) and cohesive type (well and moderately differentiated adenocarcinoma) of stomach cancers, trypsin immunoreactivity was more frequently detected in the non-cohesive type (100%) than the cohesive type (53%) (Table III).

Of 31 cases of human colon cancer tissues analyzed, trypsin immunoreactivity was detected in 32% of the cases (Table II). Immunostaining for trypsin was highly detected at the apical sides of the neoplastic glands (Fig. 6C) and the invading front of tumor cells in tubular adenocarcinoma (data not shown). There was no significant correlation between tumor type and trypsin expression in colon cancers.

Of 20 cases of human breast cancer tissues analyzed by immunostaining, no significant trypsin immunoreactivity was detected in any case (Table II).

Trypsinogen mRNA Expression in Tumor Parts and Non-Tumor Parts of Human Stomach Cancer Tissues—Our recent analyses by *in situ* hybridization and immunohistochemistry have demonstrated that trypsin is expressed in various normal human tissues such as the skin, stomach, spleen, and brain (*8*). In the digestive tract, trypsin is expressed in glandular epithelial cells. In the present study, expression of trypsinogen mRNA in tumor and non-tumor parts of human stomach cancer tissues was compared by Northern blotting with human trypsinogen-1 cDNA probe, which also hybridizes with trypsinogen-2, -3, and -4 mRNAs. Trypsinogen mRNA with a size of 0.85 kb

TABLE II. **Expression of trypsin in human stomach, colon, and breast cancer tissues as analyzed by immunohistochemical staining.**

Tumor type	Negative	Positive	Positive (%)
Stomach ($n=26$)	9	17	65.4
Colon ($n=31$)	21	10	32.3
Breast ($n=20$)	20	0	0.0

TABLE III. **Expression of trypsin in cohesive and non-cohesive types of human stomach cancers.**

Tumor type	Negative	Positive	Positive (%)
Cohesive ($n=19$)	9	10	52.6
Non-cohesive ($n=7$)	0	7	100.0

Cohesive types included 3 papillary adenocarcinomas, 13 tubular adenocarcinomas, and 3 soild-type poorly differentiated adenocarcinomas. Non-cohesive types included 3 non-solid-type poorly differentiated adenocarcinomas and 4 signet-ring cell carcinomas.

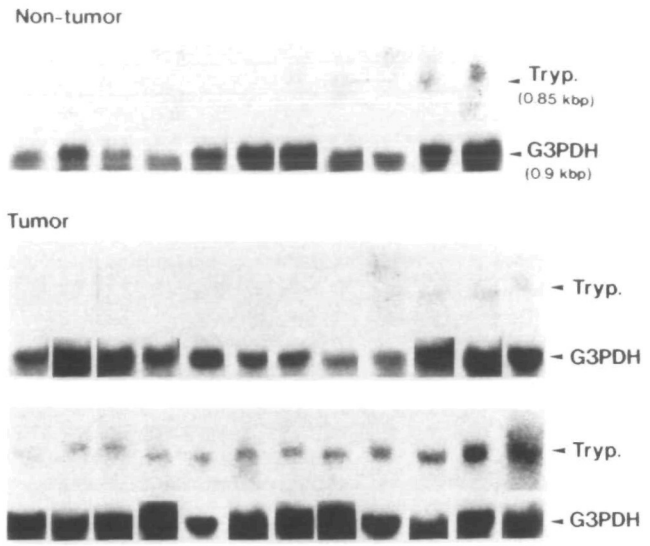


Fig. 7. **Expression of trypsinogen mRNA in tumor parts and non-tumor parts of human stomach cancers.** Total RNAs were extracted from non-tumor parts (from 11 patients) (upper panel) and tumor parts (from 24 patients) (lower panel) of human stomach cancers as described in "MATERIALS AND METHODS." Twenty micrograms of each total RNA was analyzed by Northern blotting with human trypsinogen cDNA probe. As a control, the same membranes were rehybridized with G3PDH cDNA probe. Arrowheads, 0.85 kb of trypsinogen mRNA and 0.9 kb of G3PDH mRNA.

was detected in 1 case (9.1%) out of 11 cases of non-tumor parts and in 11 cases (46%) out of 24 cases of tumor parts of stomach cancer (Fig. 7).

DISCUSSION

Past studies have shown that only a few cancer cell lines secrete trypsin(ogen)s or their related proteins *in vitro* (*2-6*). The present study with highly sensitive gelatin zymography demonstrated that over half of human colon, stomach, and breast cancer cell lines tested secreted trypsin(ogen)-1 and/or trypsin(ogen)-2. It is obvious that the trypsin(ogen)s found in the conditioned media of human cancer cell lines were not derived from bovine trypsin used at the cell harvest, because trypsin(ogen) secretion was specific to cell type and in many cases trypsinogen was secreted but not active trypsin, and because the anti-trypsin antibody used was not cross-reactive to bovine trypsin. It was also found that in most of the trypsinogen-producing stomach cancer cell lines, trypsinogen had been activated by an endogenous activator, possibly EK. Lung cancer cell lines were different from the other three types of cancers: they did not secrete trypsin(ogen)s, although they secreted trypsin-like proteins of 18 and 19 kDa. In addition, most of the colon and breast cancer cell lines and some of the stomach and lung cancer cell lines secreted an unidentified active 70-kDa serine proteinase.

Extracellular matrix proteolysis is strictly regulated in both time and space. Natural proteinase inhibitors play an important role in the regulation of the proteolytic activity. We have previously reported that human cancer cell lines secrete various kinds of trypsin inhibitors including sAPP (*16*). APP is ubiquitously expressed and functions as a potent serine proteinase inhibitor (*20*). It also contains a gelatinase inhibitor domain (*21*). The present study demonstrated that sAPP might be the most important physiological inhibitor of secreted trypsin. Essentially all cancer cell lines tested secreted sAPP into culture medium. Active trypsin quickly bound to sAPP, forming an SDS-stable complex. The bound trypsin seemed to hydrolyze the Kunitz-type proteinase domain of sAPP during zymographic reaction, because the trypsin bound to sAPP exhibited gelatinolytic activity on gelatin zymography. Excess trypsin potently degraded the trypsin-sAPP complex to lower molecular weight complexes. Similar trypsin complexes have been observed in homogenates of various rat tissues including the skin, stomach, and esophagus (*8*). These results indicate that the quantitative balance between active trypsin and sAPP is important for the proteolytic action of trypsin under various physiological and pathological conditions.

Our recent study demonstrated that trypsin is expressed at low levels in many normal non-pancreatic tissues such as epithelia of digestive organs, respiratory tracts, skin, liver, spleen, and brain (*8*). The present immunohistochemical analysis showed that trypsin was significantly synthesized in about 65% of stomach cancers and 32% of colon cancers examined. Trypsin was not detected in breast cancers, although considerable numbers of breast cancer cell lines secrete trypsinogen *in vitro*. In stomach cancers, trypsin immunoreactivity was noted in all cases of the non-cohesive type and in half of the cases of the cohesive-type. Prognosis is poor in the non-cohesive type of stomach cancers (*22*). Northern blotting analysis of stomach cancer tissues indicated that the level of trypsin expression was much higher in the cancer tissues than in non-neoplastic stomach tissues. These results, together with the frequent secretion of active trypsin from stomach cancer cell lines, suggest that trypsin expression is closely associated with the malignant growth of stomach cancers, especially the diffuse invasive growth of the non-cohesive type. This hypothesis is also supported by recent studies showing that overproduction of trypsin in human stomach cancer cell lines is associated with their high tumorigenicity or highly invasive growth in the abdominal cavity of nude mice (*14, 15*). It has also been reported that the expression of trypsinogen mRNA is associated with the malignant potential of human ovarian cancers (*6*).

Degradation of extracellular matrix components by matrix proteinases is essential for tumor cells to invade surrounding stromal tissues and capillary blood vessels. Trypsin hydrolyzes various extracellular matrix proteins such as laminin and fibronectin (*4*). It efficiently activates the latent forms of various MMPs and serine proteinases that are involved in extracellular matrix degradation (*4, 23*). Trypsin is also known to activate thrombin receptor and proteinase-activated receptor 2 (PAR-2), resulting in growth stimulation of endothelial cells (*24*) and vascular smooth muscle cells (*25*). We recently found that overexpression of trypsinogen-1 cDNA in gastric carcinoma cells stimulates both their growth and adhesion to fibronectin and vitronectin *in vitro* (*14*). It seems likely that trypsin secreted by various tumor cells contributes to their growth, invasion, and metastasis by stimulating the degradation of the extracellular matrix components and by modulating the functions of various cell-surface proteins such as growth-

factor receptors, integrins, and other precursor proteins. These possibilities are currently under investigation.

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