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Secretory Differentiation of Hamster Tracheal Epithelial Cells Increases Activation of Matrix Metalloproteinase-2

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Abstract – In chronic airway inflammatory diseases such as asthma and chronic bronchitis, it has been suggested that matrix metalloproteinases secreted from infiltrating neutrophil contribute the pathogenesis of the disease and have been a focus of intense investigation. We report here that hamster tracheal surface epithelial goblet cells (HTSE cells) produce matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2). Matrix metalloproteinase activities were investigated using [^{3}H]collagen-digestion assay and gelatin zymography. The subtype of matrix metalloproteinases expressed from HTSE cells was MMP-2 (gelatinase A), which was determined by Western blot with various subtype selective anti-matrix metalloproteinase antibodies. The MMP-2 and TIMP-2 cDNAs from HTSE cells were partially cloned by RT-PCR and they reveal more than 90% of sequence homology with those from human, rat and mouse. The collagenolytic activity was increased with the secretory differentiation of the HTSE cell and it was found that zymogen activation was responsible for the increased MMP-2 activity in HTSE cells. The results from the present study suggest that the metaplastic secretory differentiation of airway goblet cells may affect chronic airway inflammatory process by augmenting the zymogen activation of MMP-2.

Keywords □ hamster tracheal surface epithelial (HTSE) cell, goblet cell, MMP-2, TIMP-2, Secretory differentiation, zymogen activation

INTRODUCTION

Matrix metalloproteinase (MMP) are a group of more than 23 closely related endopeptidases that participate in the degradation of all the extracellular matrix proteins including collagen (Nagase and Okada, 1997).

Increasing body of evidences show that MMP may participate in the chronic airway inflammatory processes including chronic bronchitis, asthma and cystic fibrosis etc. (Yao *et al.*, 1999; D'Armiento *et al.*, 1992). It has been reported that neutrophils, alveolar macrophages and airway epithelial cells from explant culture produce MMPs (Shapiro, 1994; Yao *et al.*, 1996). Increased levels of collagenase (MMP-1) and gelatinase B (MMP-9) have been detected in bronchoalveolar lavage fluids of patients with emphysema (Finlay *et al.*, 1997a; Finlay *et al.*, 1997b). Alveolar macrophages also express another type of MMP, macrophage metalloproteinase (MMP-12) (Shapiro *et al.*, 1993). MMP-12 knockout mice do not develop emphysema and do not show the expected increase in lung macrophage after long-term exposure to cigarette smoke (Hautamaki *et al.*, 1997) again exemplifying the importance of MMPs in airway inflammatory processes.

In chronic periodontitis, it has been reported that not only connective tissues but also epithelial cells produce MMPs and tissue inhibitors of metalloproteinases (TIMPs) (Uitto *et al.*, 1998). In case of airway epithelium, it has been reported that structural cells such as bovine tracheal serous gland cells or human bronchial epithelial cells from explant cultures were shown to secrete MMPs and TIMPs (Yao *et al.*, 1996; Tournier *et al.*, 1994). It has been also suggested that these MMPs and TIMPs may participate in the regulation of airway inflammatory processes (Yao *et al.*, 1999; Vignola *et al.*, 1998). However, it is unclear whether secretory airway surface epithelial cells, such as goblet cells, produce MMPs.

Several researchers including us successfully used primary hamster tracheal surface epithelial cell (HTSE) culture system

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for the study of airway cell secretory function including mucin release *in vitro* (Wu *et al.*, 1991; Kim, 1991; Kim, 1997). HTSE cell comprises relatively pure goblet cell populations (over 95%), which is one of the major mucin-secreting cells in upper airway epithelium. HTSE culture system requires a thick collagen matrix for normal secretory function and it shows collagenolytic phenotype upon differentiation (Wasano *et al.*, 1988; Niles *et al.*, 1988). These observations prompted us to investigate whether MMPs are responsible for the observed collagenolytic activity in HTSE cells. In addition, we investigated the role of secretory differentiation on MMP regulation in HTSE cells. We found that HTSE culture expresses MMP-2 (gelatinase A) along with TIMP-2, a known physiological inhibitor of MMP-2. In addition, we report here that the secretory differentiation of HTSE cells augments zymogen activation of MMP-2.

MATERIALS AND METHODS

Primary hamster tracheal surface epithelial (HTSE) cell culture

Tracheas were obtained from male Syrian golden hamsters of 8 to 10 weeks of age. Preparation and plating of HTSE cells on thick collagen matrix were carried out as described previously (Wasano *et al.*, 1988; Kim *et al.*, 1985; Shin *et al.*, 2000). Cells were initially plated in a complete medium containing 5 % fetal bovine serum (Gibco, Gaithersburg, MD). The serum-depleted condition was achieved by gradually reducing the concentration of serum in the medium: 5 % on day 0; 2.5 % on day 1 and day 2; 1 % on day 3 and day 4; and 0 % from day 5 to day 8. Secretory function of HTSE cells was assessed by ELISA procedures against hamster airway mucin as described (Jo *et al.*, 1999).

Determination of the collagenolytic activity

The matrix digesting (collagenolytic) activity of HTSE culture spent media was assessed by [^{3}H]collagen digestion method as described by Cawston and Barett with slight modification (Cawston and Barett, 1979). In brief, [^{3}H]collagen (Amersham, Buckinghamshire, UK) was mixed with 2 mg/ml of Vitrogen (Cohesion, Palo Alto, CA) in a ratio of 1:500. Then equal volume of neutralizing buffer (0.2 M Tris-Cl., 0.4 M NaCl, 10 mM $CaCl_2$, pH 7.8) was added and 50 µl of the mixtures were dispensed onto 96 well microtest plate at 4. The [^{3}H]collagen mixtures were gellified at 37 overnight. Each well was washed twice with 150 µl of assay buffer (10 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4). Sample solution was added onto each well in a total volume of 150 µl diluted in the assay buffer. The plate was incubated for 16-20 hrs in a humid atmosphere at 37°C. Two thirds of the supernatants (100 µl) were removed and centrifuged for 10 mins at 10,000 × g. The radioactivity released from the [^{3}H]collagen gel was determined by liguid scintillation counting (Pharmacia, Wallac 1409, Sweden).

Gelatin zymography

Zymography was performed as described elsewhere with slight modification (Heussen and Dowdle, 1980). Samples were mixed with SDS sample buffer in the absence of a reducing agent and resolved by electrophoresis on 10% polyacrylamide gel containing 0.1% SDS and gelatin at a final concentration of 0.1%(w/v). Thereafter, gels were washed in 2.5% Triton X-100 for 30min to remove the SDS and then incubated for 24h at 37°C in reaction buffer (100mM glycine, pH7.6, 5mM $CaCl_2$). After staining the gel with 0.1% Coomassie Brilliant Blue R-250, gelatinolytic activities were visualized as a clear band in the uniformly stained background.

Western blot

Serum free HTSE culture spent media were treated with 2 × SDS-sample buffer at 100 for 3 min prior to electrophoresis with 8% SDS polyacrylamide gel. The resolved band was electrotransfered onto nitrocellulose (NC) membrane (Towbint *et al.*, 1979). The NC membrane was blocked with Blotto (5% Nonfat dried milk in PBS/0.2% Tween20) and then incubated at room temperature for 2 h with antibodies against various MMPs and TIMPs which was diluted appropriately in Blotto as suggested by the manufacturer (Calbiochem, San Diego, CA). After three 10 min washes with PBS-Tween, the NC membranes were incubated with peroxidase labeled goat anti-mouse IgG at room temperature for 2 h. After extensive washing with PBS-Tween, the membranes were developed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

RT-PCR

Total RNA was extracted from primary HTSE cells using the Trizol reagent (GibcoBRL, Grand island, NY). Reverse transcription was performed for 40 min at 42°C with 6 µg of total RNA using 1 unit/µl of superscript II reverse transcriptase (GibcoBRL, Grand island, NY) according to the manufacturer's instruction. Oligo $(dT)_{18}$ was used as a primer for this reaction. The samples were then heated at 94°C for 5 min to terminate the reaction. The cDNA obtained from 1.2 µg total

RNA was used as a template for PCR amplification. Oligonucleotide primers were designed based on Genebank entries for human, mouse and rat MMP-2 and TIMP-2. The following primers were used for amplification reaction:

MMP-2

forward primer : 5'-CCACATTCTGGCCTGAGCT-3'

reverse primer : 5'-TGATGCTTCCAAACTTCACAC-3'

TIMP-2

forward primer : 5'-TGCAGCTGCTCCCCGGTGCAC-3'

reverse primer : 5'-TTATGGGTCCTCGATGTCGAG-3'

PCR mixes contained 10 μl of 10 X PCR buffer, 1.25 mM of each dNTP, 100 pmol of each forward and reverse primer and 2.5 units of Tag polymerase (Takara, Shiga, Japan). The final reaction volume was 50 μl. The reaction mixture was overlaid with 50 μl of mineral oil, and heated at 94°C for 30 sec. Amplification was performed in 35 cycles at 55°C, 30 s ; 72, 1 min ; 94°C, 30 s. After the last cycle, all samples were incubated for an additional 10 mins at 72°C. The amplified PCR product was subcloned into pGEM-T vector (Promega, Madison, WI), and amplified in E.Coli DH5α cells for sequence analysis.

RESULTS

Upon confluency, HTSE cells start to digest the matrix (thick collagen gel), which is usually from day 5 in culture. The process was completed within next 4-5 days and there was no collagen matrix left on day 11 (data not shown).

For the identification of MMP subtypes responsible for the matrix digesting activity from HTSE culture spent media, Western blot with several subtype specific monoclonal anti-MMP and anti-TIMP antibodies was performed. In this study, MMP-2 and TIMP-2 immunoreactivity was observed from HTSE culture supernatants (Fig. 1A). In our experimental condition, immunoreactivity against several MMPs including MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, MMP-14 (MT1-MMP) and MMP-15 (MT2-MMP) was not observed. In addition, TIMP-1 immunoreactivity was not observed. The expression of MMP-2 and TIMP-2 from HTSE cells was further confirmed by RT-PCR with mRNA obtained from HTSE cells. In agarose gel electrophoresis, 432 and 590 bp DNA fragments were observed for MMP-2 and TIMP-2, respectively (Fig. 1B). The amplified DNA fragments were subcloned and sequenced. The nucleotide sequence identity with human, mouse and rat counterparts was 90, 91 and 90% for MMP-2 and 92, 95 and 95% for TIMP-2, respectively. Deduced amino acid sequence was more homologous showing 98% of sequence

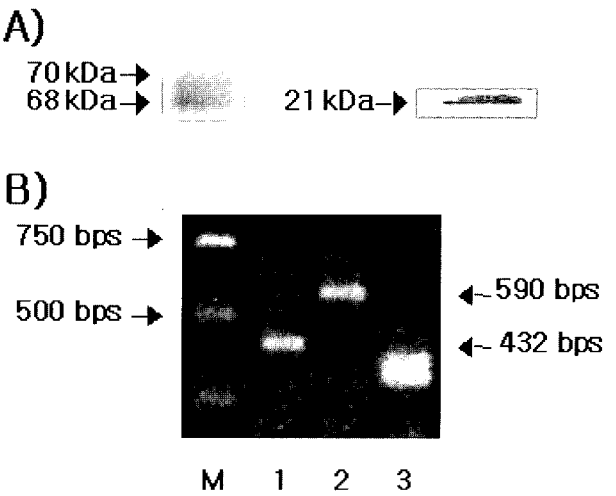

Fig. 1. A) Western blot of HTSE culture supernatnat with monoclonal antibody against MMP-2. HTSE culture supernatants were collected at culture day 5. After centrifugation, an aliquot of the supernatants were subjected to Western blot as described in materials and methods with antibodies against MMP-2 and TIMP-2. B) RT-PCR of MMP-2 and TIMP-2. HTSE cells were harvested with Trizol reagent and RNA was prepared for reverse transcription as described as materials and methods. The reverse transcribed cDNA was amplified by PCR with primer sets specific for MMP-2 and TIMP-2. The RT-PCR products were electrophoresed in 0.5 X TBE buffer on 1.2% agarose gels. M; DNA kb ladder. lane 1: MMP-2, lane 2: TIMP-2, lane 3: GAPDH.

identity (Fig. 2A and 2B). The sequences have been submitted to GeneBank (accession number AF260254 and AF260255 for MMP-2 and TIMP-2, respectively).

To determine the role of secretory differentiation of HTSE cells on MMP-2 activity, we analyzed mucin secretion and MMP activation from HTSE cells. As shown in Fig. 3A, mucin secretion from HTSE cells increased with time in culture. Usually, the secretory function was fully developed on day 5-6 in culture. In this condition, we first determined HTSE culture spent media for its collagenolytic activity using [^{3}H] collagen matrix digestion assay. In our experimental condition, even 100 U/ml of trypsin did not show collagenolytic activity while as little as 0.8 U/ml of clostridium collagenase showed appreciable collagenolytic activity. In addition, the collagenolytic activity was inhibited only by EDTA, which inhibits metalloproteinase, but not by other proteinase inhibitors (data not shown). In the present study, [^{3}H]collagen matrix digestion activity was increased along with the secretory differentiation of HTSE cells (Fig. 3B).

Next, we performed gelatin zymography to more clearly

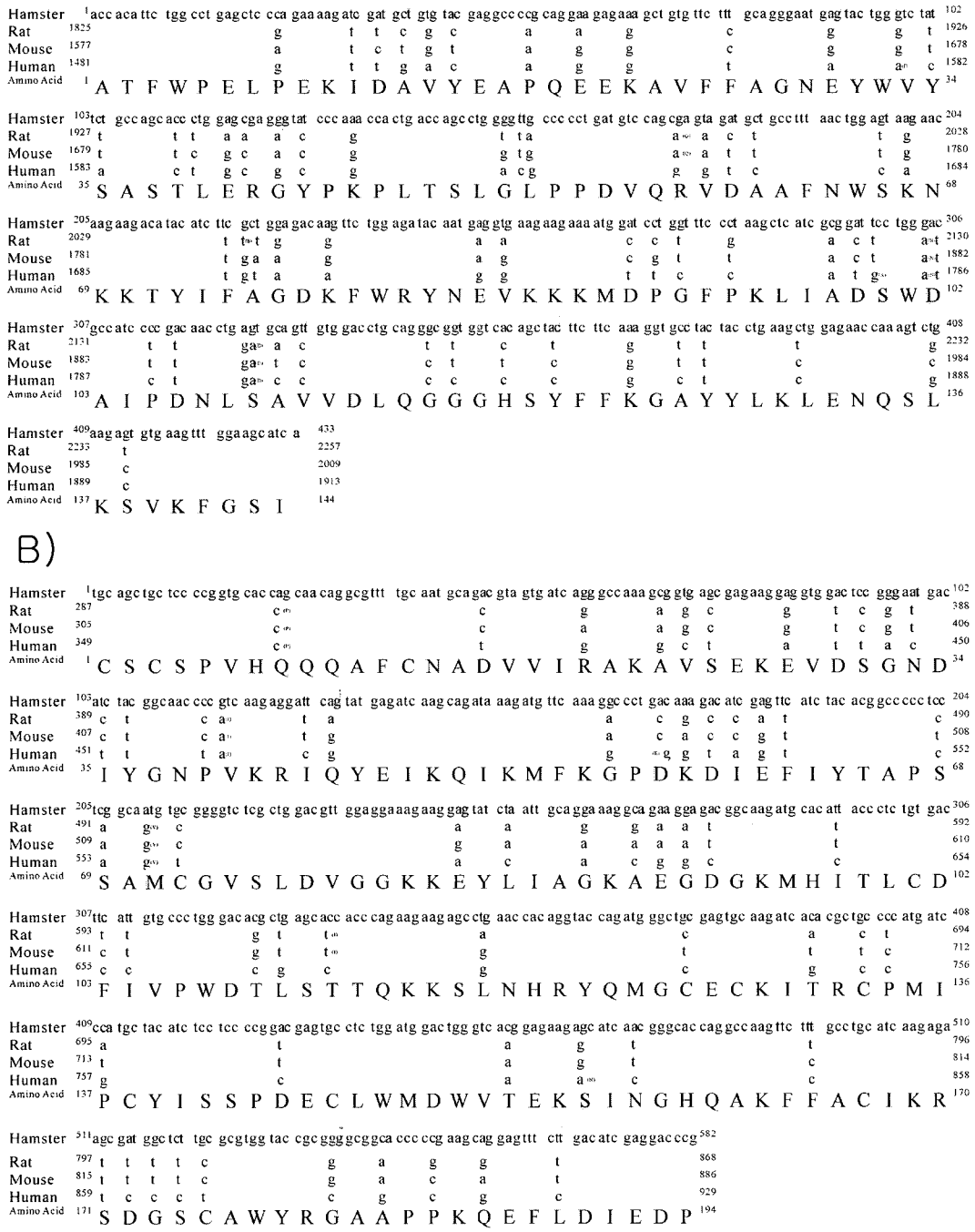

Fig. 2. The sequence and homologies of hamster MMP-2 and TIMP-2. The nucleotide sequence and the deduced amino acid sequence of hamster MMP-2 (A) and TIMP-2 (B), which was amplified by RT-PCR, were compared with those of rat, mouse and human. Different nucleotide or amino acid sequence was shaded. The overall nucleotide sequence identity was more than 90% and the amino acid sequence identity was more than 95%.

determine the activation of MMP-2 in HTSE cells (Fig. 4). When the HTSE culture spent media obtained from day 4 culture was electrophoresed, a gelatin-digesting activity, which appeared as a clear band over a blue background, was observed at Mw of 72kDa and 70kDa, which correspond to zymogen and intermediate form of MMP-2, respectively (Yao *et al.*, 1996; Tournier

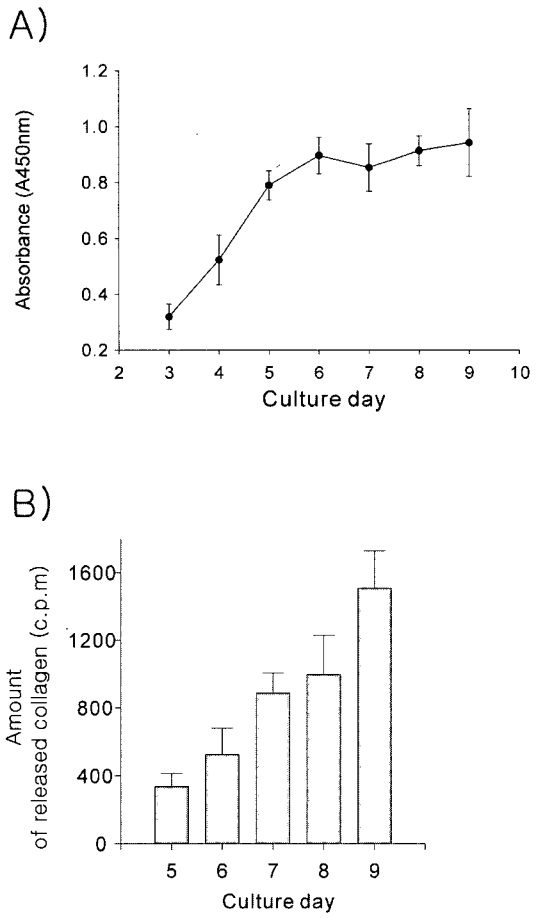

Fig. 3. Secretory differentiation and increased collagenolytic activity of HTSE cells. A) HTSE culture supernatants were collected at each culture day and an aliquot was analyzed for its mucin content by ELISA procedure using a monoclonal antibody against hamster mucin (mAb HTA). B) Alternatively, collagenolytic activity of HTSE culture supernatant was assessed by [^{3}H]collagen matrix digestion assay as described in materials and methods. Each bar represents a mean ± S.E.M (n=5).

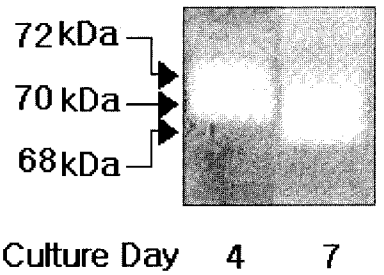

Fig. 4. Zymography of HTSE culture supernatants. At culture day 4 and 7, HTSE culture supernatants were collected and an aliquot was diluted with 2 X sample buffer without reducing reagent, and subjected to gelatin zymography as described in materials and methods. The molecular weights of positive bands were indicated by arrows.

et al., 1994; Ohuchi *et al.*, 1997; Birkedal-Hansen, 1995). With the increase of culture day, the band with Mw of 68kDa appeared, which corresponds to active form of MMP-2 (Yao *et al.*, 1996). These data suggest that zymogen activation of MMP-2 occurs with the secretory differentiation of HTSE cells.

DISCUSSION

The purpose of the present study was to identify and characterize collagenolytic enzyme expressed from primary HTSE cells and to investigate the role of secretory differentiation of HTSE cells on MMP activation.

To identify the subtypes of MMPs and TIMPs expressed from HTSE cells, Western blot analysis with various subtype selective anti-MMP and anti-TIMP antibodies were performed. In Western blot, MMP-2 bands with Mw of 70 kDa and 68 kDa are observed. The 68kDa band corresponds to the Mw of active form of MMP-2 and the 70 kDa band has been reported to be intermediate form of MMP-2 which is present in the process of activation of zymogen (Yao *et al.*, 1996; Tournier *et al.*, 1994; Ohuchi *et al.*, 1997; Birkedal-Hansen, 1995). Immunoreactivities against other MMPs including MMP-1, 3, 9, 13, 14 (MT1-MMP) and 15 (MT2-MMP) were not detected in HTSE culture media or whole cells. These data imply that the collagenolytic activity in HTSE culture supernatant was due to the presence of MMP-2. In this study, TIMP-2 immunoreactivity with Mw of 21 kDa but not TIMP-1 immunoreactivity was observd in Western blot (fig. 1). TIMP-2 exists as secreted form and inhibits predominantly MMP-2 among the various subtypes of MMPs (Yong *et al.*, 1998).

The expression of MMP-2 and TIMP-2 from HTSE cells was further confirmed by RT-PCR. The sequence analysis of the amplified DNA fragment showed closely-matching sequence homology as compared with human, rat and mouse MMP-2 and TIMP-2 (Gibbons et al., 1994; Shimizu et al., 1991; Stetler-Stevenson et al., 1990). These data suggest that hamster MMP-2 and TIMP-2 share common structural features with MMP-2 and TIMP-2 from other species. In the present study, both MMP-2 and TIMP-2 were expressed in HTSE cells, which imply TIMP-2 may contribute to the regulation of the activity of MMP-2. The simultaneous expression of MMP-2 and TIMP-2, hence the regulation of MMP-2 activity by TIMP-2, has also been reported by other researchers (Yong *et al.*, 1998).

Several researchers have reported that membrane type MMP-1 (MT1-MMP) is related to MMP-2 activation. It has

been reported that pro-MMP-2 forms a complex with TIMP-2 and the complex binds to MT1-MMP, which resides on cell surface. After being bound to MMP-2/TIMP-2 complex, MT1-MMP digests pro-peptide of MMP-2, thereby releases active MMP-2 (Sato *et al.*, 1996; Strongin *et al.*, 1995; Susan *et al.*, 1995; Murphy *et al.*, 1999). The visual inspection of the pattern of collagen matrix digestion of HTSE culture from the present study revealed islands of digestion loci on the thick collagen matrix. These observations suggest that MT1-MMP/MMP-2/TIMP-2 triad may localize the digestion of collagen matrix to the vicinity of active cells. However, in the present study, MT-1 MMP immunoreactivity was not observed in Western blot. Whether MT1-MMP is expressed from HTSE cells or not needs further clarification.

In the present study, it has been observed that HTSE cells digest the matrix (thick collagen gel) upon confluency. When assayed for its collagenolytic activity using [^{3}H]collagen matrix digestion assay, the collagenolytic activity of HTSE culture supernatant was inhibited only by EDTA, which inhibits MMPs by removing divalent cations, but not by other proteinase inhibitors such as serine proteinase inhibitor, acid-proteinase inhibitor and thiol proteinase inhibitor (data not shown). The proteinase inhibitors used in this study were as follows: phenylmethyl sulfonyl fluoride (1 mM, serine proteinase inhibitor), chymostatin (2μg/ml, serine proteinase inhibitor), leupeptin (10 μg/ml, serine proteinase inhibitor), trasylol (10 μg/ml, serine proteinase inhibitor), pepstatin (5 μg/ml serine proteinase and acid-proteinase inhibitor) and antipain (5 μg/ml, thiol proteinase inhibitor). The divalent cation dependency is reported to be typical characteristics of MMPs. The specificity of [^{3}H]collagen digestion assay was further verified by the fact that collagenase but not over 100-fold excess amount of trypsin digest [^{3}H]collagen matrix in our experimental condition (data not shown).

In this study, the increase in collagenolytic activity was well correlated with the secretory differentiation of HTSE cells (fig. 3). HTSE cells have been widely used as an model for goblet cell secretory metaplasia (Kim, 1991; Kim *et al.*, 1989). The airway mucin secretory function increased with time in culture and usually culminated at day 5 (Kim, 1991; Kim *et al.*, 1989). In this study, mucin secretory function was fully developed on day 5 and collagenolytic activity was observed from day 5 and thereafter. These data implicate that airway epithelial cells respond to chronic airway disease condition not only by inducing secretory metaplasia but also by activating MMP-2, which may result in tissue remodeling and progression of inflammation.

In airway inflammatory diseases including chronic bronchitis, asthma and emphysema, various subtypes of MMP such as MMP-1, 2, 9, 13 etc. have been found in lavage fluid of patients (Vignola *et al.*, 1998; Yaguchi *et al.*, 1998). In these inflammatory diseases, it has been known that MMPs are secreted from not only lymphocyte such as neutrophil and eosinophils, but also structural cells such as smooth muscle and endothelial cells (Murphy and Docherty, 1992; Yao *et al.*, 1997; Foda *et al.*, 1999). The immune cells secrete various MMPs such as MMP-1, 9,13 but not MMP-2. Recently, it has been reported that MMP-2 and MMP-9 are expressed from primary culture of bovine serous gland cell and human bronchial epithelial explant culture (Yao *et al.*, 1996; Tournier *et al.*, 1994). However, it has been unclear whether MMPs are expressed in tracheal epithelial goblet cells that play an import role in defense mechanism of airway by secretion of mucins. It is plausible to postulate that the expression of MMPs by airway epithelial cells may be actively involved in inflammatory disease by facilitating the infiltration of lymphocytes to airway epithelium and/or by affecting the production of reactive oxygen species. Further study will be necessary to clarify the role of MMP-2 from airway epithelial goblet cells in airway inflammatory diseases.

To more clearly investigate the activation of MMP-2 in secretory-differentiated HTSE cells, we performed zymography analysis of HTSE cell culture supernatants. The gelatin zymography of HTSE culture spent media revealed gelatinolytic bands with Mw of 72, 70 and 68 kDa (fig. 4). It has been reported that active form of MMP-2 has Mw of 68 kDa and intermediate or zymogen (pre-MMP-2) form has Mw of 70 and 72 kDa, respectively (Yao *et al.*, 1996; Tournier *et al.*, 1994; Ohuchi *et al.*, 1997; Birkedal-Hansen, 1995). Although 72 kDa band is inactive zymogen, it may be possible for this zymogen on the SDS-PAGE gel to be activated by autolysis process (Tournier et al., 1994) or by the incubation process with Triton X-100.

On culture day 4, only 72 kDa band was observed but the 68 kDa form was predominant at the later stage of culture, which indicates that zymogen activation was increased with the secretory differentiation of HTSE cells. The mechanism by which MMP-2 activation is achieved in HTSE cells is unclear yet. Whether proteolytic cleavage of proenzyme of MMP-2 by MT-1-MMP or other proteases is involved in the activation of MMP-2 is under active investigation in this laboratory.

In summary, it was identified that HTSE culture expresses MMP-2 (gelatinase A) along with the tissue inhibitors of met-

alloprotease-2 (TIMP-2), a known physiological inhibitor of MMP-2. In addition, it was observed that MMP-2 activation was increased along with the secretory differentiation of HTSE cells, which implies that it may play an important role in airway inflammatory process.

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