FEBS Letters 409 (1997) 442–448 FEBS 18713

Expression of trypsin in vascular endothelial cells

Naohiko Koshikawa[a], Yoji Nagashima[b], Yohei Miyagi[b], Hiroto Mizushima[a], Shunsuke Yanoma[c], Hidetaro Yasumitsu[a], Kaoru Miyazaki[a,*]

[a] *Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244, Japan*
[b] *Department of Pathology, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236, Japan*
[c] *Clinical Research Institute, Kanagawa Cancer Center, 54-2 Nakao-cho, Asahi-ku, Yokohama 241, Japan*

Received 26 March 1997; revised version received 28 April 1997

Abstract Proteinases produced by vascular endothelial cells are expected to play important roles in many biological processes. Here we report that human vascular endothelial cells express trypsinogen-2 mRNA and its protein product in culture. The trypsinogen production was stimulated by a tumor promoter and associated with cell growth. In situ hybridization analysis showed that the *trypsinogen* gene was significantly expressed in vascular endothelial cells around gastric tumors and in patients with disseminated intravascular coagulation (DIC). These results suggest the possible roles of endothelial cell-derived trypsin in tumor angiogenesis and abnormal blood coagulation.

Key words: Vascular endothelial cell; Trypsin; Angiogenesis; Disseminated intravascular coagulation

1. Introduction

Endothelial cells contact with blood and directly regulate many important physiological and pathological processes such as blood coagulation, angiogenesis, tumor metastasis, and control of blood pressure [1–4]. Proteinases secreted from endothelial cells are believed to be involved in these processes. Previous studies have shown that vascular endothelial cells produce two plasminogen activators (PA) and some matrix metalloproteinases (MMP) [5,6].

In a step of angiogenesis, activated endothelial cells penetrate into the vascular basement membrane, migrate through the underlying extracellular matrix (ECM), and proliferate to form new capillaries. Proteinases secreted by endothelial cells are thought to play a critical role in the degradation of the basement membrane and interstitial matrix proteins. The proteinase production by endothelial cells are stimulated by angiogenic cytokines such as basic-fibroblast growth factor (b-FGF), transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [7–9].

Vascular endothelial cells closely participate in the blood coagulation system. Under some pathological conditions, activated vascular endothelial cells initiate the extrinsic coagulation pathway [1,2]. Tissue factor on the endothelial cell surfaces binds factor VIIa, and the tissue factor-factor VIIa complex then triggers the following proteinase cascade. However, it is not clear what proteinases activate factors VII to VIIa in vivo. On the other hand, during the course of tumor metastasis, metastatic tumor cells must invade into blood vessels. Proteinases produced by endothelial cells and smooth muscle cells are expected to play some roles in the steps of intravasation and extravasation of metastatic tumor cells.

We have previously reported that some types of human cancer cells secrete pancreatic trypsinogens, which can be activated by enterokinase [10–12]. In this study, we analyzed proteinases secreted by two kinds of human vascular endothelial cells (HUVEC and MvEC), and found that vascular endothelial cells expressed trypsin in vitro and in vivo.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were prepared from fresh umbilical cords by trypsin treatment [13], and dermal microvascular endothelial cells (MvEC) were purchased from Cell Systems (Kirkland, WA). HUVEC and MvEC were maintained as described before [13]. These cultured cells were characterized as endothelial cells by their immunochemical staining for von Willebrand factor antigen and by the typical tightpacking monolayer morphology.

2.2. Gelatin zymography and immunoblotting

Gelatin zymography was performed as described before [10]. Serum-free conditioned media were prepared from confluent cultures of HUVEC and MvEC incubated for 48 h in serum-free MCDB 107 medium (Kyokuto; Tokyo, Japan). The conditioned media were concentrated about 50-fold by ammonium sulfate at 80% saturation and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) as described previously [10]. For activation of trypsinogen, 15 μl of the concentrated conditioned media (≈5 mg protein/ml) was mixed with 2 μl of 50 μg/ml enterokinase (biozyme; South Wales, UK) and 4 μl of 5 mM $CaCl_2$ and incubated at 37°C for 1 h, followed by SDS treatment. Immunoblotting was performed with rabbit polyclonal antibody against human trypsin (Athens Research and Technology; Athens, GA) by the previously reported method [10], except that the antigen was detected by the enhanced chemiluminescence method with an Amersham ECL kit (Buckinghamshire, UK).

2.3. Northern blotting and RT-PCR

Analyses of trypsinogen mRNA by Northern blotting and RT-PCR were carried out by the previously reported methods [12]. Total RNA was extracted from HUVEC, MvEC and STKM-1 cells. Twenty micrograms of each RNA sample was separated on a 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled human trypsinogen-1 cDNA probe. For RT-PCR analysis, the following two primers with an *Eco*RI linker were designed: TRY1 [5′-ACGAATTCACAAGTCCCGCATCCAG-3′] and TRY2 [5′-ACGAATTCCCACCAGAATCACCCTG-3′], which corresponded to nucleotides 200–216 and 595–611, respectively, of human trypsinogen-1 cDNA [14]. The nucleotide sequences of the two primers were

*Corresponding author. Fax: (81) 45-820-1901.
E-mail: miyazaki@yokohama-cu.ac.jp

Abbreviations: bp, base pair; DIC, disseminated intravascular coagulation; HUVEC, human umbilical vein endothelial cell(s); MMP, matrix metalloproteinase; RT-PCR, reverse transcription/polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

PII S0014-5793(97)00565-6

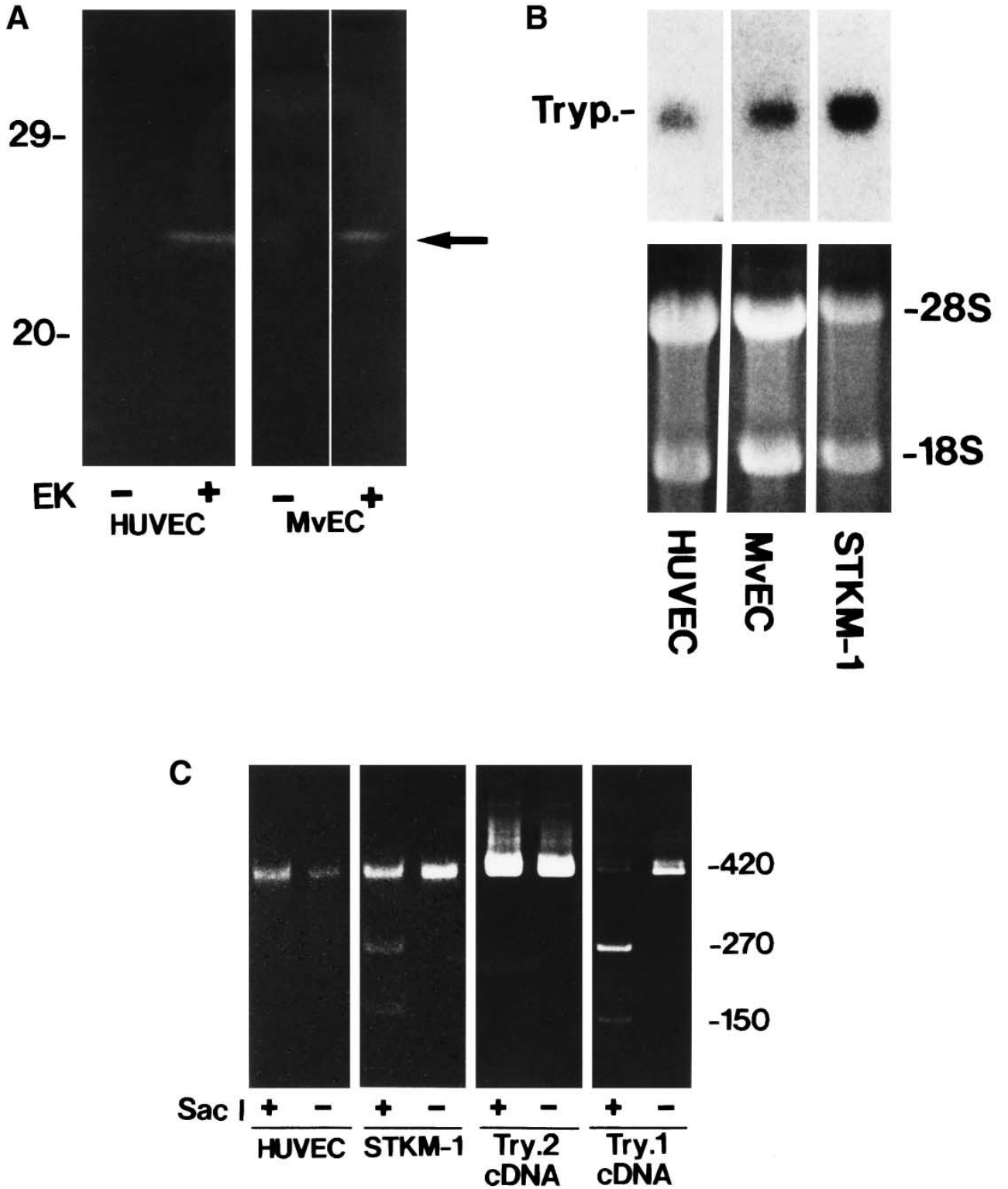


Fig. 1. Identification of trypsinogen produced by human vascular endothelial cells in culture. A: Gelatin zymography in the absence of Ca^{2+} of conditioned media of HUVEC and MvEC which were pre-incubated without (−) or with (+) enterokinase (EK). An arrow indicates the gelatinolytic band of a trypsin-like enzyme (25 kDa). B: Northern blotting analysis of trypsinogen mRNA in HUVEC, MvEC and STKM-1 (human gastric carcinoma cell line). Upper panel (Northern blot): *Tryp.* indicates the band of human trypsinogen mRNA of about 850 bases. Lower panel (ethidium bromide staining of RNAs): *Ordinate* indicates the positions of 18S and 28S ribosomal RNAs. STKM-1 cells, which produce trypsinogen-1 and -2, were used as a positive control. C: Polyacrylamide gel electrophoresis of RT-PCR product from HUVEC RNA before (−) or after (+) treatment with *Sac*I restriction enzyme. RT-PCR was done with RNAs from HUVEC and STKM-1 using a specific primer set designed for trypsinogen-1 and -2 mRNAs. Plasmid DNAs coding for human trypsinogen-1 and -2 were also amplified with the same primer set as positive controls. The amplified DNA fragment of 420 bp from human trypsinogen-1 cDNA, but not that from trypsinogen-2 cDNA, was cleaved into two fragments of 270 and 150 bp by *Sac*I. The DNA fragment from STKM-1 RNA was partially cleaved into the two fragments by the enzyme, whereas that from HUVEC RNA was not cleaved at all.

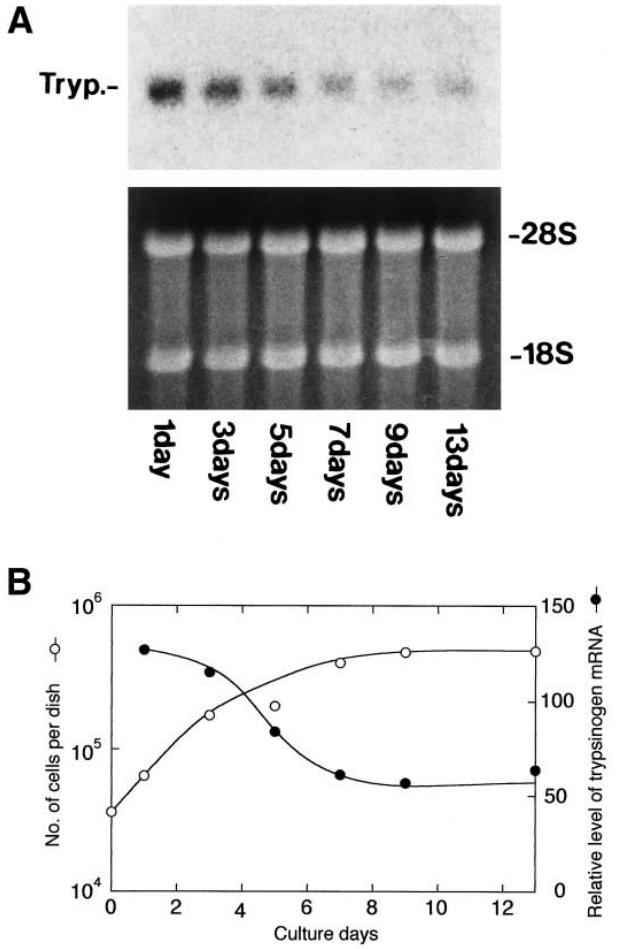
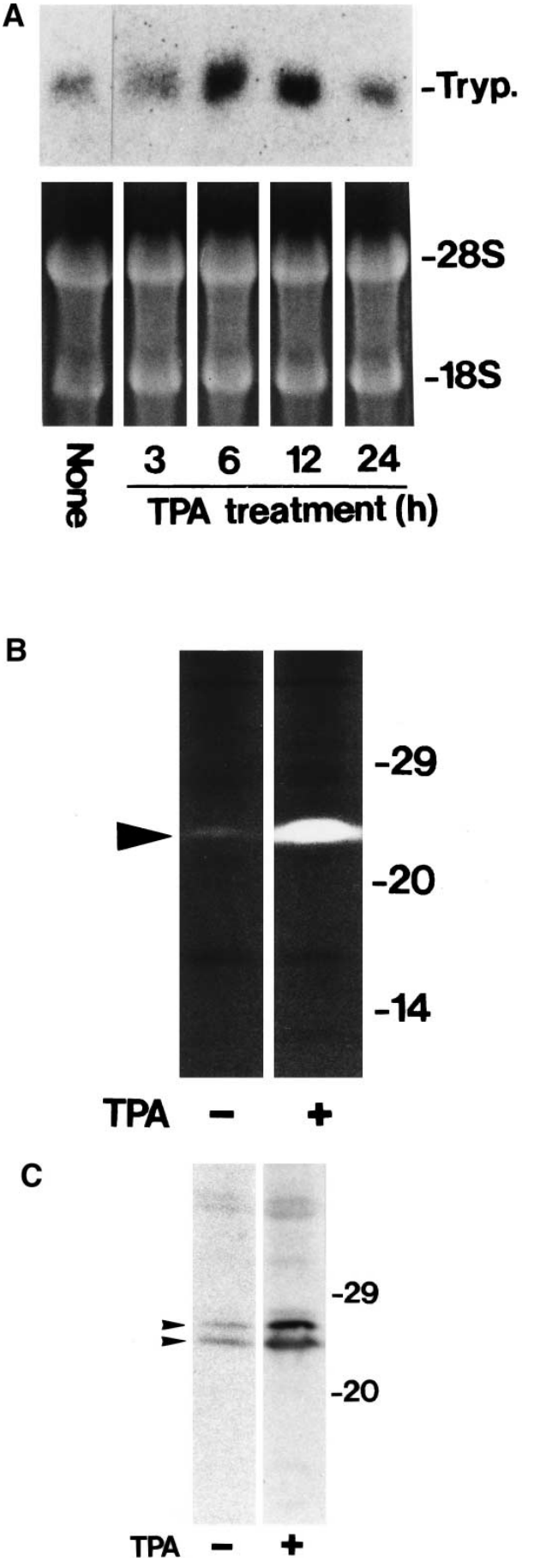


Fig. 2. Cell growth-dependent expression of *trypsinogen* gene in HUVEC. HUVEC were inoculated at a density of 3.5×10^4 in 35-mm dishes with 2 ml of MCDB 107 plus 10% fetal calf serum (FCS). After incubation for 1, 3, 5, 7, 9 and 13 days, the cells were harvested and total RNA was isolated therefrom and subjected to Northern blotting. A: Time course of trypsinogen mRNA level after cell inoculation. Upper panel: Northern blot of trypsinogen mRNA at 850 bases. Lower panel: Ethidium bromide staining pattern of 18S and 28S ribosomal RNAs. B: Growth curve of HUVEC (○) and relative level of trypsinogen mRNA (●). The level of trypsinogen mRNA was quantified with a BAS 2000 Bioimage analyzer (Fuji Film; Tokyo). Each point represents the average for duplicate cultures. Essentially the same result was obtained in a repeated experiment.

common to trypsinogens-1 and -2, but not to the other trypsinogens. Only the RT-PCR product from trypsinogen-1 transcript contained a *Sac*I cleavage site. Five micrograms of total RNA was reverse-transcrbed using Mo-MLV reverse transcriptase and then subjected to the PCR with the primers. The PCR products were digested by 40 U of *Sac*I restriction enzyme (Takara; Shiga, Japan) at 37°C for 1 h,



→

Fig. 3. Effect of TPA treatment on trypsinogen expression in HUVEC. A: Time course of trypsinogen mRNA level after TPA treatment. B: Gelatin zymography of trypsinogen secreted by HUVEC. C: Immunoblotting analysis of trypsinogen secreted by HUVEC. HUVEC were incubated with (+) or without (−) 100 ng/ml TPA in the serum-containing medium for the indicated lengths of time (A) or in serum-free medium for 2 days (B,C). Northern blotting was carried out as described in Fig. 2. Gelatin zymography and immunoblotting were carried out after the enterokinase treatment of the concentrated conditioned medium as described in Section 2.

followed by electrophoresis on a 5% polyacrylamide gel. For nucleotide sequencing, the RT-PCR product of 420 bp was electrophoretically separated and cloned into an *Eco*RI site of pGEM-3Zf(+) plasmid vector (Promega; Madison, WI). The nucleotide sequences of the cloned DNA fragments were determined by the dideoxynucleotide method with a Sequenase Version 2.0 kit (US Biochemicals; Cleveland, OH).

2.4. In situ hybridization

Surgically resected tissues were immediately fixed in 10% formalin. The paraffin-embedded sections were mounted on aminoacyl silane-coated glass slides and used for in situ hybridization. A cDNA fragment (nucleotides 131–482 in [14]) of human trypsinogen-1 was subcloned into pGEM-3Zf(+) plasmid vector. Sense and antisense RNA probes were transcribed in vitro with T7 or SP6 RNA polymerase from the linearized plasmid (cleaved by *Eco*RI and *Hin*dIII, respectively) using digoxigenin-labeled UTP according to the manufacture's manual. In situ hybridization with the RNA probes was carried out as reported previously [15]. The hybridized signals were visualized by the alkaline phosphatase reaction. The tissue sections were counterstained with methylgreen.

3. Results and discussion

3.1. Expression of trypsinogen by cultured vascular endothelial cells

To survey proteinases secreted by endothelial cells, serum-free culture media conditioned by human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (MvEC) were analyzed by gelatin zymography. The gelatin zymography without enterokinase showed a strong 64-kDa Ca^{2+}-dependent gelatinolytic activity in the two conditioned media (data not shown). This enzyme was identified as pro-gelatinase A (pro-MMP-2) by Western blotting analysis with anti-gelatinase-A antibody (data not shown). When the conditioned media were pretreated with enterokinase, which is the activator highly specific for trypsinogen [16], the zymography showed a 25-kDa Ca^{2+}-independent activity (Fig. 1A). This activity was completely inhibited by serine proteinase inhibitors such as diisopropyl fluorophosphate (DFP) and *p*-amidinophenyl methanesulfonyl fluoride (*p*-APMSF) (data not shown).

To date, four kinds of *trypsinogen* genes (*trypsinogen-1*, *-2*, *-3*, and *-4*) have been identified in humans [14,17,18], but no trypsinogens have been reported in vascular endothelial cells. To confirm that the 25-kDa enzyme was trypsin, Northern blotting analysis was carried out using a human trypsinogen-1 probe, which could detect both trypsinogen-1 and -2 mRNAs because of their high homology (93% in nucleotide sequence). Both kinds of endothelial cells expressed a *trypsinogen* gene transcript of about 850 bases (Fig. 1B). To further determine if the transcript was trypsinogen-1 or -2, reverse transcription-polymerase chain reaction (RT-PCR) was carried out. For the RT-PCR analysis, a PCR primer set was designed to have a *Sac*I restriction site in the RT-PCR product of trypsinogen-1 but not trypsinogen-2. The RT-PCR specifically amplified a 420-bp product from HUVEC RNA (Fig. 1C). When the 420-bp DNA fragment was cloned and subjected to nucleotide sequence analysis, all of three clones analyzed had a sequence identical to human trypsinogen-2. Moreover, when treated with *Sac*I restriction enzyme, the 420-bp DNA fragment was not digested although the product amplified from trypsinogen-1 cDNA was split into two fragments of 270 bp and 150 bp (Fig. 1C). This confirmed that at least the major type of the endothelial cell trypsinogen was trypsinogen-2.

Next, expression of the *trypsinogen* gene was examined in HUVEC at various cell densities by Northern blotting (Fig. 2A). HUVEC reached confluency between 7 and 9 days after seeding (Fig. 2B). The gene expression was maximal for initial 3 days, when HUVEC grew in a logarithmic growth phase, and the level gradually decreased and reached nearly the basal level on day 7 (Fig. 2A,B). Thus, the expression of the *trypsinogen* gene inversely correlated with the growth of the endothelial cells.

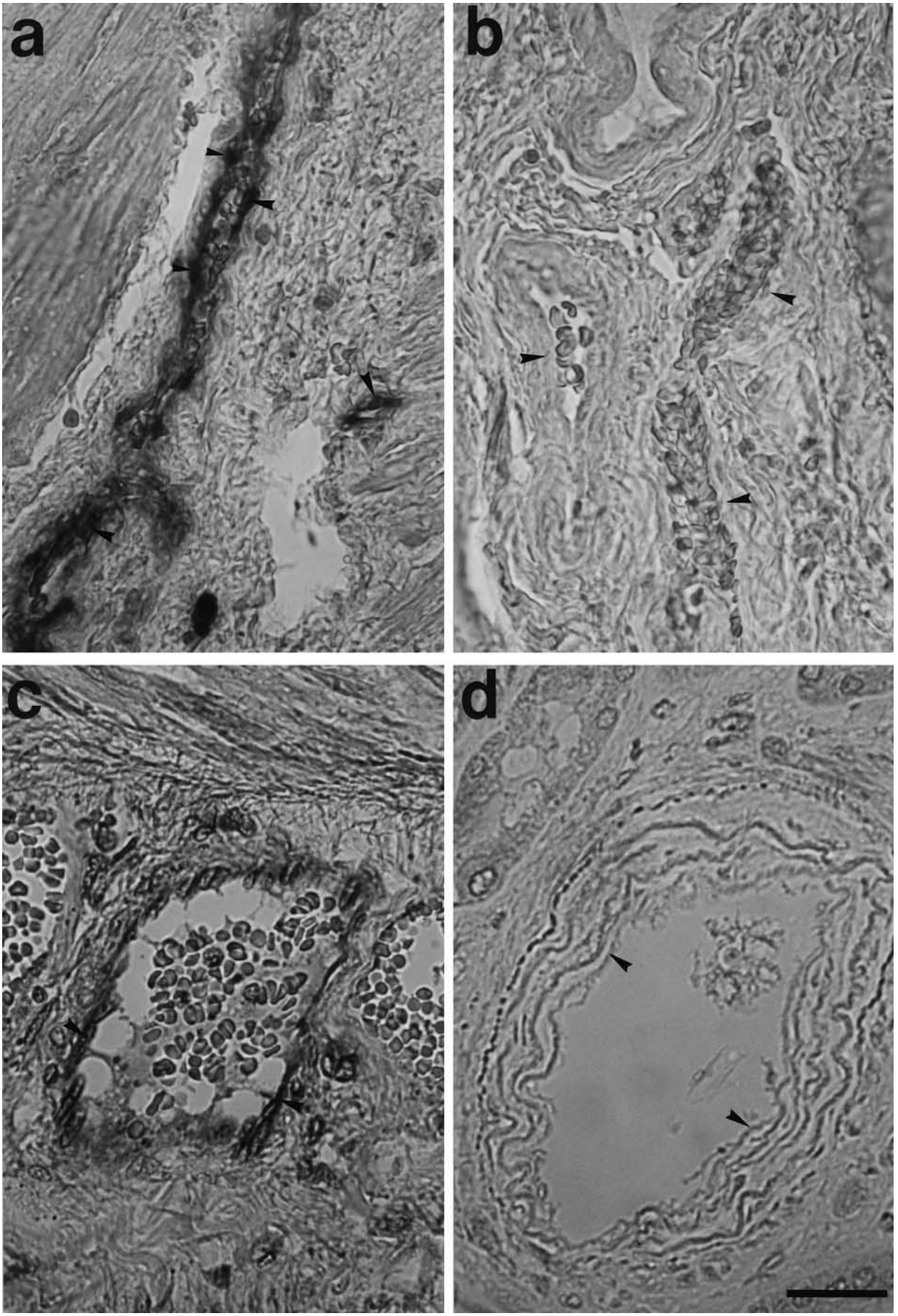
To reveal regulatory factors for the trypsinogen production, effects of various factors on HUVEC were examined. As shown in Fig. 3A,B, the tumor promoter TPA strongly stimulated the expression of *trypsinogen* gene 6–12 h after the treatment, increasing the enterokinase-dependent 25-kDa activity in the conditioned medium. When the enterokinase-treated conditioned media were analyzed by immunoblotting with rabbit polyclonal antibody against human trypsin, TPA-treated HUVEC showed much stronger immunostained bands of 26 and 25 kDa, which appeared to correspond to trypsinogen-2 and activated trypsin-2, respectively, than that of the control HUVEC, indicating that TPA increased the secretion of trypsinogen (Fig. 3C). These results suggest that protein kinase-C-dependent growth signal may up-regulate the transcription of the *trypsinogen* gene directly or indirectly. However, tumor necrosis factor-α, transforming growth factor-α, and interleukin-1β showed no significant effect on the trypsinogen expression in HUVEC (data not shown). Further experiments are required to clarify the regulatory mechanism for the expression of *trypsinogen-2* gene.

3.2. Expression of trypsinogen in vivo

To investigate the physiological and pathological roles of endothelial cell-derived trypsinogen-2, we examined its gene expression in vivo by in situ hybridization with a specific RNA probe for human trypsinogen-1 and -2 mRNAs. When a gastric carcinoma tissue was analyzed, strong staining of vascular endothelial cells was detected in small blood vessels around a tumor nest (Fig. 4A). Such staining of endothelial cells was not detected in the normal part of the tissue apart from the tumor nest (data not shown). A sense probe for trypsinogen-2 as a negative control never reacted with such endothelial cells (Fig. 4B). Similar positive signals in endothelial cells were detected in 6 cases of 7 gastric carcino-

→

Fig. 4. Detection of trypsinogen mRNA in vascular endothelial cells of human gastric carcinoma tissue and of DIC patient by in situ hybridization. All the tissues used were obtained by autopsy. Paraffin sections of the tissues were subjected to in situ hybridization for trypsinogen mRNA with antisense (a,c,d) and with sense (b) RNA probes, followed by counter-staining with methylgreen. a,b: Gastric carcinoma tissue. In (a), strong staining for the trypsinogen message is seen in vascular endothelial cells (arrowheads) of a small vein at the distance of 2–3 mm from the edge of a tumor nest. However, no signal is seen in a similar vein (arrowheads) in the negative control with the sense probe (b). c: Kidney tissue from a DIC patient with rhabdomyolysis. d: Kidney tissue from a non-DIC patient with breast cancer. Arrowheads indicate positive staining for trypsinogen mRNA of vascular endothelial cells in (c). In (d) the signal for the message is undetectable. Bar: 60 μm.


a
b
c
d

mas analyzed, but undetectable in various normal tissues (data not shown).

Vascular endothelial cells secrete various MMPs, such as gelatinase A, collagenase, stromelysin and gelatinase B, in latent proforms in response to various angiogenic factors including TPA [7–9]. These MMPs are thought to play important roles in tumor-associated angiogenesis and in tumor cell invasion through blood vessel wall. We have recently found that matrilysin (MMP-7) [19] and a small cell adhesion protein named TAF (tumor-derived adhesion factor) [20] are strongly induced in vascular endothelial cells of many human cancer tissues. Trypsin is known to degrade various extracellular matrix proteins [10] and to activate the proforms of matrilysin and many other MMPs potently [21,22]. It seems very likely that trypsin produced by tumor cell-activated endothelial cells contributes to tumor angiogenesis and tumor metastasis through pro-MMP activation and/or direct matrix degradation. In addition, we hypothesized that endothelial cell-derived trypsin might affect the blood coagulation system, because it is well known that tumor cells often activate the blood coagulation system, leading to intravascular thrombosis, and that cancer is a frequent cause of disseminated intravascular coagulation (DIC) [23]. To test this possibility, trypsinogen-2 expression in DIC patients was examined by in situ hybridization analysis. As shown in Fig. 4C, obvious positive staining for trypsinogen mRNA was detected on the endothelial cell layer of a small artery in the kidney. Similar positive signal was also seen in the the endothelial cells of intestinal wall from the same patient (data not shown). However, the signal for trypsinogen mRNA was hardly detected in a small artery in the kidney of a non-DIC patient (Fig. 4D). The signal for trypsinogen mRNA was detected in three cases of four DIC patients tested.

The systemic activation of blood coagulation system results in multifocal fibrin clots. The activation of blood coagulation system in DIC is primarily dependent on activation of the extrinsic coagulation pathway [2]. To initiate this coagulation pathway, factor VII must be activated to VIIa and then bind to tissue factor. So far, factor Xa, factor XIIa, thrombin and kallikrein have been listed as possible activators for factor VII [24,25]. Trypsin is known to activate factor VII to VIIa [26]. We have obtained data that enterokinase-treated conditioned medium of HUVEC can activate factor VII to VIIa (data not shown). Taken together, it seems possible that trypsin produced by activated endothelial cells initiates the extrinsic pathway of blood coagulation. Very recently, Liu et al. have reported that HUVEC are induced by IL-1β or LPS to express a serine proteinase of about 30 kDa which can activate prothrombin to thrombin [27]. It is also possible that trypsin acts as a prothrombin activator, although the expression of trypsin was significantly induced by neither IL-1β nor LPS in our study. Furthermore, it is important to note that there is a family of proteolytically activated, G-protein-coupled receptors with seven transmembrane domains. Thrombin receptor is activated by thrombin and mediates various cellular responses in platelets and vascular endothelial cells, such as platelet aggregation, cell growth, Ca^{2+} influx and chemotaxis [28]. Trypsin also activates the receptor but to a lesser extent than thrombin. Proteinase activated receptor 2 (PAR-2), a subtype of thrombin receptor, is effectively activated by trypsin but not by thrombin [29]. The physiological activator for PAR-2 has not been identified yet. It also seems likely that the endothelial cell-derived trypsin functions as a natural activator for PAR-2 and thrombin receptor.

There are few studies about expression and function of trypsin in non-pancreatic tissues. The present study first demonstrates that vascular endothelial cells express trypsin-2 in vivo at least under some pathological conditions. On the other hand, our immunohistochemical study has shown that trypsin is produced by normal human bronchial epithelial cells as well as bronchial carcinoma cells [30]. More recently we have found by in situ hybridization analysis that trypsin is expressed by normal epithelial cells of various tissues such as esophagus, stomach and intestine (unpublished data). These results suggest that trypsin might play important roles in the expression of normal cellular functions in various tissues and cells.

Acknowledgements: We thank Dr. S. Higashi for technical support and helpful discussion. This work was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (N.K.), and Grants-in-Aid from the Ministry of Health and Welfare and from the Ministry of Education, Culture, Sports and Science of Japan (K.M.).

References

[1] Davie, E.W., Fujikawa, K., Kisiel, W., Biochemistry 30 (1991) 10363–10370.
[2] Ruf, W., Edgington, T.S., FASEB J. 8 (1994) 385–390.
[3] Bischoff, J., Trend. Cell Biol. 5 (1995) 69–74.
[4] Folkman, J., Sem. Cancer Biol. 3 (1992) 65–71.
[5] Booyse, F.M., Scheinbuks, J., Lin, P.H., Traylor, M., Bruce, R., J. Biol. Chem. 263 (1988) 15129–15138.
[6] Unemori, E.N., Bouhana, K.S., Werb, Z., J. Biol. Chem. 265 (1990) 445–451.
[7] Mignatti, P., Tsuboi, R., Robbins, E., Rifkin, D.B., J. Cell Biol. 108 (1989) 671–682.
[8] Hanemaaijer, R., Koolwijk, P., Clercq, L.L., De Vree, W.-J.A., van Hinsbergh, V.-W.M., Biochem. J. 296 (1993) 803–809.
[9] Cornelius, L.A., Nehring, L.C., Roby, J.D., Parks, W.C., Welgus, H.G., J. Invest. Dermatol. 105 (1995) 170–176.
[10] Koshikawa, N., Yasumitsu, H., Umeda, M., Miyazaki, K., Cancer Res. 52 (1992) 5046–5053.
[11] Koshikawa, N., Yasumitsu, H., Nagashima, Y., Umeda, M., Miyazaki, K., Biochem. J. 303 (1994) 187–190.
[12] Hirahara, F., Miyagi, Y., Miyagi, E., Yasumitsu, H., Koshikawa, N., Nagashima, Y., Kitamura, H., Minaguchi, H., Umeda, M., Miyazaki, K., Int. J. Cancer 63 (1995) 176–181.
[13] Kikkawa, Y., Akaogi, K., Mizushima, H., Yamanaka, N., Umeda, M., Miyazaki, K., In vitro Cell Dev. Biol. 32 (1996) 46–52.
[14] Emi, M., Nakamura, Y., Ogawa, M., Yamamoto, T., Nishida, T., Mori, T., Matsubara, K., Gene 41 (1986) 305–310.
[15] Miyagi, Y., Kerr, S., Sugiyama, A., Asai, A., Shibuya, M., Fujimoto, H., Kuchino, Y., Lab. Invest. 72 (1995) 890–898.
[16] Kitamoto, Y., Yuan, X., Wu, Q., McCourt, D.W., Sadler, J.E., Proc. Natl. Acad. Sci. USA 91 (1994) 7588–7592.
[17] Wiegand, U., Corbach, S., Minn, A., Kang, J., Muller-Hill, B., Gene 136 (1993) 167–175.
[18] Tani, T., Kawashima, I., Mita, K., Takiguchi, Y., Nucl. Acid Res. 18 (1990) 1631.
[19] Y. Nagashima, S. Hasegawa, N. Koshikawa, A. Taki, Y. Ichikawa, H. Kitamura, K. Misugi, Y. Kihira, Y. Matuo, H. Yasumitsu, K. Miyazaki, Int. J. Cancer 1997 (in press).
[20] Akaogi, K., Okabe, Y., Sato, J., Nagashima, Y., Yasumitsu, H., Sugahara, K., Miyazaki, K., Proc. Natl. Acad. Sci. USA 93 (1996) 8384–8389.
[21] Miyazaki, K., Hattori, Y., Umenishi, F., Yasumitsu, H., Umeda, M., Cancer Res. 50 (1990) 7758–7764.
[22] Grant, G.A., Eisen, A.Z., Marmer, B.L., Roswit, W.T., Goldberg, G.I., J. Biol. Chem. 262 (1987) 5886–5889.
[23] Dvorak, H.F., Hemostasis and Thrombosis. Vol. 63, Lippincott, Philadelphia, PA, 1994.

[24] Broze, G.J., Majerus, P.W., J. Biol. Chem. 255 (1980) 1242–1247.
[25] Gjønnaess, H., Thromb. Diath. Haemorrh. 29 (1973) 633–643.
[26] Rimon, A., Benjamin, A., Katchalski, E., Biochemstry 5 (1966) 792–798.
[27] Liu, L., Rodgers, M.G., Blood 88 (1996) 2989–2994.
[28] Vu, T.-K.H., Hung, D.T., Wheaton, V.I., Coughlin, S.R., Cell 64 (1991) 1057–1068.
[29] Nystedt, S., Emilsson, K., Wahlestedt, C., Sundelin, J., Proc. Natl. Acad. Sci. USA 91 (1994) 9208–9212.
[30] Kawano, N., Osawa, H., Ito, T., Nagashima, Y., Hirahara, F., Inayama, Y., Nakatani, Y., Kimura, S., Kitajima, H., Koshikawa, N., Miyazaki, K., Kitamura, H., Human Pathol. 1997 (in press).