Tumor-Associated Trypsinogen-2 (Trypsinogen-2) Activates Procollagenases (MMP-1, -8, -13) and Stromelysin-1 (MMP-3) and Degrades Type I Collagen†

Merja Moilanen,‡ Timo Sorsa,§,|| Mathias Stenman,⊥ Pia Nyberg,‡ Otso Lindy,▽ Jaana Vesterinen,▽ Annukka Paju,# Yrjö T. Konttinen,§ Ulf-Håkan Stenman,# and Tuula Salo*,‡,||

Department of Diagnostics and Oral Medicine, Institute of Dentistry, University of Oulu, Oulu, Finland and Oulu University Hospital, Oulu, Finland, Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital (HUCH), the Orton Research Institute, and the Orthopedic Hospital of Invalid Foundation, Helsinki, Finland, Invärtes Medicine and Orton Research Foundation, Helsinki University Central Hospital, Helsinki, Finland, Department of Clinical Chemistry, Helsinki University Central Hospital, Helsinki, Finland, Institute of Biomedicine/ Biochemistry, University of Helsinki, Helsinki, Finland, and Institute of Dentistry, University of Helsinki and University Dental Clinic, Helsinki, Finland

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ABSTRACT: A critical step in cancer growth and metastasis is the dissolution of the extracellular matrix surrounding the malignant tumor, which leads to tumor cell invasion and dissemination. Type I collagen degradation involves the initial action of collagenolytic matrix metalloproteinases (MMP-1, -8, and -13) activated by MMP-3 (stromelysin-1). The role of interactive matrix serine proteinases (MSPs), including tumor-associated trypsinogens, has been unclear in collagenolysis. Now, we provide evidence that the major isoenzyme of human tumor-associated trypsinogens, trypsin-2, can directly activate three collagenolytic proMMPs as well as proMMP-3. These proMMP activations are inhibited by tumor-associated trypsin inhibitor (TATI). Furthermore, we demonstrate that trypsin-2 efficiently degrades native soluble type I collagen, which can be inhibited by TATI. However, cell culture studies showed that trypsin-2 transfection into the HSC-3 cell line did not result in MMP-1, -3, -8, and -13 activation but affected MMP-3 and -8 production at the protein level. These findings indicate that human trypsin-2 can be regarded as a potent tumor-associated matrix serine protease capable of being the initial activator of the collagenolytic MMP activation network as well as directly attacking type I collagen.

Degradation of the extracellular matrix (ECM),[1] including the basement membrane (BM), is an essential step in the modulation of different physiological and pathological processes, including tumor invasion and metastasis. In malignant processes, the delicate balance between production, activation, and inhibition of proteolytic enzymes is often irreversibly disturbed (*1*, *2*). These proteolytic enzymes belong to four classes of proteinases: (1) matrix metalloproteinases (MMPs), (2) matrix serine proteinases (MSPs), (3) cysteine proteinases, and (4) aspartic proteinases (*3*, *4*). These proteinases are thought to form different activation networks promoting ECM and BM degradation (*5*).

MMPs are a family of extracellular and cell surface-associated, highly conserved Zn^{2+}-dependent endopeptidases collectively capable of degrading essentially all ECM and BM components (*2*, *6*). MMPs are secreted as inactive zymogens, in which the Cys^{73} residue of the propeptide is coordinated into catalytic Zn^{2+} at the active site (*7*, *8*). The catalytic site can be exposed by different agents capable of dissociating the bond between cysteine and zinc. Among these agents are proteinases, including tissue and plasma proteinases or opportunistic bacterial proteinases, and different nonproteolytic agents, such as organomercurials, chaotropic agents, and oxidants (*9*).

Trypsinogens are one subgroup of matrix serine proteinases (MSPs) (*10*). The trypsinogen family contains four different isoforms, namely trypsinogen-1, -2, and -3, which are produced in the pancreas, and trypsinogen-4, which is produced in the brain (*11*, *12*). Furthermore, trypsinogen-1 and -2 have also been called tumor-associated trypsinogens-1 and -2 (TAT-1 and -2) (*13*), and they were first identified

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* To whom correspondence should be addressed. E-mail: tuula.salo@oulu.fi. Fax: +358-8-537 5560.

‡ University of Oulu.

§ Helsinki University Central Hospital (HUCH), the Orton Research Institute, and the Orthopedic Hospital of Invalid Foundation.

|| Institute of Dentistry, University of Helsinki and University Dental Clinic.

⊥ Invärtes Medicine and Orton Research Foundation, Helsinki University Central Hospital.

Department of Clinical Chemistry, Helsinki University Central Hospital.

▽ Institute of Biomedicine/Biochemistry, University of Helsinki.

[1] Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinases; MT-MMP, membrane-type MMP; trypsin-2, tumor-associated trypsin-2; TATI, tumor-associated trypsin inhibitor; MSP, matrix serine proteinase; GTG, gold thioglucose; NaOCl, sodium hypochlorite; APMA, *p*-aminophenylmercuric acetate; SDS−PAGE, sodium dodecyl sulphate−polyacrylamide gel electrophoresis; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; ECM, extracellular matrix; BM, basement membrane; PMN, polymorphonuclear neutrophil.

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and characterized from cyst fluid of human ovarian tumors (*14*). Trypsin-1 and -2 have identical N-terminal amino acid sequences, immunoreactivity, and molecular sizes as compared to their pancreatic counterparts, but substrate specificity, stability, and isoelectric points are different (*13*). Trypsin-2 is a more abundantly expressed isoform than trypsin-1. Trypsin-1 and -2 are produced by different tumor cell lines (*15*−*19*). Furthermore, trypsin-2 production has been shown to correlate with the malignant phenotype of cancer cells (*15*, *20*). Trypsins, such as MSPs, have been assumed to exert various influences promoting cancer behavior; for example, trypsins can activate progelatinase and prourokinase cascades (*13*, *21*, *22*), fragment the tissue inhibitors of MMPs (*21*), and degrade certain noncollagenous ECM components, and their overexpression can stimulate cellular growth and adhesion (*20*). Trypsinogen expression by cancer cells is often associated with elevated levels of the specific inhibitor (i.e., tumor-associated trypsin inhibitor (TATI)), which can be detected in serum and urine of various cancer patients (*23*−*25*).

Overexpression of collagenases, particularly MMP-1 and MMP-13, and to a lesser extent, MMP-8, is associated with different malignant pathologies, including melanoma, squamous cell carcinomas of skin and mouth, and breast carcinoma (*26*−*29*). MMP-3 can activate collagenolytic proMMP-1, -8, and -13 (*30*, *31*). However, it has been unclear which enzyme can activate the first latent proenzyme in the collagenolytic proMMP activation network (*32*). We have here studied the ability of the major isoenzyme of human tumor-associated trypsins, trypsin-2, to directly activate human procollagenases (MMP-1, -8, and -13) and prostromelysin-1 (proMMP-3). Additionally, we studied the ability of human trypsin-2 to directly degrade native type I collagen. Our results show that trypsin-2 can efficiently activate all these three collagenolytic proMMPs and pro-MMP-3 and may thus be the initial serine proteinase activator of the collagenolytic proMMP activation network directly or through MMP-3. Furthermore, trypsin-2 was found to be capable of directly attacking type I collagen, whose action could be inhibited by its specific inhibitor TATI similarly to the activation of proMMP-1, -3, -8, and -13.

EXPERIMENTAL PROCEDURES

Materials. MMP-1 and MMP-3 were produced and isolated as described previously (*33*, *34*). MMP-13 was expressed and purified as described previously (*35*, *36*). Human collagenase-2 (MMP-8) was purified from neutral salt extracts of isolated human neutrophils (PMNs) by a combination of conventional and affinity chromatography as described previously (*37*−*39*). Alternatively, recombinant human MMP-1 was purchased from Chemicon International Inc. (Temecula, CA), recombinant MMP-3 and MMP-8 were from Sigma Chemicals (St. Louis, MO), and recombinant human MMP-13 was purchased from Invitrotek GmbH (Berlin, Germany). Bovine pancreatic TPCK-trypsin and human neutrophil elastase were purchased from Sigma Chemicals (St. Louis, MO). Tumor-associated trypsinogen-2 (trypsin-2) was purified from serum-free conditioned medium of COLO-205 human colon adenocarcinoma cells (ATCC, CCL-222, Rockville, MD) as described previously (*16*). Tumor-associated trypsin inhibitor (TATI) was purified from urine of ovarian cancer patients (*13*, *24*). HSC-3 cells are human squamous cell carcinoma cells of tongue (JCRB, 0623, Health Science Research Resources Bank, Japan).

Proteolytic Activation of proMMPs. 15 nM trypsin-2 was mixed with each proMMP (15 μM) at various molar ratios ranging from 1:100 to 1:1000 (trypsin-2/proMMP) and incubated at 37 °C for 10−60 min as indicated for each individual experiment. Trypsin-2 was inhibited by a 4-fold amount of TATI by incubating for 60 min at 37 °C (*21*).

Electrophoresis and Protein Blotting. 8% SDS−PAGE was performed in a mini-gel system (Bio-Rad, Richmond, CA) without reduction. Reduction with 20 mM dithiothretol was used only with samples for amino acid sequencing. For sequencing purposes, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in 12.5 mM Tris−96 mM glycine−10% methanol using the Mini-Trans-Blot II apparatus from Bio-Rad.

Collagenase Assay. Collagenase activity was determined by incubating the appropriate MMPs with native soluble 1.5 μM human skin type I collagen (*37*) at 20 and 4 °C for 3−24 h. To characterize the substrate, type I collagen was also exposed to gelatinolytic but not collagenolytic recombinant human MMP-9 and -20 (*21*, *40*) and gelatinolytic and collagenolytic MMP-2 (*41*, *42*) as well as to human trypsin-2, bovine pancreatic TPCK-trypsin, and human neutrophil elastase. The cleavage products of type I collagen were analyzed by SDS−PAGE.

Aminoterminal Amino Acid Sequencing. Proteins blotted onto PVDF membrane (Bio-Rad) were visualized by staining in 0.1% Coomassie brilliant blue R-250 in 50% methanol, and appropriate protein bands were excised. Amino acid sequencing was performed in an Applied Biosystems 477A/120A pulse-liquid protein sequencer by means of Edman degradation (*43*).

Western Blot Analysis. Western blot analysis was done as described by Moilanen et al. (*29*). Concentrated serum-free cell culture medium (100-fold) from HSC-3 and HSC-3/trypsin-2 cells was diluted into 1 × sample buffer (1.25 M Tris, pH 6.8, 10% SDS, 10% glycerol, 37 μM bromophenol blue) and incubated for 20 min at 60 °C. The polyclonal antibodies used were MMP-1, MMP-3, MMP-13 (Ab 8105, Ab 811, Ab 8114, Chemicon International Inc., Temecula, CA), and MMP-8 (*44*, *45*). The MMP-8 Western blotting products were quantified with an image processing and analysis program (Scion-Image PC, Scion Corporation, Frederick, MD).

PCR, Cloning, and Sequencing. The total RNA isolated from COLO 205 cells with TRIzol reagent (Life Technologies, Grand Island, NY) was transcribed to trypsinogen-2 cDNA by Superscript II Rnase H^- reverse transcriptase (Gibco BRL Life Technologies, Paisley, Scotland). The reaction was done according to the manufacturer's instructions using 3 μg of total RNA and 2.8 pmol of trypsinogen-2 gene-specific antisense primer. The antisense primer was 5′-ATG GGA TCC TTA GCT GTT GGC AGC TAT GGT-3′, and the sense primer was 5′- CTG GCT AGC ACC ATG AAT CTA CTT CTG ATC-3′. The trypsinogen-2 PCR reaction was performed using 2 U Dynazyme EXT DNA polymerase (Finnzymes, Espoo, Finland), 150 ng of each primer, 200 μM dNTP (Promega, Madison, WI), and 0.5 mM $MgCl_2$ in a final volume of 50 μL of 1 × EXT buffer (Finnzymes, Espoo, Finland). After initial denaturation (5 min at 95 °C), 25 cycles were performed (1 min at 95 °C,

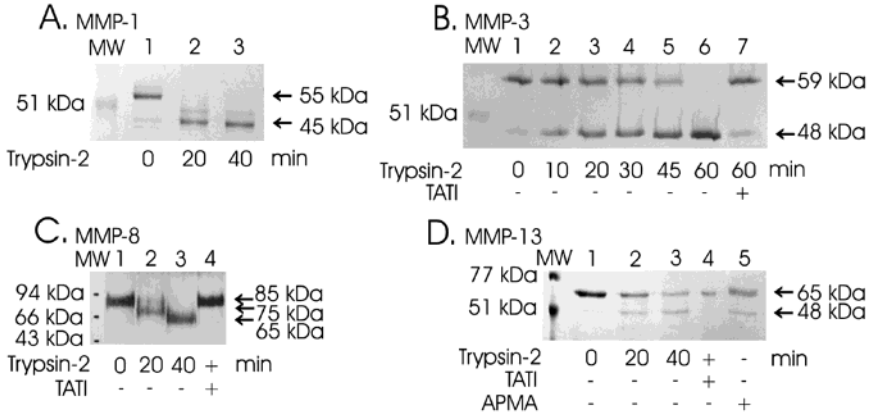

FIGURE 1: Conversion of human proMMP-1, -3, -8, and -13 into active forms by trypsin-2. Human proMMP-1, -3, -8, and -13 (15 μM) were treated with 15 nM trypsin-2 for the time intervals indicated (0−60 min) at 37 °C. For MMP-3, -8, and -13, trypsin-2 was also preincubated for 60 min at 37 °C with 60 nM TATI, and 1 mM APMA treatment is shown for MMP-13. Incubations were stopped by boiling for 5 min and analyzed on 8% SDS−PAGE. (A) ProMMP-1 (lane 1); proMMP-1 exposed to trypsin-2 for 20 (lane 2) and 40 (lane 3) min. (B) ProMMP-3 (lane 1); proMMP-3 exposed to trypsin-2 for 10 (lane 2), 20 (lane 3), 30 (lane 4), 45 (lane 5), and 60 (lane 6) min; and lane 7 as lane 6, but trypsin-2 was preincubated with TATI. (C) ProMMP-8 (lane 1); proMMP-8 exposed to trypsin-2 for 20 (lane 2) and 40 (lane 3) min; and lane 4 as lane 3 but trypsin-2 was preincubated with TATI. (D) ProMMP-13 (lane 1); proMMP-13 exposed to trypsin-2 for 20 (lane 2) and 40 (lane 3) min; lane 4 as lane 3, but trypsin-2 was preincubated with TATI, and proMMP-13 was exposed to 1 mM APMA (lane 5). Molecular weight markers (MW) are shown on the left.

1 min 15 s at 54 °C, and 3 min at 72 °C) followed by final extension of 10 min at 72 °C. The 760 bp trypsinogen-2 PCR product was purified from 1% agarose gel with a QIAEX II kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The PCR product was cloned into a pCR3.1 vector using a bidirectional TA cloning kit (Invitrogen). The trypsinogen-2-pCR3.1 vector was purified with a Quantum Prep kit (BioRad, Hercules, CA). To verify the correct orientation of the trypsinogen-2 gene in the vector and the correctness of the gene itself, sequencing was done by a ABI PRISM 377 DNA sequencer (Perkin-Elmer) using a dRhod Terminator Cycle Sequencing Ready reaction kit (Perkin-Elmer) and T7 and pCR3.1 reverse primers (Invitrogen).

Generation of Stably Transfected Cells Secreting Human Trypsinogen-2. The trypsinogen-2 construct was stably transfected into HSC-3 cells using Lipofectin Reagent (Life Technologies, Gaithersburg, MD) by following the manufacturer's instructions. A total of 1 μg of control or trypsinogen-2 plasmid and 2.5 μL of Lipofectin reagent were incubated with 30% confluent cells in 24-well dishes (Nunclon, Roskilde, Denmark) for 5 h in 250 μL of serum and antibiotic-free medium. Cells were grown in normal medium for 3 days and then placed under G418 (Life Technologies, Paisley, Scotland) selection (300 μg/mL). After the selection, the presence of trypsinogen-2 mRNA was estimated by RT-PCR before and after transfection, and the amount of trypsinogen-2 protein was measured from trypsinogen-2-transfected and control cells in serum-free culture medium in accordance with the immunofluorometric method described previously (*46*).

Cell Cultures. HSC-3 and HSC-3/trypsinogen-2 cells were grown in 1:1 DMEM and Ham's Nutrient Mixture F-12 (Gibco BRL, Life Technologies, Paisley, Scotland) with 10% heat-inactivated fetal bovine serum (FBS) (Bioclear, Holly Ditch Farm, Mile Elm, UK), 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 50 IU/mL nystatin, 0.25 μg/mL amphotericin-B, 1 mM sodium pyruvate (all supplements from Gibco BRL, Life Technologies, Paisley, Scotland), and 0.4 ng/mL hydrocortisone (Diosynth, Oss, The Netherlands). To the culture medium of trypsinogen-2-transfected cells, 300 μg/mL G418 (Gibco BRL, Life Technologies, Paisley, Scotland) was added for stable selection.

HSC-3 and HSC-3/trypsinogen-2 cells were cultured to subconfluency and washed before the experiment three times with 1 × PBS. In the cell experiment medium, FBS was replaced with 0.5% lactalbumin (Sigma Chemical Co., St. Louis, MO), and 50 ng/mL enterokinase (Boehring Mannheim, Mannheim, Germany) was added. The cells were incubated for 48 h, and the conditioned media were collected and centrifuged to remove cells and cellular debris, after which the supernatant samples were frozen at −20 °C until used. The cells were collected into Trizol (Gibco BRL, Life Technologies Inc., Roskilde, Denmark) to preserve total RNA.

RESULTS

The molecular masses of the 55 kDa proMMP-1 and the 59 kDa proMMP-3 were reduced by 10 kDa, and active forms developed after 10−60 min proteolytic treatment with trypsin-2 (Figure 1A,B). Trypsin-2 from COLO 205 medium converted the 85 kDa proMMP-8 from human PMNs and the 50 kDa proMMP-8 from HSC-3 oral squamous cell carcinoma cells into active forms of 65−75 kDa and 45 kDa, respectively (Figure 1C, Figure 2). APMA also converted the 50 kDa proMMP-8 into active forms with 10 kDa lower molecular weight (Figure 2). TATI prevented conversion by trypsin-2 (Figure 1C, Figure 2). Under similar conditions, the 65 kDa proMMP-13 was converted into an active product of 48 kDa within 20−40 min, as shown by SDS−PAGE (Figure 1D). Pretreatment of trypsin-2 with TATI prevented all these proMMP conversions into respective active forms (Figure 1B−D, Figure 2).

N-terminal analysis of trypsin-2-converted MMP species revealed that trypsin-2 cleaved MMP-1 at Lys^{56}−Val^{57}, MMP-3 at His^{82}−Phe^{83}, PMN MMP-8 at Arg^{48}−Phe^{49}, and Arg^{70}−Cys^{71} and MMP-13 at Glu^{84}−Tyr^{85}.

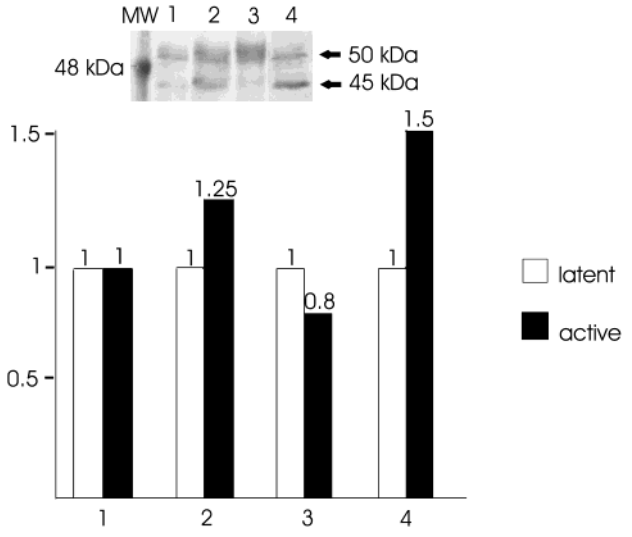

FIGURE 2: Conversion of HSC-3 cell-derived 50 kD proMMP-8 into an active form by trypsin-2 and APMA. Western immunoblotting was performed using anti-MMP-8 antibody as described in Experimental Procedures of the concentrated HSC-3 culture media. The Western blotting products were quantified with an image processing and analysis program (Scion-Image PC, Scion Corporation, Frederick, MD). Black columns represent the ratio of the active form of MMP-8 to the latent form (white columns). The media samples were exposed to buffer (lane 1); 30 nM trypsin-2 for 30 min at 37 °C (lane 2); and trypsin-2 pretreated with 120 nM TATI for 30 min at 37 °C (lane 3) or 1 mM APMA (lane 4). Molecular weight markers (MW) are shown on the left.

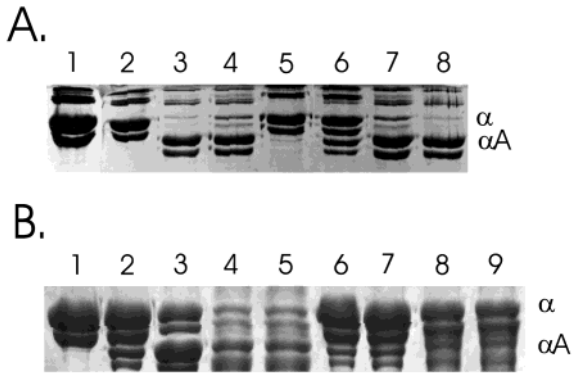

FIGURE 3: Activation of human proMMP-1, -8, and -13 by human trypsin-2. The trypsin-2-induced proteolytic activation of proMMP-1, -8, and -13, resulting in enhanced type I collagenolysis, and the inhibition of proMMP-1 activation by tumor-associated trypsin inhibitor (TATI) were studied by SDS−PAGE assay for the demonstration of collagenase activity. The specific cleavage products (αA peptides) were identified by 8% SDS−PAGE. Samples (15 μM) of native and activated latent proMMP-1, -8, and -13 were incubated with 1.5 μM type I collagen for 3 h at 20 °C. Coomassie brilliant blue staining was used. α denotes intact type I collagen monomers and αA denotes the characteristic 3/4 degradation products resulting from the action of active MMP-1, -8, and -13 on native type I collagen. (A) Type I collagen incubated with buffer (lane 1); type I collagen incubated with proMMP-1 treated with buffer (lane 2); type I collagen incubated with proMMP-1 exposed to trypsin-2 (15 nM) for 20 (lane 3) and 40 (lane 4) min at 20 °C, respectively. Trypsin-2 activity was stopped by addition of TATI (50 nM); lane 5 as lanes 3 and 4, but trypsin-2 was preincubated with TATI for 60 min at 20 °C prior to incubation with proMMP-1 and type I collagen; proMMP-1 exposed to 0.5 mM sodium hypochlorite (NaOCl) (lane 6); proMMP-1 exposed to 0.5 mM gold thioglucose (GTG) (lane 7); proMMP-1 exposed to 1 mM (APMA) (lane 8). (B) Type I collagen incubated with buffer (lane 1); type I collagen incubated with proMMP-8 and buffer (lane 2); type I collagen incubated with proMMP-8 exposed to 1 mM APMA (lane 3); type I collagen incubated with proMMP-8 exposed to trypsin-2 (15 nM) for 30 (lane 4) and 60 min (lane 5) at 37 °C. Lanes 6 and 7 as lanes 2−5 but with proMMP-13.

Without activation, latent human proMMP-1, -8, and -13 did not degrade or degraded only slightly native type I collagen (Figure 3). Pretreatment of proMMP-1, -8, and -13 by trypsin-2 increased the capacity of these interstitial collagenases to generate the specific cleavage products of type I collagen αA and αB peptides representing the characteristic 3/4 and 1/4 degradation products, as demonstrated by SDS−PAGE (Figures 3 and 4). Pretreatment of trypsin-2 with TATI prevented the proMMP-1 activation, and no formation of cleavage products was observed (Figure 3A). Exposure of proMMP-1 to known nonproteolytic proMMP activators [APMA, gold thioglucose (GTG), and sodium hypochlorite (NaOCl)] also resulted in procollagenase activation, as demonstrated by SDS−PAGE assay for MMP-1, showing the formation of specific cleavage products (Figure 3A).

When trypsin-2 was incubated with human native soluble type I collagen at 20 and 4 °C for 24 h, trypsin-2 degraded collagen efficiently, generating degradation products of multiple lower molecular size at both incubation temperatures (Figure 4). Pretreatment of trypsin-2 with TATI prevented the degradation of type I collagen by trypsin-2 at 20 and 4 °C. Trypsin-2 induced multiple degradation of native type I collagen clearly different from classical MMP-8-induced specific degradation generating the characteristic 3/4 (αA) and 1/4 (αB) cleavage products at both 20 and 4 °C (Figure 4). As expected, trypsin-2 and MMP-8 degraded native type I collagen more efficiently at 20 than at 4 °C. Human neutrophil elastase and TPCK-treated bovine pancreatic trypsin did not degrade native soluble type I collagen (not shown).

Furthermore, we transfected the cDNA of trypsin-2 into a malignant human tongue squamous cell carcinoma cell line (HSC-3) to study the role of trypsin-2 in proMMP-1, -3, -8, and -13 activations in cell culture conditions. After transfection, the trypsin-2 mRNA levels were elevated in the HSC-3 cell line, and the immunofluorometric measurements of secreted trypsin-2 were clearly elevated as compared to the control cells transfected with pCR3.1 vector (*47*). The secreted trypsinogen-2 in the cell culture media was activated into catalytically competent trypsin-2 by the addition of enterokinase (*47*, *48*). Western immunoblotting revealed that MMP-1 and -13 secreted by the transfected cells were mainly in their latent 50 and 60 kDa proforms, respectively (Figure 5A,D). Amount of MMP-13 protein production was low, and the 60 kDa band was seen weakly. The production of MMP-3 and -8 protein was decreased in trypsin-2-transfected cells as compared to normal HSC-3 cells, and no immunoreactive bands could be seen in transfected cells (Figure 5B,C).

DISCUSSION

Our results show that human trypsin-2 can proteolytically activate human proMMP-1, -3, -8, and -13. The interstitial collagenases (MMP-1, -8, and -13) have been regarded as essential to initiate type I collagen degradation. MMP-3 is the key regulator of this collagenolytic MMP network since it can activate human proMMP-1, -8, and -13 (*30*, *31*, *35*). Since all extracellular MMPs are synthesized and released

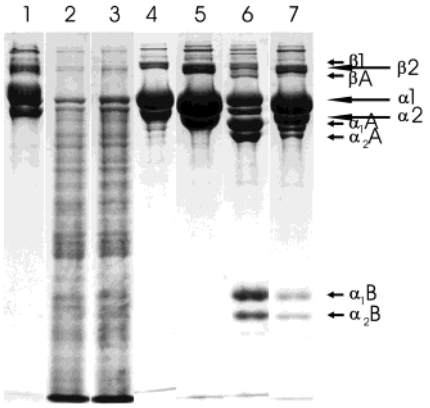

FIGURE 4: Degradation of native type I collagen by human trypsin-2 and MMP-8 at 20 and 4 °C. Trypsin-2 (15 nM) and MMP-8 (15 μM) were incubated with 1.5 μM native soluble type I collagen for 24 h at 20 and 4 °C. Type I collagen incubated with buffer (lane 1); type I collagen incubated with trypsin-2 at 20 °C (lane 2); type I collagen incubated with trypsin-2 at 4 °C (lane 3); type I collagen incubated with trypsin-2 pretreated (60 min at 37 °C) with a 4-fold amount of TATI at 20 °C (lane 4); type I collagen incubated with trypsin-2 pretreated (60 min at 37 °C) with a 4-fold amount of TATI at 4 °C (lane 5); type I collagen incubated with 1 mM APMA-treated MMP-8 at 20 °C (lane 6); type I collagen incubated with 1 mM APMA-treated MMP-8 at 4 °C (lane 7). Coomassie brilliant blue staining and 8% SDS−PAGE were used. β1 and β2 denote intact type I collagen dimers, and βA denotes the characteristic 3/4 degradation products of type I collagen dimers. α1 and α2 denote intact type I collagen monomers, and α_1A, α_2A, α_1B, and α_2B denote the characteristic 3/4 and 1/4 cleavage products, respectively, of type I collagen monomers resulting from the action of active neutrophil collagenase on type I collagen. Note the multiple-size cleavage pattern of native type I collagen resulting from trypsin-2 action, which was prevented by TATI, at both 20 and 4 °C.

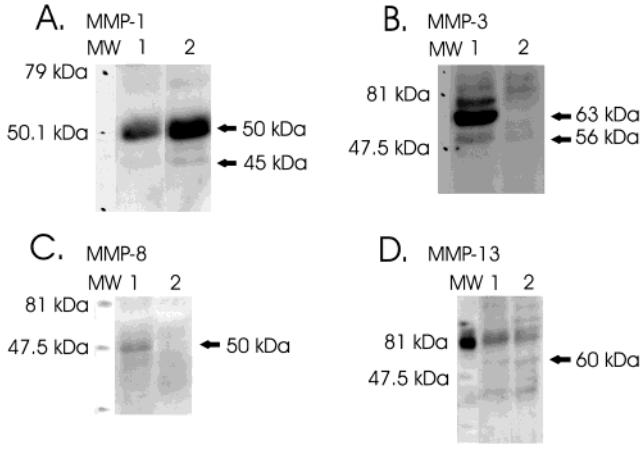

FIGURE 5: Comparison of MMP-1, -3, -8, and -13 protein production of trypsin-2 transfected and untransfected HSC-3 cells. ECL-Western blot analysis was used; the subconfluent cell cultures were incubated in serum-free media with 0.5% lactalbumin and 50 ng/mL enterokinase. The cells were incubated for 48 h, and the conditioned media were collected. A total of 20 μL of 100-fold concentrated media was loaded per lane. The following antibodies were used in Western blot: polyclonal antibodies: MMP-1, MMP-3, MMP-13 (Ab 8105, Ab 811, Ab 8114, Chemicon International Inc., Temecula, CA), and MMP-8 (*44*, *45*). (A) MMP-1; (B) MMP-3; (C) MMP-8; and (D) MMP-13. Lane 1 means, in all panels, conditioned media of untransfected HSC-3 cells, while lane 2 represents conditioned media of trypsin-2-transfected HSC-3 cells. Molecular weight markers (MW) are shown on the left.

by cells as inactive latent proforms (*2*), the initial activation of the first proenzyme of the proMMP activation network evidently requires the action of the other (i.e., serine type) matrix proteinases (*32*). In this regard, we have previously shown that trypsin-2 activates proMMP-9 in vitro at the lowest molar ratio reported so far and also activates partially, although less efficiently, proMMP-2 (*21*). These in vitro findings were further supported by the present findings that showed the trypsin-2 levels to be significantly associated especially with proMMP-9 but not with proMMP-2 activation in human ovarian tumor cyst fluids in vivo (*22*). In this study, we have shown that, in addition to progelatinases (MMP-2 and -9) and proenamelysin (MMP-20) (*40*), trypsin-2 activates proMMP-1, -3, -8, and -13 at a molar ratio of 1:1000 in a time-dependent manner. The molecular masses of proMMP-1, -3, -8, and -13 were processed by trypsin-2 to be approximately 10 kDa lower; thus, all the three human interstitial procollagenases and stromelysin-1 were efficiently proteolytically processed into their active counterparts. These processings could be prevented by preincubation of trypsin-2 with its endogenous and specific inhibitor TATI.

N-terminal sequence analysis of the activated MMPs revealed that either part or all of the propeptide domain was released by human trypsin-2 treatment. MMP-1 was processed by the cleavage of the Lys^{56}−Val^{57} peptide bond, which is in line with trypsin specificity (*49*), leading to a partially activated but catalytically competent form of MMP-1; the conserved PRCGVPD sequence motif, which is responsible for the latency of MMPs (*6*), was not removed from the enzyme. MMP-3 and MMP-13, similarly to gelatinase B (*21*), were processed downstream of the PRCGVPD sequence by cleavage of His^{82}−Phe^{83} and Glu^{84}−Tyr^{85}, indicating the formation of completely activated forms of these enzymes. Human PMN MMP-8 was processed by trypsin-2 at two tryptic sites, Arg^{48}−Phe^{49} and Arg^{70}−Cys^{71}, located upstream and in the PRCGVPD latency motif, respectively. These cleavages result in partial activation of MMP-8. The activation of proMMP-3 and proMMP-13 by trypsin-2 obviously involves an autoproteolytic process since trypsin-2 is not able to cleave at the carboxyterminal site of histidine or glutamic acid (*49*). Thus, the initial sites of MMP-3 and -13 cleaved by trypsin-2 remain unidentified in respect of its specificity. The corresponding His^{82}−Phe^{83} peptide bond of proMMP-3 cleaved by trypsin-2 is also cleaved by bovine chymotrypsin (*50*). TPCK-treated bovine trypsin also induces cleavage of proMMP-13 similar to human trypsin-2 between Glu^{84} and Tyr^{85} (*35*). The 85 kDa inactive MMP-8 was initially cleaved at the Arg^{48}−Phe^{49} peptide bond by bovine trypsin and further activated cleavage of Arg^{70}−Cys^{71} similarly to human trypsin-2 (*51*).

Our present results demonstrated that human trypsin-2 could directly and efficiently activate latent proforms of interstitial procollagenases (proMMP-1, -8, and -13) and their efficient activator prostromelysin-1 (proMMP-3). Thus, trypsin-2 exerts potent effects to be identified as the matrix serine proteinase (MSP) that may be the initial triggering proteolytic activator of the collagenolytic proMMP activation network (*32*). Nonetheless, the cell culture findings showing that trypsin-2 cDNA transfection into a malignant human oral squamous cell tongue carcinoma cell line (HSC-3) did not result in elevated activation of proMMP-1, -3, -8, and -13 secreted by these cells. Protein production of MMP-3 and -8 was markedly decreased in trypsin-2-transfected HSC-3 cells. Furthermore, based on our preliminary results

using TaqMan PCR analysis, expression of MMP-1, -3, and -13 mRNA decreased clearly after trypsin-2 transfection in HSC-3 cells (personal communication, H. Palosaari). In light of our previous findings, we have shown that trypsin-2 cDNA transfection into these malignant cell lines leads to elevated proMMP-9 but not proMMP-2 activation (*47*). Overall, these findings seem to indicate that human trypsin-2 is an efficient initial activator of many proMMPs (progelatinases, procollagenases, prostromelysin-1, and proenamelysin) but eventually prefers proMMP-9 in vivo (*21*, *22*, *47*).

Previously, PMNs have been considered the only cellular source of glycosylated 85 kDa MMP-8, which is synthesized during early PMN differentiation and stored within specific granules to be finally released on PMN degranulation (*52*). However, recent studies have shown cytokine- and growth factor-inducible de novo expression of less glycosylated 50−55 kDa MMP-8 from human chondrocytes, synovial fibroblasts, endothelial cells, pulpal odontoblasts, bronchial and gingival sulcular epithelial cells, Jurkat T leukemia cells, epithelial tumor cells, plasma cells, and oral squamous cell carcinoma cells (*29*, *53*−*60*). Furthermore, cultured human mononuclear phagocytes exposed to interleukin-1β were recently shown to express and release 75 kDa proMMP-8 and active 55 kDa MMP-8 (*61*). We wished to clarify whether the 50 kDa form of MMP-8 secreted by human oral squamous cell represents the proform of the mesenchymal cell type MMP-8 or an already activated form of larger molecular size (75−85 kDa) MMP-8. Therefore, we exposed the 50 kDa MMP-8 present in the culture media of HSC-3 cells to an optimal organomercurial MMP activator (APMA) as well as trypsin-2. APMA and trypsin-2 converted the oral carcinoma cell-derived 50 kDa proMMP-8 into a 45 kDa MMP-8 active form. These data indicate that the 50 kDa forms represent a proform of MMP-8 expressed by oral carcinoma cells (*29*), which thus can be activated by both APMA and trypsin-2. Thus, trypsin-2 can also be considered a potential pathophysiological proteolytic activator of 50 kDa proMMP-8 from oral carcinoma cells.

The mechanisms of interstitial collagen degradation have been a topic of considerable interest since the native triple-helical conformation or state type I collagen is resistant to attack by most mammalian proteolytic enzymes (*6*, *62*). The initiation of the extracellular breakdown of triple-helical native type I collagen at neutral pH is accomplished by the action of at least three specific interstitial collagenases: collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13); gelatinase A (MMP-2) and MT1-MMP (MMP-14) can also degrade native triple-helical type I collagen similarly to MMP-1, -8, and -13 (*2*, *41*, *63*). These collagenolytic MMPs initially cleave the type I collagen triple helix at a specific Gly-Leu/Ile bond to generate the characteristic 3/4 (αA) and 1/4 (αB) degradation products, which, at body temperature, after loosing their triple-helical conformation and becoming randomly coiled, can then be further degraded by gelatinases as well as other serine proteinases, such as neutrophil elastase (*64*). To the best of our knowledge, there has so far been no published reports on human trypsin-2 cleavages on native human triple-helical type I collagen. We found in the present study that, at neutral pH and at both 20 and 4 °C, trypsin-2 cleaved native soluble human type I collagen, generating multiple lower molecular size degradation products. The collagenolytic activity of human trypsin-2 was not due to a contaminating MMP or any other serine proteinase since it was efficiently inhibited by TATI, a specific trypsin-2 inhibitor (*25*) at both 20 and 4 °C. The type I collagen substrate in the native triple-helical conformation was not degraded by gelatinolytic but not collagenolytic MMP-9 and MMP-20 but was also degraded by collagenolytic MMP-1, -8, and -13. The native human skin type I collagen was not degraded by human neutrophil elastase or TPCK-treated bovine pancreatic trypsin. After the native triple-helical type I collagen had been rendered into randomly coiled gelatin by heating, it could be efficiently degraded by the tested gelatinolytic MMPs. These results indicate that trypsin-2 exerts a potential to directly attack native type I collagen. Thus, our data point to trypsin-2 as a previously unsuspected participant in type I collagen breakdown.

Taken together, the present findings demonstrated that trypsin-2 can, directly or indirectly, through stromelysin-1, initiate and activate the collagenolytic proMMP network as well as directly attack native type I collagen. Thus, the expression and action of trypsin-2, especially in human tumors, appears to be a new or additional mechanism contributing to their invasiveness and tissue-destructive potential. Since the inhibition of trypsin-2 by TATI has been shown to reduce the pericellular extracellular matrix proteolysis and invasion by tumor cells (*48*), this mechanism may be a potential target for future tumor therapy.

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