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Production of Soluble Matriptase by Human Cancer Cell Lines and Cell Surface Activation of Its Zymogen by Trypsin

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Abstract The membrane-bound serine proteinase matriptase, which is often released from the plasma membrane of epithelial and carcinoma cells, has been implicated to play important roles in both physiological and pathological conditions. However, the regulatory mechanism of its activity is poorly understood. In the present study, we examined expression and activation state of soluble matriptase in 24 human cancer cell lines. Soluble matriptase was detected in the conditioned media from all of 5 colon and 4 breast carcinoma cell lines and 8 of 10 stomach carcinoma cell lines tested. Only two of five lung cancer cell lines released the matriptase protein into the culture media. Out of the five matriptase-negative cell lines, two cell lines expressed the matriptase mRNA. Among 24 cancer cell lines tested, 13 cell lines secreted trypsin in an active or latent form and all of them released matriptase. Most of the 24 cell lines released a latent, single-chain matriptase of 75 kDa as a major form, as well as low levels of complex forms of an activated two-chain enzyme with its specific inhibitor HAI-1. Thus, these soluble matriptases appeared to have little proteolytic activity. Treatment of stomach and colon cancer cell lines with epidermal growth factor stimulated the release of matripatase/HAI-1 complexes. In cancer cell lines secreting active trypsin, however, matriptase was released mostly as an inhibitor-free, two-chain active form. Trypsin seemed to activate the membrane-bound, latent matriptase on the cell surface. These results suggest that matriptase and trypsin cooperatively function for extracellular proteolysis. J. Cell. Biochem. 95: 632–647, 2005.

Key words: serine proteinase; matriptase; trypsin; HAI-1; cancer

It has been widely accepted that extracellular proteolysis is critical for the processes of tumor invasion and metastasis [Nagase and Woessner, 1999; Nelson et al., 2000; Seiki and Yana, 2003]. Degradation of extracellular matrix is essential for tumor cells to invade basement membranes, stromal tissues, and blood vessels. Various matrix metalloproteinases (MMPs) are thought to play a major role in the matrix degradation, but serine proteinases including plasminogen activators, plasmin and trypsin are also involved in the tumor progression. There is accumulating evidence that these proteinases contribute to tumor malignancy by not only degrading extracellular matrix proteins but also modulating the functions of various extracellular proteins, including cell surface receptors [Egeblad and Werb, 2002; Seiki and Yana, 2003]. For example, the membrane-bound MMP MT1-MMP has been reported to cleave CD44 [Kajita et al., 2001], syndecan-1

Abbreviations used: CBB, Coomassie Brilliant Blue R-250; EGF, epidermal growth factor; HAI-1, hepatocyte growth factor activator inhibitor-1; MMP, matrix metalloproteinase; phosphate-buffered saline (PBS), Ca^{2+}- and Mg^{2+}-free PBS.

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[Endo et al., 2003], and integrin αv [Deryugina et al., 2002] on the cell surface, and the basement membrane protein laminin-5 [Koshikawa et al., 2000], thereby enhancing tumor cell migration. The serine proteinase trypsin, which is secreted by many types of human carcinoma cells in vitro and in vivo [Koshikawa et al., 1992; Miyata et al., 1999], activates protease-activated receptor-2 (PAR-2), stimulating integrin-dependent cell adhesion and proliferation of human gastric carcinoma cells [Miyata et al., 2000].

Recently, a new type of membrane-bound serine proteinase, matriptase (or membrane type serine proteinase-1/MT-SP1), has been characterized [Lin et al., 1997]. This enzyme was initially found as a trypsin-like serine proteinase secreted by human breast cancer cells [Shi et al., 1993] and later purified as a complex with HGF activator inhibitor-1 (HAI-1) from human milk [Lin et al., 1999b]. cDNA cloning revealed that matriptase is a type II transmembrane serine proteinase with multiple extracellular domains [Takeuchi et al., 1999; Lin et al., 1999a]. Matriptase is expressed in normal epithelial tissues such as the skin, stomach, colon, kidney, breast, ovary,and pancreas, but not in mesenchyma [Oberst et al., 2001, 2002, 2003a]. Experiments with matriptase-deficient mice have shown that matriptase plays critical roles in the epidermal barrier function, hair follicle development,and thymic homeostasis [List et al., 2002]. On the other hand, matriptase and its inhibitor HAI-1 are co-expressed in cancer tissues of the breast, ovary and cervix and also by some stomach and colon carcinoma cell lines in vitro [Oberst et al., 2001, 2002]. Matriptase degrades extracellular matrix proteins and activates urokinase-type plasminogen activator (uPA), HGF, and PAR-2 [Lee et al., 2000; Takeuchi et al., 2000]. These activities of matriptase, as well as it distribution, suggest that it may play a role in the growth, invasion, and metastasis of human carcinoma cells [Aimes et al., 2003; Suzuki et al., 2003]. However, the regulatory mechanism of matriptase activity has not fully been clarified. We previously reported that a variety of human carcinoma cells secrete a 70-kDa serine proteinase, provisionally identified as a soluble form of matriptase, as well as active and/or latent forms of trypsin [Hirahara et al., 1998; Miyata et al., 1999]. In this study, we investigated the production of the soluble form of matriptase by various human cancer cell lines and its interaction with trypsin.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Human stomach cancer cell lines used here were adenocarcinoma (MKN-28, STKM-2, STKM-1 (clone S4), MKN-45, MMK-74, NUGC-3), adenosquamous carcinoma (MKN-1); unspecified carcinoma (AZ521), signet-ring cell carcinoma (KATO-III), and choriocarcinoma (SCH). Human colon adenocarcinoma cell lines used were WiDr, COLO-201, DLD-1, SW480, and RCM-1. Human breast carcinoma cell lines used were BT-20, MMK-29, MDA-MB-157, and MCF-7. Human lung carcinoma cell lines used were adenocarcinoma (A549), small cell carcinoma (YLC-KKI), squamous cell carcinoma (VMRC-LCP), and giant cell carcinoma (Lu-99 and Lu-65). The sources and culture conditions of these cell lines were described previously [Miyata et al., 1999]. DMEM/F12 or RPMI 1640 (Invitrogen, Carlsbad, CA) was used as a basal culture medium for each cell line.

Preparation of Serum-Free Conditioned Media of Human Cancer Cell Lines

Cells were grown in 90-mm culture dishes with DMEM/F12 or RPMI 1640 medium containing 10% fetal calf serum to confluence, rinsed twice with the Ca^{2+}- and Mg^{2+}-free PBS, and incubated in the serum-free basal culture medium overnight. After the culture medium was replaced with the fresh serum-free medium, the cultures were further incubated for 2 days. The serum-free conditioned media were collected, centrifuged to remove cell debris, dialyzed against pure water at 4°C, and freeze-dried. The dried protein powder was dissolved in a 1/100 volume to the initial conditioned medium of 20 mM Tris-HCl (pH 7.5) buffer and used as a 100-fold concentrated conditioned medium. Protein concentrations of the conditioned media were measured using Bradford protein assay reagents (Bio-Rad, Hercules, CA).

Immunoblotting for Matriptase

Matriptase was routinely analyzed by immunoblotting with a monoclonal antibody against human matriptase (M32) [Lin et al., 1999b; Benaud et al., 2001]. In some experiments, another mouse monoclonal antibody (M69), which recognizes the two-chain, active form of matriptase [Benaud et al., 2001, 2002a,b], was

used. Concentrated conditioned media containing 15 μg protein were resolved by SDS–AGE on 10% polyacryamide gels under nonreducing conditions unless otherwise specified, transferred onto Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and subsequently probed with a matriptase-specific monoclonal antibody (M32 or M69). A biotinylated rabbit anti-mouse-IgG antibody (Vector Laboratories, Burlingame, CA) was used as a secondary antibody for M32, and a HRP-conjugated, goat anti-mouse-IgG antibody (Amersham Biosciences, Buckinghamshire, UK) was used as a secondary antibody for M69. Immuno-reactive bands with M32 were detected by the alkaline phosphatase method, and those with M69 were detected by the enhanced chemiluminescence (ECL) method (Amersham Biosciences).

Gelatin Zymography

Zymographic analysis of gelatinolytic activity was carried out on 10% or 12.5% polyacrylamide gels containing 1 mg/ml gelatin as described before [Koshikawa et al., 1992; Shi et al., 1993; Miyata et al., 1999]. After SDS–PAGE under nonreducing conditions, the gels containing separated proteins were incubated in a renaturation buffer consisting of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 2.5% Triton X-100 for 1.5 h and then in a 50 mM Tris-HCl (pH 7.5) buffer supplemented with 0.5 mM EDTA for 30 min to eliminate MMP activities. Finally, the gels were incubated at 37°C for 16 h in a reaction buffer, consisting of 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$ to detect the gelatinolytic activity of matriptase. The resultant gels were stained with Coomassie Brilliant Blue R-250 (CBB) [Shi et al., 1993]. In some experiments, trypsinogen present in conditioned media was activated to trypsin by incubating the protein sample with 500 ng/ml of enterokinase (Biozyme Laboratories, Blaenavon, Great Britain) at 37°C for 1 h, and the activated trypsin was analyzed by the gelatin zymography [Miyata et al., 1999].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses of Matriptase and HAI-1 mRNAs

Total RNAs were isolated from cultured human cancer cells using the TRIzol reagent (Invitrogen), reverse-transcribed to cDNAs by Mo-MLV reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's protocol, and used as the templates to amplify a matriptase cDNA fragment by the PCR. Primers used for the PCR of matriptase message were 5′-CACCCCTTCTTCAATGACTTCACC-3′ (nucleotide no. 2134–2157, sense) and 5′-TACCCCAGTGTTCTCTTTGATCC-3′ (nucleotide no. 2579–2601, antisense). After initial denaturation at 94°C for 5 min, 30 cycles of incubation at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 1 min were performed to amplify the cDNA fragment of matriptase. HAI-1 primers used were 5′-ATGTGCCTCGCATCCAAC-3′ (nucleotide no. 745–762, sense) and 5′-TCAGAGGGGCCGGGTGGTGT-3′ (nucleotide no. 1572–1590, antisense) [Parr et al., 2004]. The PCR was performed with 28 cycles of denaturation at 94°C for 40 s, annealing at 58°C for 60 s, and elongation at 72°C for 90 s.

Expression of Exogenous Trypsinogen-1 Gene in MKN-45 Cells

An expression vector containing the full-length human trypsinogen-1 cDNA, pEF-TRY-CITE-neo, was previously prepared by Miyata et al. [1998]. The trypsinogen-1 expression vector was transfected into MKN-45 cells using the lipofectin reagent (Invitrogen), and stable transfectants highly expressing the trypsinogen gene (Tryp-MKN-45) were selected and cloned as described before [Miyata et al., 1998].

Assay of Matriptase Activity

The enzyme activity of matriptase was assayed at 37°C in 200 μl of 50 mM Tris-HCl (pH 7.5) buffer containing 0.1 M NaCl, 0.01% (v/v) Briji 35, 0.01% (w/v) bovine serum albumin and 5 mM $CaCl_2$, using a fluorescent peptide substrate (2 nM Boc-Glu-Ala-Arg-MCA) (Peptide Institute, Osaka, Japan) [Lee et al., 2000]. After incubation for an appropriate length of time, the fluorescent intensity of the reaction mixture was measured with excitation at 360 nm and emission at 480 nm, using a Cytofluor 2350 fluorometer (Millipore; Bedford, MA). To assay matriptase activity selectively, concentrated conditioned media (2.5 μg protein/3 μl) containing both matriptase and trypsin were first incubated with 160 μg ovomucoid (Sigma) in 200 μl of 20 mM Tris-HCl (pH 7.5) buffer containing 0.5 M NaCl, 0.1% CHAPS, and 0.01% Brij 35 for 5 min, which inhibits trypin activity selectively [Yamasaki et al., 2003], and then with Sepharose beads conjugated with the anti-

matriptase antibody M32 (2.5 μg IgG/40 μl beads) at 4°C for 4 h. The incubated beads were precipitated by centrifugation and washed three times with 200 μl of the same Tris-HCl buffer. Matripatase bound to the beads was eluted with 0.1 M glycine-HCl buffer (pH 2.8), neutralized, and subjected to the activity assay, zymography and immunoblotting. In some experiments, the conditioned media (2.5 μg protein) were incubated with ovomucoid (80 μg) as above and then directly incubated with the reaction mixture containing the enzyme sample.

RESULTS

Release of Matriptase by Human Stomach Cancer Cell Lines

Matriptase has been purified as a soluble enzyme from human milk [Lin et al., 1999b] and from the conditioned media of human breast cancer cells [Lin et al., 1997]. We analyzed soluble matriptase present in the conditioned media of various types of human cancer cell lines both by immunoblotting with the monoclonal antibody M32 [Lin et al., 1999b; Benaud et al., 2001] and by gelatin zymography [Shi et al., 1993]. The immunoblotting analysis of 10 stomach cancer cell lines revealed that at least 8 cell lines released matriptase at relatively high levels, while the other two cell lines, MKN-1 and AZ521, released it at very low levels, if at all (Fig. 1A). In most cell lines, matriptase was separated into a doublet around 75 kDa as a major component and two minor bands of 110 and 95 kDa, the latter two of which appeared to correspond to the complexes of matriptase and it inhibitor HAI-1 [Lin et al., 1997, 1999b]. The 75-kDa doublet bands seemed to be produced by glycosylation or other post-translational modifications. On the other

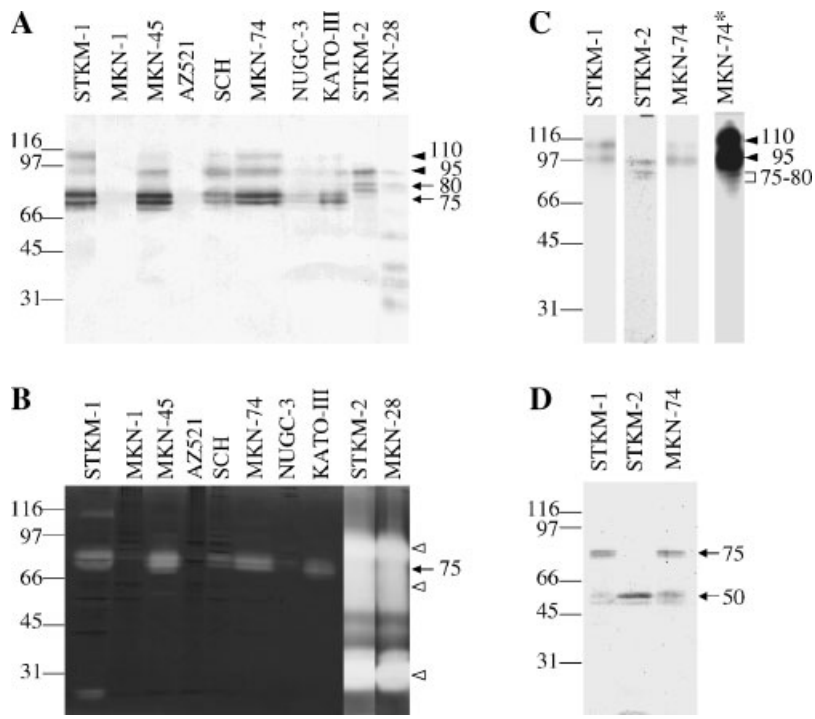

Fig. 1. Analysis of matriptase in conditioned media of 10 human stomach cancer cell lines. **A**: Immunoblotting with the anti-matriptase monoclonal antibody M32 under nonreducing conditions. Serum-free conditioned media were prepared from the confluent cultures of 10 stomach cancer cell lines, and the concentrated samples containing 15 μg protein were applied to the immunoblotting as described in Materials and Methods. Arrows, matriptase with apparent molecular sizes of 80 and 75 kDa; arrowheads, matriptase/HAI-1 complexes. **B**: Gelatin zymography. The same samples were applied to gelatin zymography under the conditions described in Materials and Methods. Arrow, gelatinolytic activity of 75-kDa matriptase; open arrowheads, activities due to trypsin. The two upper arrowheads correspond to the complexes of trypsin with soluble forms of β-amyloid protein precursor. **C**: Immunoblotting under nonreducing conditions with the antibody M69, which specifically recognizes the two-chain, active matriptase. In the left three lanes, chemiluminescence detection of immuno-reactive bands was performed by exposing the membrane to an X-ray film for 10 min. In the fourth lane (MKN-74*), the exposure time for the MKN-74 conditioned medium was increased to 50 min to detect a 75-kDa active matriptase. **D**: Immunoblotting under reducing conditions with the M32 antibody. Arrows indicate the 75-kDa single-chain, latent matriptase and a 50-kDa heavy chain of the two-chain, active enzyme.

hand, the conditioned media of STKM-2 and MKN-28 showed a doublet around 80 kDa instead of the 75-kDa doublet, as well as the 95-kDa band. In addition, the MKN-28 conditioned medium contained lower molecular weight, presumably degraded forms of matriptase.

Matriptase activity in the conditioned media was also analyzed by zymography on gelatin-containing gels (Fig. 1B). In order to detect serine proteinases selectively, the gels containing separated proteins were pre-incubated with 0.5 mM EDTA for 30 min, and then incubated in a reaction mixture containing 5 mM $CaCl_2$ [Shi et al., 1993]. Under these conditions, MMP activities were not detected. The cell lines that produced the 75-kDa, immuno-reactive band of matriptase showed a gelatinolytic activity at a similar position. Gelatinolytic activity was barely detected at the positions corresponding to the 110- and 95-kDa bands, shown in Figure 1A. The conditioned media of AZ521 and MKN-1 showed little gelatinolytic activity. The conditioned media of STKM-2 and MKN-28 showed strong and broad gelatinolytic activities at 23–25 kDa and 50–100 kDa. As previously reported [Miyata et al., 1999], these activities were thought to be due to active trypsin and its complexes with β-amyloid protein precursor (APP). STKM-1, which is known to secrete trypsinogen [Koshikawa et al., 1992], showed a faint gelatinolytic activity of active trypsin at 23 kDa.

To characterize the immuno-reactive bands shown in Figure 1A, the conditioned media of three cell lines (MKN-74, STKM-1 and STKM-2) were further analyzed by immunoblotting under different conditions. When conditioned media containing 15 μg protein were analyzed by immunoblotting with the anti-matriptase antibody M69, which specifically recognizes the two-chain, active enzyme [Benaud et al., 2001], the 95-kDa band and the 80-kDa doublet in STKM-2 showed immuno-reactivity to this antibody (Fig. 1C). In MKN-74 and STKM-1 cells, the 110- and 95-kDa bands showed immuno-reactivity to the M69 antibody. Although these cell lines showed gelatinolytic activity at 75 kDa or higher in Figure 1B, any immuno-reactive band was not detected at the corresponding position by the antibody M69. However, when the chemiluminescence detection was enhanced by a prolonged exposure time, faint immuno-reactive bands were detected at 75–80 kDa, in addition to the intense bands at 95 and 110 kDa (Fig. 1C, lane 4). Furthermore, these conditioned media were analyzed by the immunoblotting under reducing conditions with the anti-matriptase antibody M32 (Fig. 1D). The conditioned media of MKN-74 and STKM-1 showed immuno-reactive bands at 75 and 50 kDa, while that of STKM-2 showed a band only at 50 kDa. The results shown in Figures 1A-D indicated that the 75-kDa band in MKN-74 and STKM-1 (Fig. 1A) was a single-chain, latent enzyme, while the 110- and 95-kDa bands were complexes of the activated two-chain enzyme, presumably with HAI-1 [Lin et al., 1999b]. In addition, it was concluded that STKM-2 cells released only the active two-chain enzyme in either the major, 80-kDa noncomplexed form or the minor, 95-kDa form complexed with HAI-1. Both the 80-kDa enzyme and the two-chain enzyme in the 110- and 95-kDa complexes were thought to be composed of a 50-kDa subunit and a smaller subunit, which was undetectable with the M32 antibody.

Release of Matriptase by Colon, Breast, and Lung Cancer Cell Lines

When five colon cancer cell lines were analyzed as above, all the cell lines showed a relatively strong matriptase doublet at 75 kDa, by both immunoblotting and zymography (Figs. 2A,B). The conditioned medium of DLD-1 cells contained the highest level of matriptase. COLO-201 cells secreted active trypsin, as detected by zymography (Fig. 2B).

Like colon cancer cell lines, the conditioned media of four breast cancer cell lines all showed the 75-kDa matriptase band and additional minor bands (Fig. 2C,D). However, the complexes of matriptase and HAI-1 were barely detected in these cell lines. MDA-MB-157 cells secreted a low level of active trypsin, as detected by zymography (Fig. 2D).

When five lung cancer cell lines were analyzed, only two cell lines, LU-99 (giant carcinoma) and VMRC-LCP (squamous cell carcinoma), showed the 75-kDa matriptase band by immunoblotting (Fig. 2E), as well as some gelatinolytic activities (Fig. 2F).

The results of the four types of human cancer cell lines are summarized in Table I. Most of the stomach, colon, and breast cancer cell lines released soluble forms of matriptase, whereas only two of five lung cancer cell lines released matriptase. Past studies have also shown the release of soluble matriptase from MKN-45 cells [Ihara et al., 2002] and expression of matriptase

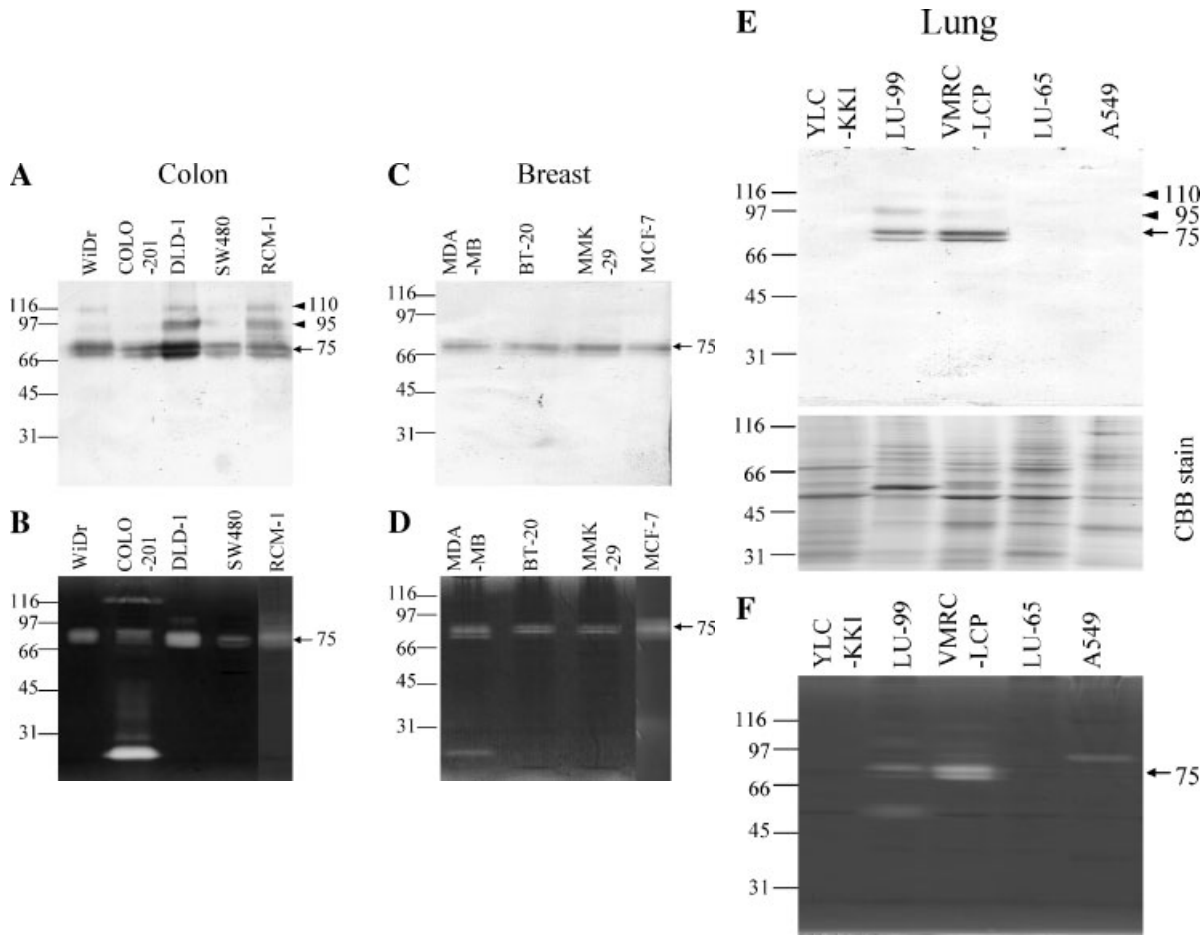

Fig. 2. Analysis of soluble matriptase released by human colon, breast, and lung cancer cell lines. Conditioned media of colon (**A**, **B**), breast (**C**, **D**), and lung (**E**, **F**) cancer cell lines were analyzed by immunoblotting with the M 32 antibody (A, C, E) and by gelatin zymography (B, D, F). In (E, **lower panel**), the conditioned media of five lung cancer cell lines were separated by SDS–PAGE, and the separated proteins were stained with CBB as the internal loading controls. Experimental conditions are the same as in Figure 1.

message in MDA-MB and MCF-7 cell lines [Oberst et al., 2001].

We have previously analyzed expressions of trypsin(ogen) in various human cancer cell lines by immunoblotting and gelatin zymography, following enterokinase treatment [Miyata et al., 1999]. Of 24 cell lines tested in this study, the following 13 cell lines secreted trypsin as a mature form and/or a zymogen (trypsinogen): MKN-28, STKM-1, MKN-74, STKM-2, COLO-201, DLD-1, WiDr, SW480, KATO-III, MDA-MB, BT-20, MCF-7, and MMK-29. All of the tripsin(ogen)-expressing cell lines also expressed matriptase, while half of the other cell lines expressed matriptase (Table II).

TABLE I. Summary of Analyses of Soluble Matriptase in Four Types of Human Cancer Cell Lines

	Matriptase	
Cell lines	Immunoblotting (%)	Gelatinolytic activity (%)
Stomach	8/10 (80)	8/10 (100)
Colon	5/5 (100)	5/5 (100)
Breast	4/4 (100)	4/4 (100)
Lung	2/5 (40)	2/5 (40)

The results were summarized from the data shown in Figures 1 and 2.

Expression of Matriptase mRNA and HAI-1 mRNA

As shown above, matriptase was barely detected in the conditioned media of two stomach cancer cell lines (AZ521 and MKN-1) and three lung cancer cell lines (YLC-KKI, LU-65, and A549). To examine the relationship between the release of matriptase and gene expression, the expression of matriptase mRNA in most

TABLE II. Comparison Between Expressions of Soluble Matriptase and Trypsin(ogen)

	Matriptase		
Trypsin(ogen)	Positive	Negative	Total
Positive	13	0	13
Negative	6	5	11
Total	19	5	24

$P < 0.05$.
The results were summarized from the data shown in Figures 1 and 2 for matriptase expression and the previously published data [Miyata et al., 1999] for trypsin(ogen) expression.

of the 24 cell lines was analyzed by RT-PCR. Some of the results are shown in Figure 3. The three cell lines, AZ521, YLC-KKI, and LU-65, barely expressed the matriptase mRNA (data not shown), but MKN-1 and A549 intermediately or weakly expressed the message (Fig. 3A). In MKN-1 and A549, matriptase was undetectable not only in the condition media (Fig. 3B) but also in their cell lysates (data not show). To test the possibility of rapid degradation of matriptase, we included a mixture of proteinase inhibitors (4-(2-aminoethyl)-benzenesulfonyl-fluoride (AEBSF), aprotinin, bestatin, E-64, leupeptin, pepstatin, and TAPI) in the culture medium. Even in the presence of these inhibitors, however, matriptase protein was detected neither in the condition media nor in the cell lysates (data not shown). The results of other cell lines shown in Figure 3 also indicated that the matriptase mRNA level was not well correlated with the level of soluble enzyme in the conditioned media. For example, Lu-99 and MKN-74 weakly expressed the matriptase message RNA, whereas they produced the soluble protein at very high levels.

HAI-1 is known to be often co-expressed with matriptase in normal epithelial cells and carcinoma cells [Oberst et al., 2001; Parr et al., 2004].

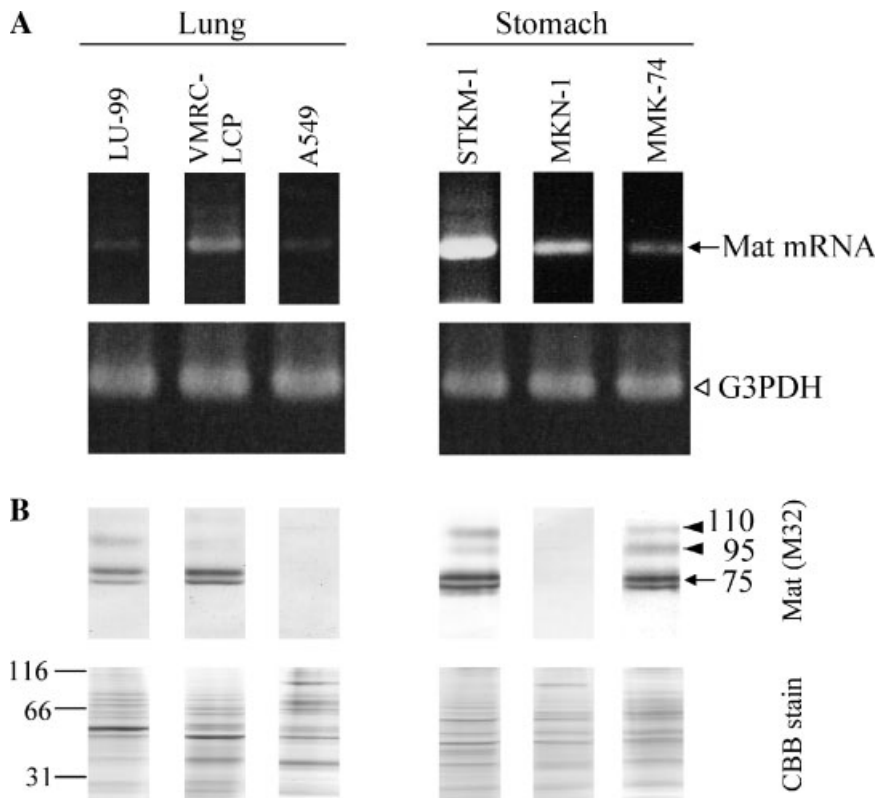

Fig. 3. Comparison between expression of matriptase mRNA and release of soluble forms of matriptase in three lung and three stomach cancer cell lines. **A**: Analysis of matriptase mRNA by RT-PCR. Total RNAs (5 μg each) from Lu-99, VMLC-LCP, A549, STKM-1, MKN-1, and MKN-74 cells were reverse-transcribed, and one-fifth of the transcripts was amplified by PCR, and then the PCR products were applied to gel electrophoresis. Other experimental conditions are described under Materials and methods. Arrow, amplified DNA fragment of matriptase message (**upper panel**); open arrowhead, amplified DNA fragment of glyceraldehydes-3-phosphate dehydrogenase (G3PDH) message (**lower panel**). **B**: Immunoblotting of matriptase in the conditioned media of the six cell lines with the monoclonal antibody M32 (**upper panel**). The lower panel indicates CBB-stained patterns of the total proteins as internal loading controls. Experimental conditions and symbols are the same as in Figure 1.

We also examined expression of HAI-1 message in selected 10 cell lines. All the cell lines more or less expressed HAI-1, but there was no apparent correlation between the expressions of HAI-1 and matriptase (data not shown). Among three breast cancer cell lines tested, HAI-1 expression was very low in MMK-29 and MDA-MB, but intermediate in BT-20. Therefore, the apparent lack of matriptase-HAI-1 complexes in these cell lines (Fig. 2C) cannot be fully explained by the level of HAI-1. The stomach cancer cell line MKN-74 and the lung cancer cell line LU-99 showed very high levels of HAI-1 expression, whereas the colon cancer cell line DLD-1, which highly produced soluble matriptase, expressed a low level of HAI-1.

Effects of Growth Factors on Release of Matriptase

It has been reported that EGF promotes the redistribution of activated matriptase at the sites of membrane ruffling [Benaud et al., 2002a]. EGF may also affect the release of soluble matriptase from cell membrane. In this study, we investigated effects of three growth factors (EGF, platelet-derived growth factor, insulin) on the mRNA expression and release of soluble enzyme by the gastric carcinoma cell line STKM-1. None of the three growth factors significantly affected the mRNA level (Fig. 4A, upper panel). However, EGF increased the level of the 110-kDa band in the conditioned medium (Fig. 4A, center panel). The increased release of the 110-kDa complex was more evident as analyzed by immunoblotting with the M69 antibody specific for the two-chain enzyme (Fig. 4A, lower panel). A similar stimulatory effect of EGF was reproduced in the colon cancer cell line WiDr (Fig. 4B). EGF treatment increased the levels of 110- and 95-kDa complex forms of matriptase in the conditioned medium. However, this effect was not evident in the stomach cancer cell lines MKN-74 and the breast cancer cell line MMK-29 (data not shown). These results suggested that EGF promoted the activation of pro-matriptase on the cell surface and/or the release of the activated matriptase in some types of cancer cells.

Activation of Matriptase by Trypsin

Human carcinoma cells often produce another type of serine proteinase, pancreatic trypsin, in vitro and in vivo [Miyata et al., 1999; Ichikawa et al., 2000]. In the present study, two gastric carcinoma cell lines secreting high

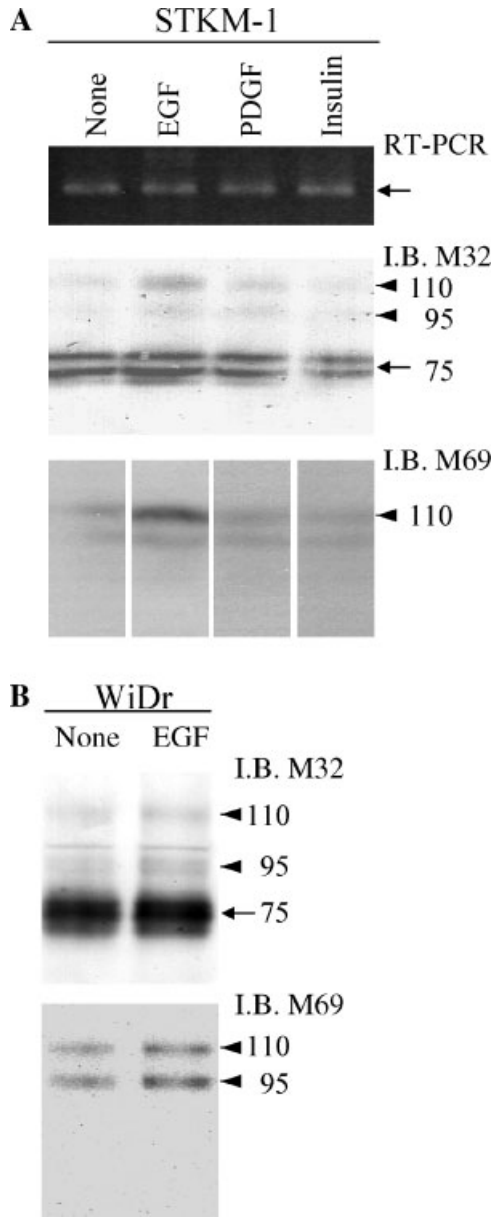

Fig. 4. Effects of growth factor treatments of STKM-1 and WiDr cells on expression of matriptase message and soluble enzyme. **A**: STKM-1 cells were grown to confluence in 90-mm culture dishes with serum-containing RPMI-1640 medium, washed with PBS and incubated for 2 days in serum-free medium supplemented without (none) or with 30 ng/ml EGF, 2 ng/ml platelet-derived growth factor (PDGF), or 5 μg/ml insulin. From the resultant cultures, cells and conditioned media were collected. Matriptase mRNA in the cell lysates was analyzed by RT-PCR (**upper panel**). Soluble forms of matriptase present in the conditioned media were analyzed by immunoblotting with the M32 antibody (**center panel**) and the M69 antibody (**lower panel**). Arrowheads, complexed forms of matriptase; arrows, 75-kDa single-chain matriptase. **B**: Effect of EGF treatment of WiDr cells on release of soluble matriptase was analyzed as above. **Upper panel**, immunoblotting with the M32 antibody; **lower panel**, immunoblotting with the M69 antibody. Other experimental conditions are described in Figure 3 and Materials and Methods.

levels of active trypsin, MKN-28 and STKM-2, released the active, two-chain matriptase into the culture medium, suggesting that trypsin might activate the latent matriptase (Fig. 1A). To confirm this possibility, we examined the effect of soybean trypsin inhibitor on the release of the active matriptase in the culture of MKN-28 cells (Fig. 5A). The addition of soybean trypsin inhibitor to culture medium, which inhibited the activation of trypsin (Fig. 5A, right panel), completely changed the electrophoretic profile of released matriptase (Fig. 5A, left panel). In the presence of the inhibitor, the 80-kDa two-chain form, as well as some lower molecular weight forms, disappeared, and instead the 75-kDa single-chain form was detected as a major band.

We also examined the inverse effect by activating trypsinogen that was endogenously produced by STKM-1 gastric carcinoma cells (Fig. 5B). When the culture of STKM-1 cells was treated with the trypsinogen activator enterokinase, the 80-kDa two-chain matriptase, degraded forms with lower molecular weights, and the 95-kDa complexed form were produced, instead of the 75-kDa single-chain form (Fig. 5B, left panel).

To eliminate the possibility that enterokinase might activate matriptase, we further investigated the effect of trypsin on matriptase activation, using MKN-45 cell lines which had been transfected with a trypsinogen-1 cDNA or a control vector. The trypsinogen-transfectant (Tryp-MKN-45) of MKN-45 cells, but not the

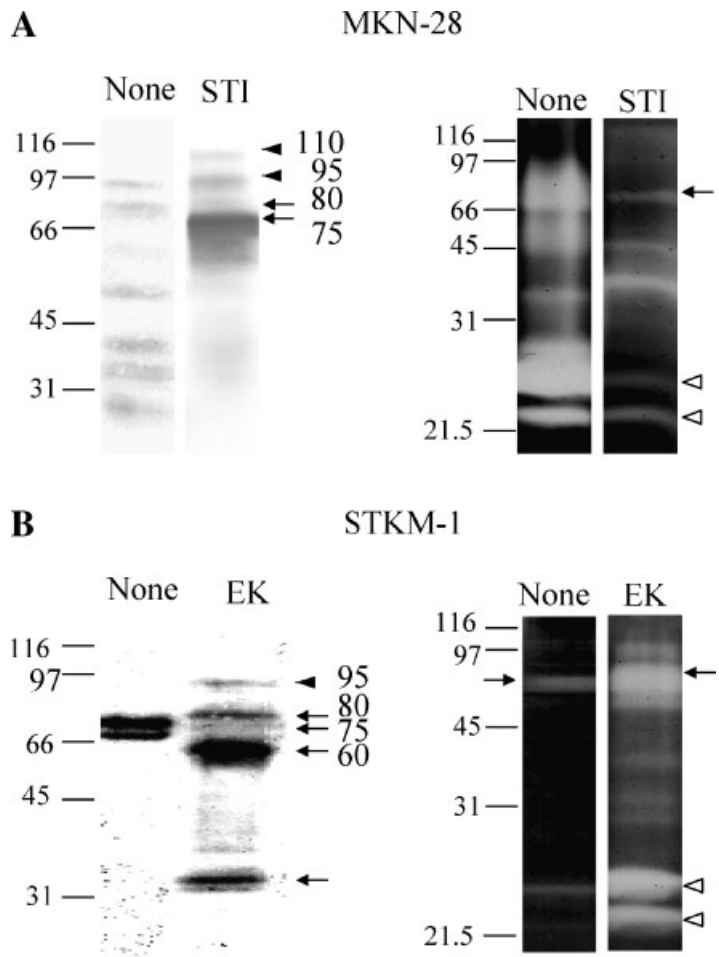

Fig. 5. Effects of inhibition and activation of endogenous trypsin(ogen) on release of soluble matriptase. **A**: Confluent cultures of MKN-28 cells, which secreted active trypsin, were incubated without (none) or with 100 μg/ml soybean trypsin inhibitor (STI) (Sigma, Louis, MO) in serum-free RPMI-1640 medium for 2 days. **B**: Confluent cultures of STKM-1cells, which secreted trypsinogen but little active trypsin, were incubated without (none) or with 500 ng/ml enterokinase (EK) in serum-free RPMI-1640 medium for 2 days. The conditioned media of the resulting cultures were collected and applied to immunoblotting with the monoclonal antibody M32 (**left panels**) and to gelatin zymography (**right panels**). Other experimental conditions are described in Figure 1.

control transfectant (Mock-MKN-45), released a high level of trypsinogen (Fig. 6A). When the culture was incubated in the presence of exogenous enterokinase, the molecular size of the major matriptase was 75 kDa in the Mock-MKN-45 culture, but 80 kDa in the Tryp-MKN-45 culture as analyzed by immunoblotting (Fig. 6B). In addition, the Tryp-MKN-45 culture released matriptase forms of 95 and 60 kDa. When the conditioned media were analyzed by gelatin zymography, following removal of active trypsin, Tryp-MKN-45 cells showed a strong gelatinolytic activity at 80 kDa, which appeared to have a slightly lower migration rate than the activity in Mock-MKN-45 cells (Fig. 6C). In the immunoblotting analysis with the anti-matriptase antibody M69, all of the 95-, 80- and 60-kDa bands in the Tryp-MKN-45 culture were reactive to the active-matriptase-specific antibody, whereas only trace reactivity was observed at 95 kDa in the Mock-MKN-45 culture (Fig. 6D). When the conditioned media were boiled in the presence of SDS and then analyzed by the immunoblotting with the matriptase antibody M32, the 95- and 60-kDa bands in Tryp-MKN-45 cells disappeared, and 50- and 35-kDa bands appeared, in addition to the major band of 80 kDa (Fig. 6E). This strongly suggested that both the 95- and 60-kDa bands were complexes of active matriptases with HAI-1. The partially degraded, 35-kDa matriptase species appeared to form the 60-kDa complex with HAI-1. Taken together, these data indicated that trypsin might activate the matriptase zymogen

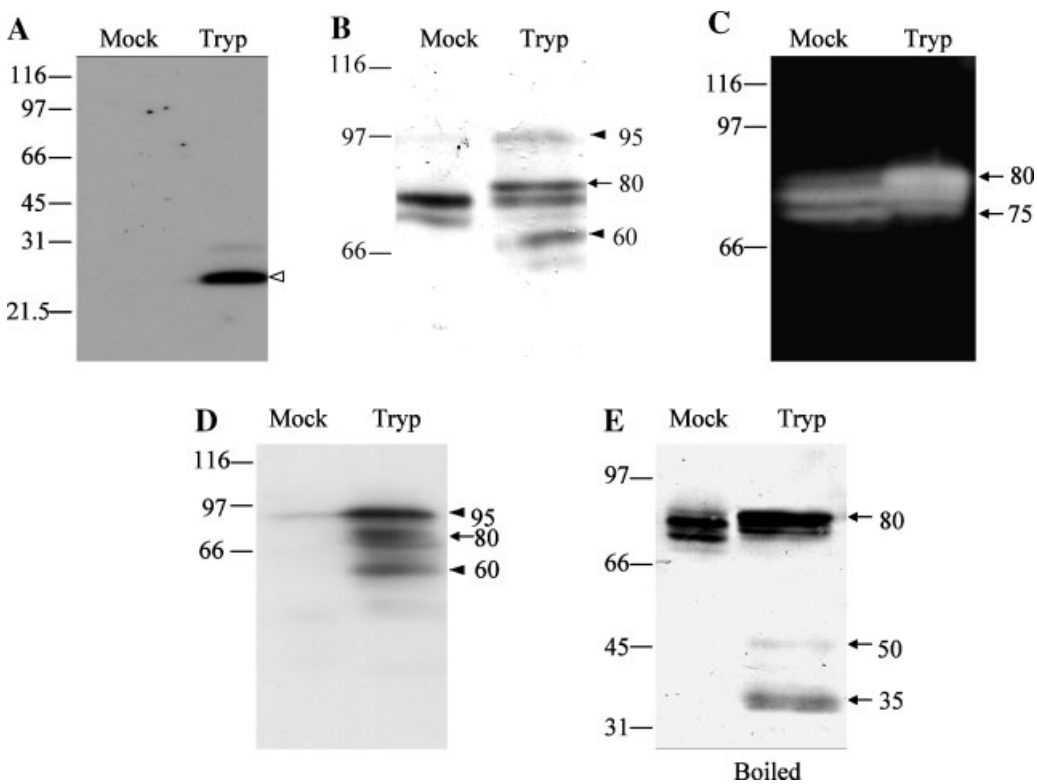

Fig. 6. Effect of enterokinase treatment on release of soluble forms of matriptase from MKN-45 gastric carcinoma cells over-expressing exogenous trypsinogen. MKN-45 cells transfected with a human trypsinogen cDNA (Tryp-MKN-45; Tryp) or with a control vector (Mock-MKN-45; Mock) were grown to confluence in 90-mm culture dishes and incubated with 500 ng/ml enterokinase in serum-free RPMI-1640 medium for 2 days. The conditioned media of the two cultures were collected and applied to immunoblotting with a monoclonal antibody against human trypsin (Chemicon, Temecula, CA) (**A**), immunoblotting with the anti-matriptase antibody M32 (**B**, **E**), gelatin zymography (**C**), and immunoblotting with the active matriptase-specific antibody M69 (**D**). In (C), the condition media were flowed through an affinity column conjugated with the anti-trypsin antibody to remove trypsin, concentrated and applied to the electrophoresis. In (E), the concentrated conditioned media were mixed with the SDS sample buffer without reducing reagent and boiled for 5 min to dissociate the matriptase/HAI-1 complexes and then applied to the electrophoresis. Other experimental conditions are described in Figure 1 and in Materials and Methods. Open arrowhead in A, trypsinogen of 25 kDa; closed arrowheads, matriptase/HAI-1 complexes; arrows, matriptases free of HAI-1.

and produced the noncomplexed, two-chain matriptase. It was also concluded that exogenous enterokinase was not able to activate matriptase.

To further confirm that the latent matriptase is activated by trypsin, we tried to assay the enzymatic activity of matriptase in conditioned media from the entrerokinase-treated cultures of Mock-MKN-45 and Tryp-MKN-45 cells. After the conditioned media were shortly incubated in the presence or absence of an excess amount of ovomucoid, which inhibits trypsin activity but not matriptase activity [Yamasaki et al., 2003], the enzyme activity was assayed using the synthetic peptide substrate Boc-Glu-Ala-Arg-MCA (a factor XIa substrate). As shown in Figure 7A, the conditioned medium of Mock-MKN-45 cells showed little hydrolytic activity regardless of the presence or absence of ovomucoid, whereas that of Tryp-MKN-45 cells showed a high activity even in the presence of ovomucoid. To eliminate residual trypsin activity completely, matriptase was immunoprecipitated with the matriptase antibody M32 from the conditioned media. The antibody efficiently precipitated matriptase, and no trypsin activity was detected in the immunoprecipitates by gelatin zymography (Fig. 7B). When the immunoprecipitates were assayed for the enzyme activity, Tryp-MKN-45 cells again showed a much higher activity than Mock-MKN-45 cells (Fig. 7C). These results confirmed that the latent matriptase was in fact activated by trypsin.

Trypsin-Dependent Activation of Matriptase on Cell Surface

To determine whether or not trypsin directly processes the 75-kDa, soluble form of matriptase zymogen to the two-chain, active matriptase in culture medium, the cell-free conditioned media of Mock-MKN-45 and Tryp-MKN-45 cells were incubated in the presence or absence of enterokinase and then analyzed by immuoblotting (Fig. 8). When the conditioned medium of Mock-MKN-45 cells was incubated, the amount of the 75-kDa latent matriptase was barely changed during 6-h incubation, regardless of the presence or absence of enterokinase. However, the matriptase in the conditioned medium of Tryp-MKN-45 cells almost completely disappeared by

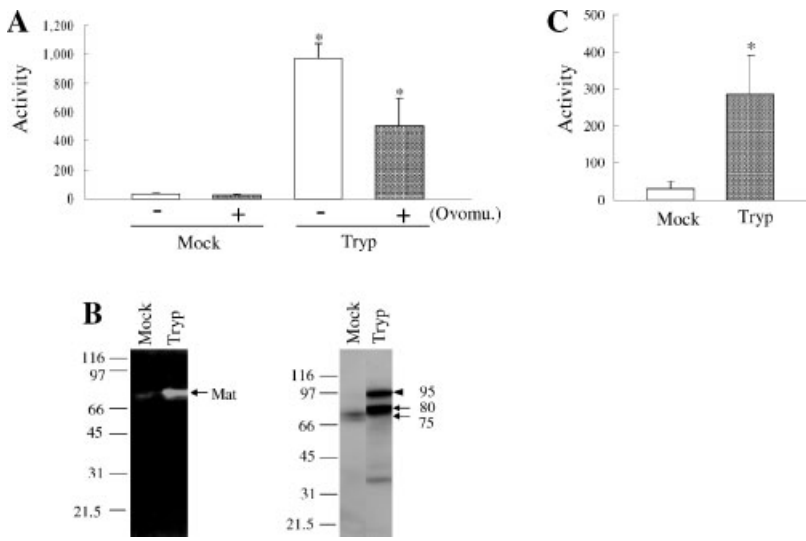

Fig. 7. Matriptase activity in conditioned media of Mock-MKN-45 and Tryp-MKN-45 cells after enterokinase treatment. **A**: Conditioned media were prepared from Mock-MKN-45 (Mock) and Tryp-MKN-45 (Tryp) cells, which had been treated with enterokinase as described in Figure 6. The conditioned media were further treated with (+) or without (−) an excess amount of ovomucoid for 5 min, which is known to inhibit trypsin activity selectively, and their enzyme activities were assayed by incubating with the synthetic peptide substrate Boc-Glu-Ala-Arg-MCA (a factor XIa substrate) for 30 min. Ordinate indicates fluorescent intensity produced by the hydrolysis of the substrate. Each value represents the mean ± SD of the hydrolytic activities obtained from triplicate cultures. The asterisk (*) indicates statistical significance ($P < 0.05$). **B**: To eliminate the trypsin activity completely, matriptase was immunoprecipitated with the anti-matriptase antibody M32 from the ovomucoid-treated condtitioned media of Mock-MKN-45 and Tryp-MKN-45 cells. The resultant immunoprecipitates were analyzed by gelatin zymography (**left panel**) and immunoblotting with the anti-matriptase antibody M32 (**right panel**). **C**: The immunoprecipitates from (B) were assayed for the enzyme activity by incubating with the enzyme substrate for 60 min, as described in (A). Other experimental conditions are described in Materials and Methods.

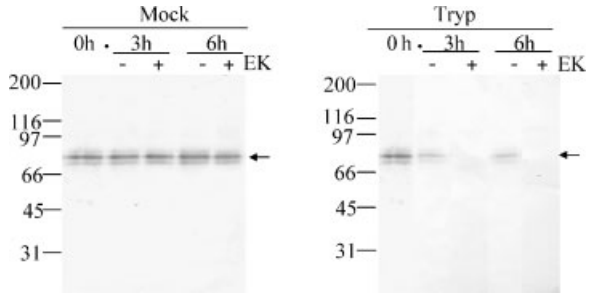

Fig. 8. Degradation of single-chain matriptase by trypsin in conditioned medium. Serum-free conditioned media were prepared from Mock-MKN-45 (Mock) and Tryp-MKN-45 (Tryp) cells and concentrated as described in Figure 6. Both conditioned media were incubated with (+) or without (–) 500 ng/ml enterokinase (EK) for 3 and 6 h. The incubated samples, together with untreated samples (0 h), were applied to immunoblotting with the M32 antibody to detect the 75-kDa matriptase. The enterokinase treatment activated trypsinogen present in the conditioned medium of Tryp-MKN-45 cells. Arrows indicate the 75-kDa, single-chain matriptase.

3-h incubation in the presence of enterokinase. Even in the absence of enterokinase, the amount of matriptase in Tryp-MKN-45 cells was significantly reduced by the incubation for 3 h. These results indicate that the 75-kDa, soluble form of latent matriptase is not directly activated by trypsin, and rather it is degraded. The degradation of matriptase in the absence of enterokinase might be due to trypsin auto-activated during the incubation.

Next, we examined the activation of matriptase on the cell surface by trypsin. Tryp-MKN-45 and Mock-MKN-45 cells were incubated in the presence of enterokinase, and then their membrane fractions were analyzed by immunoblotting and gelatin zymography. There was no apparent difference in total membrane proteins between Tryp-MKN-45 and Mock-MKN-45 cell lines (Fig. 9A). However, immunoblotting with the M32 antibody revealed that the total amount of a major membrane matriptase was much higher in Mock-MKN-45 cells than Tryp-MKN-45 cells (Fig. 9B). Significantly, when the M69 antibody was used for detection, the two-chain, active matriptase appeared to be more abundant in the membrane fraction of Tryp-MKN-45 cells than that of Mock-MKN-45 cells (Fig. 9C). Furthermore, we determined whether there was active trypsin on the membrane of Tryp-MKN-45 cells. Immunoblotting with an anti-trypsin antibody detected 70- and 55-kDa bands in the membrane fraction of

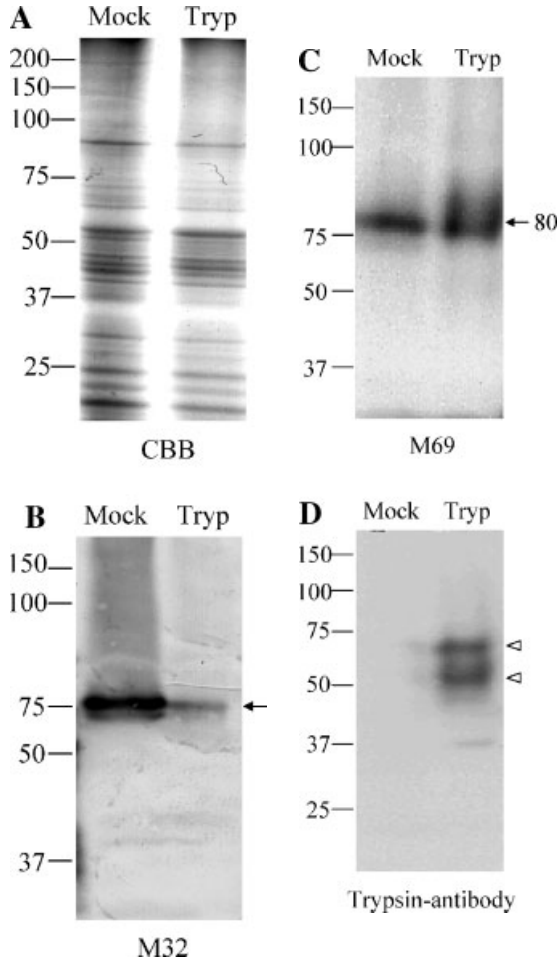

Fig. 9. Activation of matriptase by trypsin on cell surface. Mock-MKN-45 cells (Mock) and Tryp-MKN-45 cells (Tryp) were incubated with 500 ng/ml enterokinase in serum-free RPMI-1640 medium for 2 days as described in Figure 6. The resultant cells were collected, and their membrane fractions were prepared according to the two-phase method with 1% Trixton-114 [Bordier, 1981]. The membrane fractions were applied to SDS–PAGE, followed by CBB staining as internal loading controls (**A**), nonreducing immunoblotting with the anti-matriptase antibody M32 to detect total matriptase (**B**), immunoblotting with the anti-matriptase antibody M69 to detect the active matriptase (**C**), and immunoblotting with a rabbit anti-human-trypsin(ogen) polyclonal antibody (Athens Research and Technology, Athens, GA) to detect membrane-bound trypsin (**D**). Arrows indicates the single-chain, latent matriptase (B) or the two-chain, active matriptase (C). Open arrowheads indicate trypsin (D).

Tryp-MKN-45 cells but not in Mock-MKN-45 cells (Fig. 9D). These bands were presumed to be the complex forms of trypsin with HAI-1 or with partially degraded forms of β-APP. All these results strongly suggest that trypsin activates a membrane-bound matriptase zymogen on the cell surface. The lower amount of total matrip-

tase in the membrane fraction of Tryp-MKN-45 cells seems to result from the efficient activation and release of membrane matriptase by trypsin (see Figs. 6B,D).

DISCUSSION

The present study demonstrated that most of the human carcinoma cell lines tested produced high levels of soluble matriptase. Soluble matriptase was detected in conditioned media from all of 5 colon and 4 breast carcinoma cell lines and 8 of 10 stomach carcinoma cell lines tested. Notably, only 2 of 5 lung carcinoma cell lines released the matriptase protein into the culture media. Of the 5 matriptase-negative cell lines, two cell lines (MKN-1 and A549) intermediately or weakly expressed matriptase mRNA as determined by RT-PCR, but the protein product was undetectable even in their membrane fractions. It is unknown whether the lack of matriptase protein in the two cell lines is due to its rapid degradation or to the failure of its protein synthesis. Even in other cell lines, the expression level of matriptase mRNA was not well correlated with the amount of soluble matriptase. It seems likely that many factors affect the shedding and stability of matriptase. In this context, it has been reported that the glycosylation of matriptase by GnT-V greatly increases its stability against trypsin, chymotrypsin, or other proteinases [Ihara et al., 2002, 2004].

It has previously been reported that matriptase is released from normal epithelial cells and carcinoma cells in both a single-chain zymogen and complex forms of a two-chain matriptase with HAI-1 [Lin et al., 1997; Benaud et al., 2002a]. Consistent with these studies, we found that many human carcinoma cell lines released to the culture media a latent, single-chain matriptase of approximately 75 kDa as a major component, and the 95- and 110-kDa complexes of a two-chain form with two different sizes of HAI-1. In addition, they released a weak activity at 75 kDa or higher as detected by gelatin zymography. This weak gelatinolytic activity might be due to a trace amount of active matriptase released from the matriptase-HAI-1 complexes due to equilibrium in the conditioned media or by SDS–AGE. The two forms of matipatase/HAI-1 complexes showed little activity on zymography. It is noted that the complex forms were barely detectable in any of four breast cancer cell lines tested in this study. Since one of them appreciably expressed HAI-1 message, there may be a mechanism by which the matriptase zymogen is not efficiently activated in these cell lines. However, the low levels of matipatase/HAI-1 complexes are unlikely to be a common feature in breast carcinoma cells, because the release of matipatase/HAI-1 complexes from some breast carcinoma cell lines has been reported [Lin et al., 1997; Benaud et al., 2002a].

EGF promotes membrane ruffling and the redistribution of active matriptase to the membrane ruffles [Benaud et al., 2002a]. In the present study, the treatment of cancer cells with EGF increased the secretion of matripatase/HAI-1 complexes in two of four cell lines tested, but it did not produce the noncomplexed, active matriptase. The mechanism of matriptase shedding and its physilogical relevance have poorly been understood. Recently, Oberst et al. [2003b] reported a transactivation mechanism of matriptase on the surface of BT549 breast cancer cells. In this model, a 95-kDa, full-length latent matriptase on the cell surface is first cleaved at an NH_2-terminal site by an unidentified enzyme, and then the cleaved matriptase, while still remaining anchored to the membrane, forms an activation complex with HAI-1 through the interaction between their noncatalytic domains, leading to the autoactivation of the enzyme and its release from the membrane. Thus, HAI-1 acts not only as the inhibitor of active matriptase but also as an essential component for the matriptase activation. These activities of HAI-1 are similar to those of TIMP-2, which not only plays an essential for the activation of MMP-2 (gelatinase A) by MT1-MMP on the cell surface but also regulates the activity of activated MMPs [Kinoshita et al., 1998]. Although it is unknown whether the matriptase/HAI-1 complexes are formed before or after the shedding from the cell surface, there seems to be little chance for the released matriptase to exert proteolytic activity, unless the soluble zymogen is activated.

The present study demonstrated that only cancer cell lines producing active trypsin released high levels of the inhibitor-free, active matriptase. The activation of matriptase occurred even when trypsinogen-producing cells were treated with exogenous enterokinase to activate trypsinogen. Consistent with recent reports [Ihara et al., 2002, 2004], however, trypsin degraded the soluble, latent matriptase

in a cell-free conditioned medium (Fig. 8). A similar degradation was obtained when a latent matriptase purified from conditioned medium was incubated with trypsin (data not shown). On the other hand, the data shown in Figure 9 indicated that the membrane-bound latent matriptase was activated by trypsin. All these results strongly suggest that trypsin activates the membrane-bound matriptase zymogen on the cell surface, but it rather degrades the released matriptase zymogen in extracellular space. The mechanism of the membrane-bound matriptase activation by trypsin remains to be clarified.

The activation of matriptase by trypsin seems to be clearly different from the matriptase activation in trypsin-nonproducing cell lines, which barely released the noncomplexed, active matriptase activation into conditioned media. It seems likely that in tumor cells without trypsinogen expression, matriptase activity is restricted to a close vicinity of cell surface, whereas trypsin-secreting tumor cells can release the inhibitor-free, soluble matriptase. Of 24 cancer cell lines tested in this study, 13 cell lines secreted trypsinogen or trypsin, and all of them expressed matriptase (Table II). Two cell lines (STKM-2 and MKN-28) secreted active trypsin at high levels, and three cell lines (COLO-201, STKM-1, and MDA-MB) did at low levels. It has been reported that trypsin(ogen) is frequently expressed in the cancer tissues of the stomach, colon, and ovary [Hirahara et al., 1998; Miyata et al., 1999]. In addition, active trypsin is often detected in various tissues [Koshikawa et al., 1998]. Therefore, the aberrant expression of matriptase and trypsin in vivo may accelerate matrix degradation by their direct actions and through activation of other proteinases, such as urokinase and MMPs. They can also activate HGF, protease-activated receptpor-2 (PAR-2), and presumably other functional proteins in the tumor cell vicinity. These proteolytic activities are likely to promote tumor cell growth and invasion. We initially expected that matriptase, on the cell surface or in a soluble form, might activate trypsinogen to trypsin. However, cell lines expressing high levels of matriptase, such as STKM-1 gastric carcinoma cells and MKN-45 cells transfected with trypsinogen cDNA, did not efficiently activate trypsinogen. Therefore, membrane-bound serine proteinases other than matriptase may be responsible for the activation of trypsinogen in STKM-2 and MKN-45 cells.

Many studies have suggested that matriptase is involved in tumor progression. However, the exact role of matriptase still remains unclear. Oberst et al. [2001] reported that matriptase and HAI-1 are co-expressed by many breast cancer cell lines, as well as immortalized mammary epithelial cell lines. Their expression is well correlated with the expression of the epithelial marker E-cadherin, but it is not associated with the expression of a marker of the mesenchymal phenotype (vimentin) and the in vitro invasive potential of cancer cells. Matriptase and HAI-1 are also co-expressed by both carcinoma cells and normal epithelial cells in many human cancer tissues of the breast, ovary, and colon [Oberst et al., 2001]. Another study reports that expression of the HGF receptor, c-Met, matriptase, and HAI-1 is correlated with poor prognosis of node-negative breast cancer patients [Kang et al., 2003]. Therefore, even if matriptase is involved in tumor progression, the activation state of matriptase, rather than its protein level, seems to be critical. HAI-1 obviously plays a key role in the regulation of its enzyme activity. It has been shown that ovarian cancers at the advance stage (III/IV) frequently express matriptase but rarely express its inhibitor (HAI-1) [Oberst et al., 2002]. Other studies also suggest that the imbalance between matriptase and HAI-1 is important for the tumor development, especially tumor invasion and metastasis [Johnson et al., 2003; Santin et al., 2003]. It was also reported that the suppression of matriptase with an antisense-matriptase cDNA inhibited receptor-bound pro-urokinase (uPA) activation in vitro, and thereby reduced tumor growth in vivo and showed a decreased ability of invasion in vitro [Suzuki et al., 2004]. However, it is unknown whether or not the noncomplexed, active matriptase is released in vivo. In the present study, the noncomplexed, active matriptase was found only in trypsin-expressing cell lines. In the other cell lines tested, the two-chain enzyme was mostly associated with HAI-1. We have previously shown that expression of trypsin is associated with elevated tumor growth and tumor malignancy in experimental tumors [Kato et al., 1998; Miyata et al., 1998] and in clinical cancers [Hirahara et al., 1998; Ichikawa et al., 2000]. The present study suggests the possibility that the two serine proteinases, trypsin and matriptase, cooperatively contribute to tumor progression.

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