Pergamon

European Journal of Cancer, Vol. 34, No. 7, pp. 1117–1123, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(98)00077-X

Original Paper

Production of Trypsins by Human Gastric Cancer Cells Correlates with their Malignant Phenotype

Y. Kato,[1] Y. Nagashima,[2] N. Koshikawa,[3] Y. Miyagi,[2] H. Yasumitsu[3] and K. Miyazaki[3]

[1]Department of Oral Biochemistry, Kanagawa Dental College, 82 Inaoka-cho, Yokosuka 238-0003, [2]Department of Pathology, Yokohama City University School of Medicine; and [3]Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, Japan

Proteolytic degradation of extracellular matrix is a critical step in tumour invasion and metastasis. To examine the role of trypsin in tumour dissemination, we cloned two variants (S4 and R3 cells) from STKM-1, a trypsinogen 1-producing diffuse gastric cancer cell line. Western blot analysis with anti-trypsin antibody showed that 26 and 24 kDa proteins were highly detected in S4 conditioned medium (CM) in comparison to R3 CM. In addition to the 26 and 24 kDa proteins, 25 and 23 kDa bands, which correspond to enterokinase-activated trypsin, were found only in S4 CM. When the CMs of the two clones were treated with enterokinase, the 25 and 23 kDa trypsin activities in S4 CM were effectively increased as compared with R3 CM. When the two clones were inoculated intraperitoneally (i.p.) into nude mice, S4 cells strongly invaded the liver, pancreas and peritoneum and killed the hosts more rapidly than R3 cells: the 50% survival time was 50 days for S4 and 82 days for R3 cells. These results suggest that trypsin production is associated with the invasive growth of STKM-1 gastric cancer cells.

Key words: trypsin, invasion, extracellular matrix, RT-PCR
Eur J Cancer, Vol. 34, No. 7, pp. 1117–1123, 1998

INTRODUCTION

DIFFUSE GASTRIC carcinoma often invades through the posterior wall of the stomach and then disseminates into the abdominal cavity. Degradation of the extracellular matrix (ECM) is an essential step for dissemination of tumour cells. Many studies have shown the mechanism of ECM degradation by various matrix metalloproteinases (MMPs) [1–5]. Some serine proteinases have been considered to support matrix degradation by activating the latent forms of MMPs. For instance, the plasminogen–plasminogen activator (PA) cascade has been well studied [6–8] and trypsin and kallikrein are also able to activate some MMPs [9, 10]. Koivunen and colleagues [11] reported trypsinogen isoenzymes, called tumour-associated trypsinogen 1 and 2 (TAT-1 and TAT-2, respectively), from cyst fluid of an ovarian tumour patient. These proteins were also detected in some tumour cell lines such as colon carcinoma, fibrosarcoma and leukaemia [12, 13]. Moreover, we found that two gastric cancer cell lines, MKN28 and STKM-1, secreted trypsinogens, plasminogen and kallikrein but not MMPs [14, 15]. In these cell lines, tumour cell-derived trypsins may play a major role in the degradation of ECM in the process of tumour invasion. To examine this possibility, we cloned two sublines from parental STKM-1 cells with high and low trypsin secretion and compared their growth in the abdomen.

Correspondence to Y. Kato.
Received 7 Apr. 1997; revised 7 Oct. 1997; accepted 10 Oct. 1997.

MATERIALS AND METHODS

Cell culture

STKM-1 cell line, a human gastric cancer was a kind gift from S. Yanoma, Kanagawa Cancer Center, Yokohama, Japan. Autoclavable RPMI 1640 (Nissui, Japan) supplemented with 10% fetal bovine serum (FBS, ICN Biochemicals, Japan) was used as a culture medium. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air [14, 15].

Cloning of STKM-1 clones

Cell suspension containing approximately 100 cells was inoculated into 100 mm plastic culture dishes (Sumitomeo

Bakelite, Tokyo, Japan). After 2 weeks in culture, each colony was isolated by an aluminum cylinder and harvested by trypsinisation.

Preparation of conditioned medium (CM)

Cells were grown to confluence in 100 mm culture dishes containing 10 ml of RPMI 1640 supplemented with 10% FBS. Then the cultures were washed twice with Dulbecco's Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and once with serum-free RPMI 1640. The CM was collected three times every second day (first CM, day 0–2; second CM, days 2–4; third CM, days 4–6) after the removal of serum. The CMs were clarified by sequential centrifugation at 800×g for 15 min and 1,500×g for 30 min, 4°C. The supernatant was dialysed against pure water and concentrated by freezing-dry. The resultant dry sample was dissolved in 333 µl of PBS (final 30-fold concentrated).

Western blot analysis

The 30-fold concentrated CM (5 µl) to be analysed was mixed with an equal volume of SDS sample buffer consisting of 4% (w/v) SDS, 125 mM Tris-HCl (pH 6.8), and 10% (v/v) glycerol and then electrophoresed without prior heating in boiling water. After the electrophoresis, the separated proteins were transferred on to Immobilon-P transfer membranes (Millipore, Bedford, Massachusetts, U.S.A.) in 25 mM Tris, 192 mM glycine and 20% methanol in a Bio-Rad Mini Transblot apparatus (Richmond, California, U.S.A.). The blotted filters were blocked with PBS containing 0.05% (v/v) Tween 20 and 5% (w/v) skimmed milk at 37°C for 1.5 h, and then washed with PBS supplemented with 1% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20 (PBS-BSA-Tween 20). They were incubated overnight at room temperature with an anti-trypsin antibody (Athens Research and Technology, Athens, Greece), which had been 1,000-fold diluted with PBS-BSA-Tween 20. After washing the filters with PBS-BSA-Tween 20, they were incubated for 1.5 h at room temperature with biotinylated anti-rabbit IgG (H + L) (Vector Laboratories, Bulingame, California, U.S.A.) as the second antibody (1,000-fold dilute). Then they were incubated with 0.1 unit/ml alkaline phosphatase avidin D (Vector Laboratories) (1,000-fold dilute) for 30 min at room temperature. Finally, the filters were stained with 175 µ/ml 5-bromo-4-chloro-3-indolyl phosphate and 337.5 µg/ml nitroblue tetrazolium (Boehringer Mannheim, Mannheim, Germany) in buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$). The molecular makers used were rabbit muscle phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 66,200), hen egg white ovalbumin (M_r 42,699), bovine carbonic anhydrase (M_r 29,000), soybean trypsin inhibitor (M_r 21,500), and hen egg lysozyme (M_r 14,000).

Zymographic analysis

Analysis of gelatinolytic activity by gelatin-zymography has been previously reported [3, 16]. The 30-fold concentrated CMs (5 µl) to be analysed were mixed with an equal volume of SDS sample buffer and then electrophoresed without prior heating in boiling water. The proteinases were separated on 12.5% gels containing 0.1% gelatin. The proteinases separated on gels were renatured in 2.5% Triton X-100 containing 50 mM Tris-HCl (pH 7.5) and 0.1 M NaCl at room temperature for 1 h, followed by incubation in 50 mM Tris-HCl (pH 7.5) containing 10 mM $CaCl_2$ and 0.02% NaN_3 at 37°C for 20 h. The resultant gels were stained with Coomassie brilliant blue R-250. The molecular makers used were described above.

Trypsinogen activation

The 30-fold concentrated CMs (5 µl) were incubated with 0.5 µg of porcine enterokinase (Biozyme, South Wales, U.K.) in 10 mM dimethylglutric acid (pH 5.6)/10 mM $CaCl_2$ for 10 min at 37°C. At the end of incubation time, the reaction mixtures were mixed with an equal volume of SDS sample buffer as described above.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from each clone of STKM-1 cells according to the standard guanidine and urea method using Ultraspec RNA Isolation System (Biotex Laboratories, Houston, Texas, U.S.A.). Total RNA (2 µg) was reverse-transcribed at 37°C for 60 min to the first strand cDNA with 100 pmol of the random primer (Takara, Tokyo, Japan) using 200 units of Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (GIBCO BRL, Gaithersburg, Maryland, U.S.A.). The used primer set [upstream primer: 5′ACGAATTCACAAGTCCCGCATCCAG3′ and downstream primer: 5′ACGAATTCCCACCAGAATCACCCTG3′] was designed from the sequence of human trypsinogen 1 [17]. They were kindly provided by E. Miyagi of Yokohama City University, Japan. The PCR amplification was carried out by mixing the above primers and reverse transcriptase reaction product in 2.5 units of *Taq* polymerase (Takara). The design of PCR was as follows: denature, 94°C for 1 min; annealing, 54°C for 1 min; extension, 72°C for 2 min. The PCR product was electrophoresed on the 2% agarose in 1×TAE buffer at a constant voltage of 100. After electrophoresis, the resultant gel was stained by ethidium bromide.

Digestion of PCR products by restriction enzymes

To classify trypsinogen cDNA, PCR products were digested by either *Sac* I or *Pst* I. Ten µl of PCR product were mixed with 4 U of the restriction enzyme and incubated at 37°C for 1 h. Each cleavage site is shown in Figure 1 [17–19]. The ethidium bromide-stained bands were measured by laser scanning and analysed by Macintosh soft, NIH Image 1.55.

Assay of tumour growth in nude mice

Cell suspension containing 1×10^7 cells was injected intraperitoneally (i.p.) and subcutaneously (s.c.) into Balb/c nu/nu mice aged 3 and 6 weeks old (Japan SLC, Shizuoka, Japan), respectively. All mice were maintained with diet and water *ad libitum* in a clean room kept at 24°C with a light cycle from 0700 to 1930. The tumour volume (cm^3), established in the s.c., was calculated according to the following formula: $4\pi/3X(r1 + r2)^3/8$, where r1 is the longitudinal radius and r2 the transverse radius.

RESULTS

Trypsin(ogen) secretion

We have previously purified and identified trypsinogens secreted from STKM-1 cells [14, 15]. To study the role of trypsins in the tumour invasion, STKM-1 clones with high and low capabilities of trypsin secretion were isolated from the parental cell line. Figure 2 shows the time course of enzyme production by two clones as analysed by Western blotting with antibody against human trypsin. The CMs were

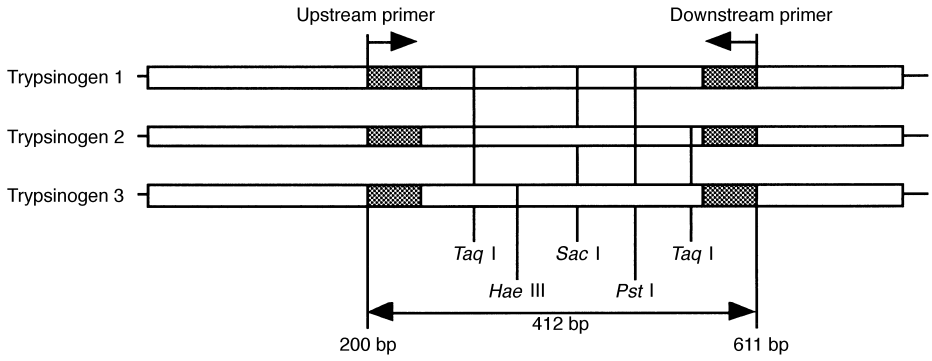

Figure 1. Comparison of restriction sites in trypsinogens.

collected three times every second day after removal of serum. Two immunoreactive bands of 26 and 24 kDa were highly detected in the second and third CMs of S4 cells as compared with R3 CMs. In the third CM of S4 cells, additional bands of 25 and 23 kDa appeared, but not in any stage of R3 CM tested.

Next, we attempted to distinguish these four bands into trypsinogen and active trypsin. When each CM was incubated with the trypsinogen activator enterokinase prior to Western blotting analysis, the 26 and 24 kDa bands were converted to 25 and 23 kDa, respectively (Figure 3a). When the CM was analysed by gelatin-zymography, the enterokinase treatment increased gelatinolytic activities of 25 and 23 kDa (Figure 3b). These results suggest that the 26 and 24 kDa bands corresponded to trypsinogens, and the 25 and 23 kDa bands were active trypsins. As previously reported, it is likely that the 26 and 25 kDa proteins corresponded to two-chain forms, and the 24 and 23 kDa proteins corresponded to one-chain forms.

The effect of enterokinase on the activities of trypsins was also analysed in the three CMs collected. When zymography was performed without enterokinase treatment, the 25 kDa activity was detected in the second and third CMs of S4 cells, but hardly in R3 CMs (Figure 4). Although enterokinase-treated R3 CMs showed gelatinolytic activity, it was far lower than the corresponding S4 CMs. Plasmin-like activity of 70 kDa was found in the first CMs from both clones at similar levels. Besides the 70 kDa activity, 64 and 52 kDa activities were detected in the second and third CMs of S4, but not in the R3 CM without enterokinase treatment. However, when each CM was treated with enterokinase, the 64 and 52 kDa activities appeared in the second and third R3 CMs. The gelatinolytic activities of trypsins were much higher than that of plasmin-like activities. Overall, production of trypsins in the third CM of S4 cells was 2.2-fold higher for the gross amount and more than 70-fold higher for the active form than that of R3 cells as based on cell number. The results described above suggest that S4 cells secrete trypsinogens and trypsins more than R3 cells.

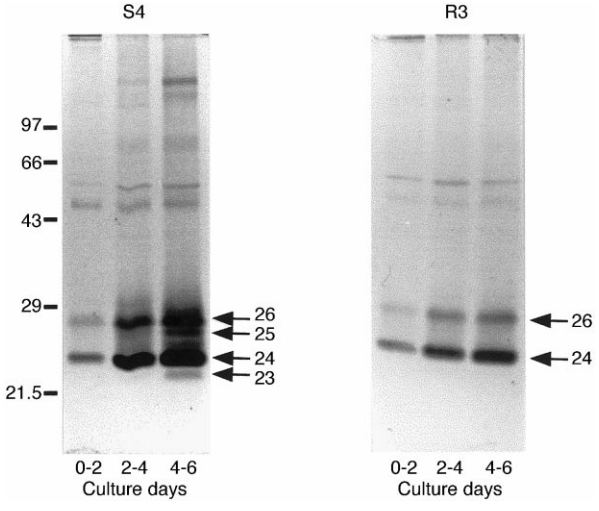

Figure 2. Western blotting of trypsin(ogen)s secreted by STKM-1 clones. CMs were collected three times every second day after removal of serum. The CMs were concentrated 30-fold, and 5 μl (≈8–10 μg protein) of them was analysed by Western blotting with anti-trypsin antibody. The number of viable cells was 10.8×10^4 cells/cm^2 (day 0), 11.3×10^4 cells/cm^2 (day 2), 9.2×10^4 cells/cm^2 (day 4), and 11.1×10^4 cells/cm^2 (day 6) in S4 cells and 10.4×10^4 cells/cm^2 (day 0), 13.0×10^4 cells/cm^2 (day 2), 9.3×10^4 cells/cm^2 (day 4) and 10.1×10^4 cells/cm^2 (day 6) in R3 cells. Ordinate, molecular weight in thousand; Arrow, trypsin(ogen)s.

Expression of trypsinogen mRNAs

Expression of trypsinogen mRNA in two STKM-1 clones was determined by RT-PCR analysis. By laser scanning of ethidium bromide-stained gel, trypsinogen cDNA product of 412 bp was highly and consistently detected in S4 cells

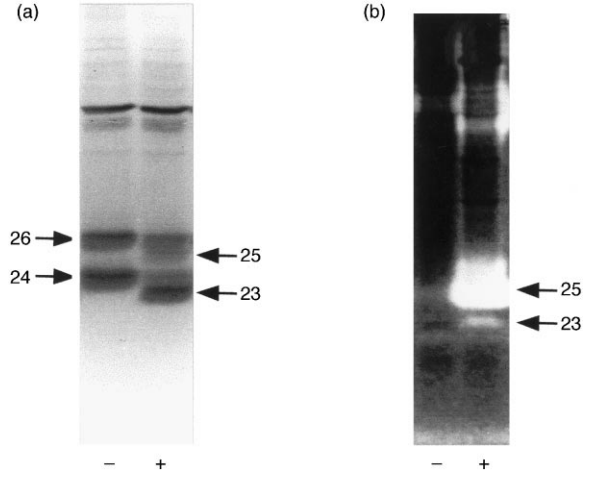

Figure 3. Activation by enterokinase of trypsinogens secreted by STKM-1 (S4) cells. The CM was incubated with (+) or without (–) 0.5 μg of porcine enterokinase in 10 mM dimethylglutric acid (pH 5.6)/10 mM $CaCl_2$ for 10 min at 37°C. (a) Western blotting using anti-trypsin antibody. (b) Gelatin-zymography. Ordinate, molecular weight in thousand; Arrow, trypsin(ogen) molecules (a) and activities (b).

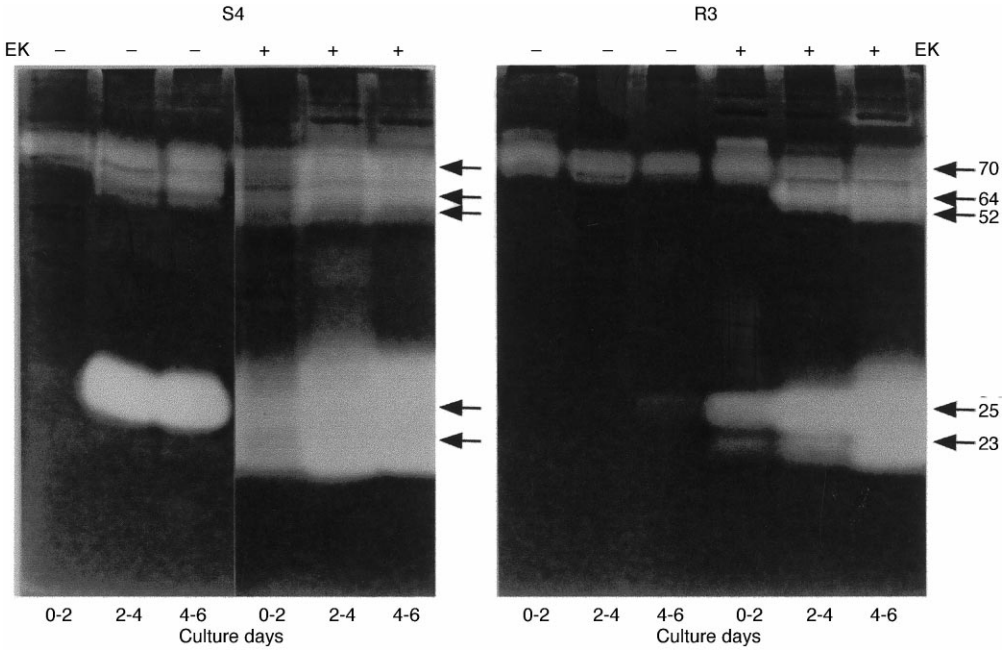

Figure 4. Gelatinolytic activities in CMs of two STKM-1 clones before and after enterokinase treatment. Each CM was collected three times every second day and concentrated. Samples (5 μl, ≈8–10 μg protein) were incubated with (+) or without (–) porcine enterokinase. Gelatinolytic activities were analysed by gelatin-zymography. The number of viable cells was 10.8×10^4 cells/cm^2 (day 0), 11.3×10^4 cells/cm^2 (day 2), 9.2×10^4 cells/cm^2 (day 4) and 11.1×10^4 cells/cm^2 (day 6) in S4 cells and 10.4×10^4 cells/cm^2 (day 0), 13.0×10^4 cells/cm^2 (day 2), 9.3×10^4 cells/cm^2 (day 4) and 10.1×10^4 cells/cm^2 (day 6) in R3 cells. EK, enterokinase; Arrows, positions of gelatinolytic activities.

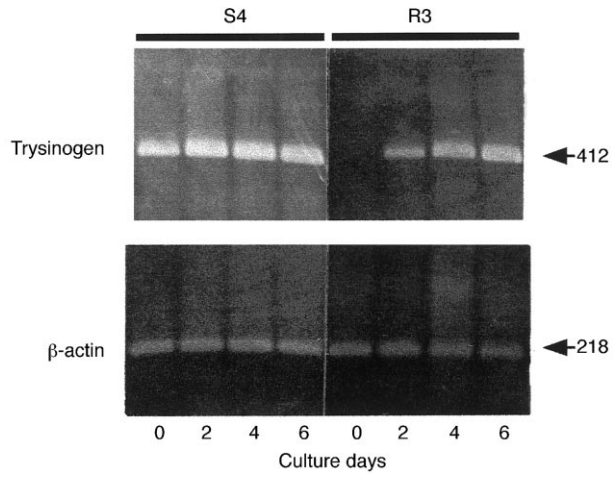

Figure 5. Expression of trypsinogen mRNA from STKM-1 clones. Total RNA from each clone was extracted by guanidine and urea method, and was analysed by RT-PCR. Arrows, base pair size.

(Figure 5). In R3 cells, the cDNA fragment was hardly detected on day 0, but became detectable after day 2. To determine the molecular species of trypsin, the PCR product was digested by restriction enzymes. As shown in Figure 1, two primers were designed to include *Sac* I and *Pst* I cleavage sites in the amplified sequence of trypsinogen 1 cDNA. *Sac* I site was not included in the trypsinogen 2 cDNA sequence. *Pst* I site was present in trypsinogen 1 and 2 but not in trypsinogen 3. The PCR products from both clones were completely digested by *Pst* I, indicating that both trypsinogens were not trypsinogen 3 (Figure 6). *Sac* I digested 8% of the PCR product from S4 cells and 38% of that from R3 cells. This suggests that both STKM-1 clones express trypsinogen 2 as a major form and trypsinogen 1 as a minor form and that the proportion of trypsinogen 2 is higher in S4 cells than R3 cells.

Growth of STKM-1 clones in nude mice

When S4 and R3 cells were cultured *in vitro*, their cell size, shape, and growth rate were similar (data not shown). However their growth rates *in vivo* were quite different. At 3 weeks

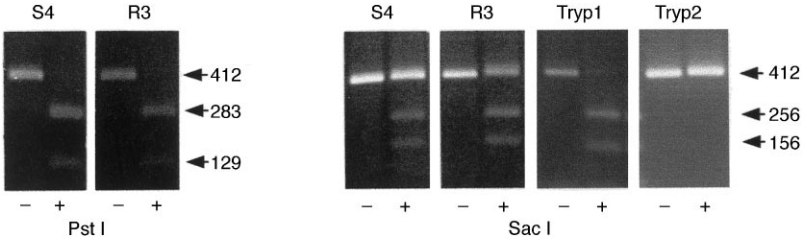

Figure 6. Digestion of PCR products with (+) or without (–) restriction enzymes, *Pst* I and *Sac* I. Total RNA from each clone was extracted and obtained by RT-PCR analysis. Arrows, base pair size; *Tryp* 1, Trypsinogen 1 cDNA; *Tryp* 2, Trypsinogen 2 cDNA.

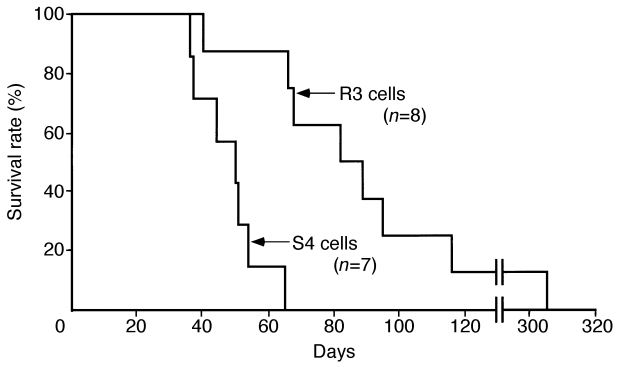

Figure 7. Survival curves of nude mice injected with two STKM-1 clones. S4 or R3 cells (1×10^7 cells/mouse) were injected i.p. into male Balb/c nu/nu mice. Statistical analysis was done according to the Cox–Mantel method. $P<0.01$.

after s.c. injection of S4 and R3 clones (1×10^7 cells) into male nude mice, the incidence of tumorigenicity was 5 of 5 (100%) and 2 of 4 cases (50%), respectively. The mean tumour volume was $3.2\pm2.1\,cm^3$ for S4 cells and $0.13\pm0.22\,cm^3$ for R3 cells within 3 weeks ($P<0.05$). Furthermore, growth of the two clones in the abdominal cavity was also examined by injecting 1×10^7 cells (as a model of experimental dissemination into abdominal cavity) into nude mice. We found metastasis in the pancreas, liver, peritoneum and mesoterium. This incidence was 3 of 7 mice (43%) for S4 cells and 2 of 8 mice (25%) for R3 cells. S4 cells killed hosts more rapidly than R3 cells: 50% survival time was 50 days for S4 and 82 days for R3 cells (Figure 7). When the mice were killed after 35 days after i.p. injection (the day when the first mouse was killed by S4 cells, see Figure 7 but not R3 cells), S4 cells had invaded the mesoterium.

Trypsin production in transplanted tumour cells

Tumour tissues in the abdominal cavity, established by S4 and R3 cells, were obtained to estimate the production of trypsins by immunocytochemistry technique. The number of immuno-reactive cells were higher in S4 cells than R3 cells (data not shown). Moreover, active forms of trypsins were detected from CMs of the transplanted tumour cells that were cultured by the explanted culture technique. High activity was found in the S4 cells as compared with that of R3 cells (Figure 8). No MMP activities, induced by transplantation, were seen. Therefore, it is strongly suggested that trypsins act on the degradation of ECM and are responsible for invasive growth of some kinds of gastric cancer cells.

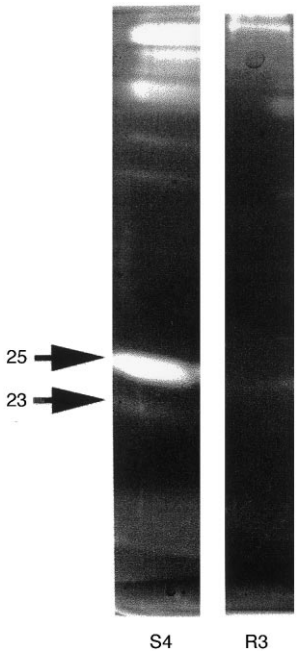

Figure 8. Trypsin secretion of transplanted tumour tissues derived from two STKM-1 clones. Tumour tissues from S4 or R3 cells were re-cultured according to explanted culture method. Grown cells were maintained in serum-free RPMI1640. The CMs were collected and concentrated by freezing-dry. Each concentrated CM including 2.6 μg of protein was analysed by gelatin-zymography. Arrow, trypsin activities (molecular weight in thousand).

DISCUSSION

Proteolytic activation and hydrolysis are considered to be localised on the cell surface at invadopodia, which are sites of cell invasion into the ECM. LaBombardi and colleagues [20] reported that a trypsin-like enzyme is localised on the plasma membrane of Walker-256 carcinosarcoma. Furthermore, Santibanez and colleagues [21] showed that urokinase type-PA (u-PA) is the major proteolytic enzyme in cell membranes and u-PA is able to activate pro-collagenase. Activation of MMPs through the plasminogen-PA cascade has also been reported by others [7, 22]. Besides the cascade, it has been shown that trypsin activates pro-gelatinase B [23] and pro-collagenase [24, 25]. Moreover, trypsin is capable of degrading some ECM components [14]. The present study suggests that invasion of S4 cells may be due to the ability to degrade ECM directly and indirectly via MMP activation.

At least three trypsin cDNAs have been cloned [17–19]. According to these sequences, we confirmed the isoforms of the trypsinogen expressed in two STKM-1 variants. When the PCR-amplified product from each clone was treated with *Sac* I restriction enzyme, the product from R3 cells was effectively digested, whereas that from S4 cells was less digested. *Pst* I completely digested both products. Therefore, it is likely that 92% of total trypsinogens in S4 cells and 62% of that in R3 cells are trypsinogen 2. The parental STKM-1 cell line produced trypsinogen 1 predominantly [15]. S4 cells showed a greater invasive capability than the parent and R3 cell lines. Therefore, trypsinogen 2 may play a more important role in tumour invasion than trypsinogen 1. This hypothesis is supported by previous observations. Koivunen and colleagues [26] reported that trypsinogen 2 is the predominant isozyme in malignant cyst fluid of ovarian cancer patients. Although trypsin is capable of activating interstitial collagenase, the cleavage sites in the propeptides of pro-MMPs by trypsin 1 and 2 are different. For example, the cleavage site of pro-collagenase by trypsin 1 is located at R^{36}-N^{37} bond and that by trypsin 2 at K^{66}-V^{67} and R^{72}-C^{73} bonds [24, 25]. To produce the fully active form of pro-collagenase, propeptide (F^1 to Q^{80}) must be removed. The trypsin 2-cleaving site locates more close to Q^{80}-F^{81} bond than the trypsin 1-cleavage site. Therefore, it seems likely that trypsin 2 is more effective than trypsin 1 for activation of pro-collagenase. Although trypsin, plasmin and leukocyte elastase activate pro-matrilysin, only trypsin can fully activate it, with plasmin and leukocyte elastase only able to produce 50% activation [27].

Trypsinogens act in the stomach after activation by enterokinase. This enzymatic cascade may contribute in the degradation of ECM by tumour derived trypsinogens. It may also be possible to activate trypsinogens by autocatalysis. Trypsinogen 2 is known to autoactivate more rapidly than trypsinogen 1 in acidic condition (pH 5.6) in the presence of Ca^{2+}, which inhibits the autoactivation of trypsinogen 1 [28]. Indeed, S4 CM contained active forms of trypsin (see Figure 4). It is well known that the pH in tumour tissues is lower than in normal tissues. Glycolysis and tumour necrosis cause a lower pH in tumour tissues and induce gelatinase B secretion [3, 16, 29, 30]. Therefore, the production of trypsinogen 2 may be involved in a proteolytic cascade to activate gelatinase B and other production of pro-MMPs.

Clinical data have shown proteolytic activity in gastric cancer. Sier and colleagues [31] reported that expression of both gelatinase A and B were high in gastric carcinoma and correlated well with prognosis. We also found gastric cancer cell lines which secreted two gelatinases along with trypsins [14]. According to Ohtani and colleagues [32], MT1-MMP mRNA of gastric cancer, which has been considered an activator for gelatinase A, was found in 33 of 39 cases, but diffuse-type gastric adenocarcinoma showed lower levels than other types. This might support the possibility that trypsin-gelatinase B cascade is more responsible than MT-MMP-gelatinase A cascade for invasive growth in the diffuse-type gastric cancer. Our preliminary unpublished results have shown that introducing trypsin cDNA into mouse B16-F1 melanoma cells, which has low levels of gelatinase B production in acidic culture conditions, see reference [3], positively affected their tumorigenicity. Thus, it is possible that trypsins act by activation of MMPs *in vivo*, but it seems important to determine the contribution of each trypsin isoform to MMP activation and tumour invasion because of slightly different biochemical properties such as the isoelectric point and substrate specificity between two isoforms of trypsinogen [11, 33].

Recently, we have found the expression of trypsinogen in clinical samples of ovarian carcinomas [34] and human bladder carcinoma (data not shown). However, secretion of trypsins by cultured tumour cells has rarely been reported [14]. Zymographic analysis has been extensively used for detection of MMPs. Although most MMPs are produced as latent proforms, pro-MMPs can show proteolytic activity on zymography because of their activation by SDS. However, trypsinogens cannot show this activity on zymography. As shown in Figure 3, the treatment of trypsinogen with enterokinase produced trypsin activity on the zymogram, indicating that the proteolytic activation of trypsinogen is essential for its detection by zymography.

In the present study, we showed that (a) the production of trypsinogens was higher in S4 cells than in R3 cells; (b) the active form of trypsinogen was present in S4 CM only; (c) the expression of trypsinogen genes was higher in S4 cells than in R3 cells; and (d) S4 cells invaded into the liver after i.p. injection into nude mice and killed the hosts faster than R3 cells. These results suggest that trypsin production is associated with the invasive growth of STKM-1 cells in the abdominal cavity of nude mice.

1. Liotta LA, Trygvasson K, Garbisa S, Hart I, Folts CM, Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 1980, **284**, 67–68.
2. Miyazaki K, Ashida Y, Kihira Y, Mashima K, Yamashita J, Horio T. Transformation of rat liver cell line by Rous sarcoma virus causes loss of cell surface fibronectin, accompanied with secretion of metallo-proteinase that preferentially digests the fibronectin. *J Biochem Tokyo* 1987, **102**, 569–582.
3. Kato Y, Nakayama Y, Umeda M, Miyazaki K. Induction of 103-kDa gelatinase/type IV collagenase by acidic culture conditions in mouse metastatic melanoma cell lines. *J Biol Chem* 1992, **267**, 11424–11430.
4. Sato H, Takino T, Okada Y, *et al.* A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* 1994, **370**, 61–65.
5. Fini ME, Girard MT, Matsubara M, Bartlett JD. Unique regulation of the matrix metalloproteinase, gelatinase B. *Invest Ophthalmol Vis Sci* 1995, **36**, 622–633.
6. Liotta LA, Goldfarb RH, Brundage R, Siegal GP, Terranova V, Garbisa S. Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. *Cancer Res* 1981, **41**, 4629–4636.
7. Reich R, Thompson EW, Iwamoto Y, *et al.* Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. *Cancer Res* 1988, **48**, 3307–3312.
8. Turner MA, Palefsky JM. Urokinase plasminogen activator expression by primary and HPV 16-transformed keratinocytes. *Clin Exp Metastasis* 1995, **13**, 260–268.
9. Eeckhout Y, Vaes G. Further studies on the activation of procollagenase, the latent precursor of bone collagenase. Effects of lysosomal cathepsin B, plasmin and kallikrein, and spontaneous activation. *Biochem J* 1977, **166**, 21–31.
10. Watanabe H, Nakanishi I, Yamashita K, Hayakawa T, Okada Y. Matrix metalloproteinase-9 (92 kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. *J Cell Sci* 1993, **104**, 991–999.
11. Koivunen E, Huhtala M-L, Stenman U-H. Human ovarian tumor-associated trypsin. *J Biol Chem* 1989, **264**, 14095–14099.
12. Koivunen E, Ristimaki A, Osman S, Vuento M, Stenman U-H. Tumor-associated trypsin participates in cancer cell-mediated degradation of extracellular matrix. *Cancer Res* 1991, **51**, 2107–2112.
13. Koivunen E, Saksela O, Itkonen O, Osman S, Huhtala M-L, Stenman U-H. Human colon carcinoma, fibrosarcoma and leukemia cell lines produce tumor associated trypsinogen. *Int J Cancer* 1991, **47**, 592–596.
14. Koshikawa N, Yasumitsu H, Umeda M, Miyazaki K. Multiple secretion of matrix serine proteinases by human gastric carcinoma cell lines. *Cancer Res* 1992, **52**, 5046–5053.
15. Koshikawa N, Nagashima Y, Yasumitsu H, Umeda M, Miyazaki K. Identification of one- and two-chain forms of trypsinogen 1 produced by a human gastric adenocarcinoma cell line. *Biochem J* 1994, **303**, 187–190.
16. Kato Y, Ozono S, Shuin T, Miyazaki K. Slow induction of gelatinase B mRNA by acidic culture conditions in mouse metastatic melanoma cells. *Cell Biol Int* 1996, **20**, 375–377.
17. Emi M, Nakamura Y, Ogawa M, *et al.* Cloning, characterization and nucleotide sequences of two cDNAs encoding human pancreatic trypsinogens. *Gene* 1986, **41**, 305–310.
18. Tani T, Kawashima I, Mita K, Takiguchi Y. Nucleotide sequence of the human pancreatic trypsinogen III cDNA. *Nucleic Acids Res* 1990, **18**, 1631–1631.
19. Kang J, Wiegand U, Muller-Hill B. Identification of cDNAs encoding two novel rat pancreatic serine proteinases. *Gene* 1992, **110**, 181–187.
20. LaBombardi VJ, Shaw E, DiStefano JF, Beck G, Brown F, Zucker S. Isolation and characterization of a trypsin-like serine proteinase from the membranes of Walker 256 carcino-sarcoma cells. *Biochem J* 1983, **211**, 695–700.
21. Santibanez JF, Martinez JG. Membrane-associated procollagenase of leukemic cells is activated by urokinase-type plasminogen activator. *Leuk Res* 1993, **17**, 1057–1062.
22. Gross JL, Moscatelli D, Jaffe EA, Rifkin DB. Plasminogen activator and collagenase production by cultured capillary endothelial cells. *J Cell Biol* 1982, **95**, 974–981.

23. Okada Y, Gonoji Y, Naka K, *et al.* Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT1080 human fibrosarcoma cells. *J Biol Chem* 1992, **267**, 21712–21719.
24. Grant GA, Eisen AZ, Marmer BL, Roswit WT, Goldberg GI. The activation of human skin fibroblast procollagenase. Sequence identification of the major conversion products. *J Biol Chem* 1987, **262**, 5886–5889.
25. Crabbe T, O'Connell JP, Smith BJ, Docherty AJP. Reciprocated matrix metalloproteinase activation: a process performed by interstitial collagenase and progelatinase A. *Biochemistry* 1994, **33**, 14419–14425.
26. Koivunen E, Itkonen O, Halila H, Stenman UH. Cyst fluid of ovarian cancer patients contains high concentrations of trypsinogen-2. *Cancer Res* 1990, **50**, 2375–2378.
27. Imai K, Yokohama Y, Nakanishi I, *et al.* Matrix metalloproteinase 7 (Matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymatic properties. *J Biol Chem* 1995, **270**, 6691–6697.
28. Colomb E, Figarella C, Guy O. The two human trypsinogens. Evidence of complex formation with basic pancreatic trypsin inhibitor-proteolytic activity. *Biochim Biophys Acta* 1979, **570**, 397–405.
29. Hatanaka M, Hanafusa H. Analysis of a functional change in membrane in the process of cell transformation by Rous sarcoma virus; alteration in the characteristics of sugar transport. *Virology* 1970, **41**, 647–652.
30. Bonfil RD, Medina PA, Gomez DE, *et al.* Expression of gelatinase/type IV collagenase in tumor necrosis correlates with cell detachment and tumor invasion. *Clin Exp Metastasis* 1992, **10**, 211–220.
31. Sier CFM, Kubben FJGM, Ganesh S, *et al.* Tissue levels of matrix metalloproteinases MMP-2 and MMP-9 are related to the overall survival of patients with gastric carcinoma. *Br J Cancer* 1996, **74**, 413–417.
32. Ohtani H, Motohashi H, Sato H, Seiki M, Nagura H. Dual overexpression pattern of membrane-type metalloproteinase-1 in cancer and stromal cells in human gastrointestinal carcinoma revealed by *in situ* hybridization and immunoelectron microscopy. *Int J Cancer* 1996, **68**, 565–570.
33. Rinderknecht H, Geokas MC. Anionic and cationic trypsinogens (trypsins) in mammalian pancreas. *Enzyme* 1973, **14**, 116–130.
34. Hirahara F, Miyagi Y, Miyagi E, *et al.* Trypsinogen expression in human ovarian carcinomas. *Int J Cancer* 1995, **63**, 176–181.

Acknowledgements—We are grateful to Dr E. Miyagi for providing a primer set for RT-PCR for trypsinogens. This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan.