EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 29, No 1, 71-79, March 1997

Membrane activator of the 72 kDa type IV collagenase in malignant breast carcinoma patients: Expression of membrane-type 1-matrix metalloproteinase (MT1-MMP)

Kyoung-Ja Chai,[1,4] Hee-Jin Lee,[1] Su Jin Lee[3] and Kyoung-Sik Lee[2]

1 Department of Biochemistry and Molecular Biology and the Institute of Genetic Science, Yonsei University College of Medicine, Seoul 120-752, Korea
2 Department of General Surgery, Yonsei University College of Medicine Seoul, Korea
3 Department of Genetic Engineering, Hallym University, Chunchon, Korea
4 Corresponding author

Accepted 10 March 1997

Abbreviations: MT1-MMP, membrane-type-1 metalloproteinase; APMA, aminophenyl-mercuric acetate; RT-PCR, reverse transcriptase-polymerase chain reaction

Abstract

In this study, we determined proMMP-2 activating capacity of membrane extract prepared from the tissue of invasive ductal carcinoma of breast by zymogram gel analysis. We compared the effect of membrane extract on the activation of the latent type IV collagenases with that of the organic mercurial compound (eg, APMA)-induced self cleavage of the latent type IV collagenases. We also compared the expression levels of MT1-MMP between invasive carcinoma and normal tissue by Western blot, Northern blot and semi-quantitative RT-PCR analysis. Our result demonstrated that the specificity of processing by breast carcinoma membrane activator corresponds to the specificity of MT1-MMP, which clearly showed the conversion of 72-kDa proMMP-2 to the activated form while APMA processed both 72- and 92-kDa proMMPs to their activated forms. MT1-MMP protein and mRNA were expressed both in invasive carcinoma and normal tissues, and the expression levels in both tissues were comparable. Quantitative analysis of the mRNA level by RT-PCR revealed that the difference of MT1-MMP mRNA between carcinomas and normal tissues was not statistically significant on Wilcoxon signed-ranks test ($P > 0.05$). The results from the study on the expression of MT1-MMP gene suggest that the cellular activation of MMP-2 in breast tissue, requires additional effects in addition to up-regulation of MT1-MMP.

Keywords: invasive ductal carcinoma of breast, membrane-type-1 matrix metalloproteinase (MT1-MMP), proMMP-2 activator

Introduction

Breast cancer, like most solid tumors is usually the metastatic disease rather than the primary tumors, that causes death. However, the mechanism involved in breast cancer cell invasion and metastasis has not been known. The proteinases implicated in tumor invasion include components of the urokinase receptor/urokinase/plasminogen system and several members of the matrix metalloproteinases (MMPs) family (He *et al*., 1989; Vassalli and Pepper, 1994). MMPs, especially MMP-2 (gelatinase A/72 kDa-type IV collagenase) have been implicated in invasive and metastatic tumor progression due to its ability to degrade type IV collagen which is a major component of basement membrane, a primary barrier for tumor cell migration (Liotta *et al*., 1991; Stetler-Stevenson *et al*., 1993; Tryggvason *et al*., 1993). Recent studies reported that the expression and activation of the latent proMMP-2 were significantly enhanced in malignant breast tissues (Monteagudo *et al*., 1990; Davies *et al*., 1993; Brown *et al*., 1993a; Chai *et al*., 1994) and occurrence of active MMP-2 species is related to tumor grade or spreading of the breast carcinoma (Davies *et al*., 1993). Other studies also showed that increased levels of the activated form of MMP-2 correlated with the invasive phenotype to a great extent than the expression of MMP-2 alone (Azzam *et al*., 1993; Brown *et al*., 1993b). The studies suggest that proMMP-2 activator may be a central key substance triggering the breast cancer cell invasion. Non-physiological activation of both latent type IV collagenases (proMMP-2 and 92-kDa proMMP-9/gelatinase B) by organomercurial compound (aminophenylmercuric acetate, APMA) has been well characterized (Stetler-Stevenson *et al*., 1989) and various putative physiological activators such as serine proteinases are known for the activation of other members of MMPs including proMMP-9 (Okada *et al*., 1988, 1992; Nagase *et al*., 1992; Suzuki *et al*., 1992). However, the physiological activator for proMMP-2 has not been known despite the various efforts. It was only known to be related to the plasma membrane-associated components (Kleiner and Stetler-Stevenson., 1993; Monsky *et al*., 1993; Strongin *et al*., 1993). Recently, membrane-

bound MMP (MT1-MMP) cDNA was cloned (Sato *et al*., 1994) and it was shown that the recombinant MT1-MMP protein can act as a proMMP-2 specific activator in *in vitro* studies (Kinoshita *et al*., 1996). In this study, we investigated the implication of MT1-MMP for activation of latent MMP-2 in invasive ductal carcinoma of the breast to disclose a possible target molecule which is responsible for the invasion and metastasis of these cells.

Materials and Methods

Materials

Triton X-100 and 114, phenylmethylsulfonyl fluoride (PMSF), aprotinin, APMA, ethidium bromide, SDS, gelatin, gelatin-agarose, Brij 35 and sodium azide were from Sigma (St. Louis, MO). Bradford reagent, Coomassie blue R-250 and protein molecular weight marker came from Bio-Rad. Deoxynucleotide triphosphate (dNTP), Quick prep Micro mRNA purification kit and T7 DNA sequencing kit were purchased from Pharmacia (Uppsala, Sweden). Tri Reagent was obtained from MRC (Cincinnati, OH). ECL kit, Rediprime random primer labelling kit, Nylon membrane (Hybond N^+) and [α-^{32}P] dCTP (3000 Ci/mmol) were obtained from Amersham (Buckinghamshire, UK). SuperScript^{TMII} RNase H^- Reverse Transcriptase, RT buffer and dithiothreithol were purchased from GibcoBRL. *Taq* polymerase was obtained from Poscochem (Korea). Gene clean purification kit was obtained from Bio 101 (Vista, CA). PCR primers were from Korea Biotech (Korea). Anti-MT1-MMP mAb (113-15E7) and 1.2 kb human MT1-MMP cDNA were provided by Dr. H. Sato (Kanazawa University, Ishikawa, Japan). Human β-actin cDNA clone HHC 189 was from ATCC.

Biopsies (surgical specimens)

Twenty one invasive ductal carcinoma tissue of breast, 2 or 3 histological grade and sixteen adjacent paired normal tissues were obtained by surgical operation from patients at Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. The samples were rapidly frozen in liquid nitrogen and stored at -100˚C freezer. Tissues were diagnosed as invasive ductal carcinoma and normal tissues at the department of Pathology at Severance Hospital, Yonsei University College of Medicine, Seoul, Korea.

Fractionation of plasma membrane protein from the tissue samples

Ten cases were used for the preparation of plasma membrane protein extraction. One gram of pooled tissues of 5 cases from both tumor (T2) and normal (N) samples, and another 5 cases (T1) from only tumor tissues were homogenized in 4 ml of 8.5% sucrose, 50 mM NaCl and protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin). Plasma membrane fractions of tissue samples were prepared by the method of Strongin *et al*. (1993), using a discontinuous sucrose gradient centrifugation and Triton-X 114 extraction. The protein content in the extract was assayed by the Bradford method (1976), kept frozen at -80˚C until use. For another pooled tissues of 5 cases, paired normal tissues were not available.

Effects of the invasive ductal carcinoma membrane extract and APMA on the activation of exogenous type IV collagenases

Gelatin agarose-binding pool of serum-starved HT1080 fibrosarcoma cell supernatant was prepared by gelatin-agarose affinity column chromatography (Stetler-Stevenson *et al*., 1989) followed by dialysis against 50 mM Hepes, 1 mM $CaCl_2$, 0.05% Brij 35, PH 7.2, and was used as a source of exogenous latent type type IV collagenases. The fractions of plasma membrane protein isolated from invasive carcinoma tissues (T2) (0, 10, 20, and 50 μg) were mixed with 200 ng of latent type IV collagenases in 25 mM Hepes/KOH (PH 7.5) containing 0.1 mM $CaCl_2$ and incubated for 2 h at 37˚C. The reaction was terminated by the addition of sample buffer, and the samples were analyzed by gelatin zymography (Brown *et al*., 1990). In order to examine the effect of APMA on the processing of type IV collagenases, 1 mM APMA was mixed with 200 ng of latent type IV collagenases as described above, incubated for 2 h at 37˚C and the sample was subjected to gelatin zymography.

Western blot analysis of MT1-MMP protein

Fifty μg of plasma membrane protein extracted from the pooled invasive carcinoma and normal tissue was dissolved in 50 μl reducing SDS-PAGE sample buffer, subjected to SDS-PAGE in 12% polyacrylamide gels under reducing conditions. The separated proteins were transferred to a nylon membrane using a electrotransfer system (Hoefer Scientific Instrument, San franscisco, CA). The membrane was blocked with 10% non-fat dried milk in PBS for 2 h at room temperature and blotted with the anti-MT1-MMP mAb, 3 μg/ml in blocking solution at room temperature overnight. After extensive washing with TBS (20 mM Tris-HCl, PH 7.6, 137 mM NaCl), the membrane was reproved with anti-rabbit IgG conjugated with horse radish peroxidase (1:5000 in TBS) for 1 h at room temperature. Bands were localized with the enhanced chemiluminescence (ECL) detection system. Two separate preparations of carcinoma tissues membrane (T1 and T2) were loaded for comparison of the level of MT1-MMP protein in normal tissue membrane preparation (N).

Northern blot analysis of MT1-MMP mRNA

Total RNA was extracted from the frozen tissue samples

of 2 invasive ductal carcinoma of breast and paired normal tissues using Tri-Reagent according to the manufacturer's instructions. RNA yield and purity were checked by spectrophotometric determinations at 260 and 280 nm. Twenty μg of RNA was applied to 2.2 M formaldehyde/1% (w/v) agarose gel electrophoresis, transferred onto a nylon membrane (Hybond N^+) by means of capillary method (Sambrook *et al*., 1989) and exposed to UV by using UV cross linker (Hoefer, Scientific Instrument, CA). The blot was then hybridized with 1.2 kb human MT1-MMP cDNA labeled with [α-^{32}P] dCTP (3000 Ci/mmol) using rediprime random primer labelling system. The blots were washed to a final stringency of 0.1 × SSPE at 65˚C. Equalities in loadings were checked by reprobing the blot with 0.9 kb human actin cDNA (Adams *et al*., 1991). Hybridization signals were counted by Phospho-Image analyzer (Fuji, BAS 1000, Japan). Relative hybridization signal for invasive carcinoma was presented as ratio of the value between tumor and paired normal tissue (T/N).

RT-PCR analysis of MT1-MMP mRNA

RNA extraction: Poly(A^+) RNA was extracted from the frozen tissue samples of 9 invasive ductal carcinoma of breast and paired normal tissue samples using Quick Prep Micro mRNA purification kit according to the manufacturer's instructions. RNA yield and purity were checked by spectrophotometric determinations at 260 and 280 nm.

RT-PCR: The semi-quantitative assay to measure MT1-MMP mRNA levels using RT-PCR was that used by Onisto *et al*., (1995). First strand cDNA was synthesized from one μg of mRNA in a 40 μl reaction mixture containing 1 × RT reaction buffer, 1 mM dNTP, 0.3 μg of random hexamer, 10 mM dithiothreitol, and 200 units of Superscript II reverse transcriptase. The reaction mixture was incubated at 42˚C for 1 h and heated at 95˚C for 5 min to terminate the reverse transcription.

For PCR amplification, 1st strand cDNA was diluted in water to 1:5 for MT1-MMP or 1:50 for internal standard, β2-microglobulin (β2-MG). The linear range-finding experiments using serially diluted cDNA solutions were performed to determine the range of cDNA dilutions over which PCR amplification was linear for each species. The quantity of RNA indicated to amplify the cDNA in PCR refers to the generated cDNA derived from the quoted RNA amount. To amplify MT1-MMP cDNA, 3 doses of cDNA dilutions (20, 40 and 80 ng) which were within the linear range of amplification were used for PCR amplification. Aliquots of cDNA dilutions were subjected to 25 cycles of PCR in 50 μl of 1 × buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 1.25 unit of *Taq* polymerase and 0.5 μM of each sense and antisense primers. The reaction was carried out in a PCR system (Perkin Elmer, 2400, Branchburg, NJ). Each cycle contained a denaturation step at 94˚C for 1 min, primer annealing step at 61˚C for 1 min, extension step at 72˚C for 2 min and a final extension step at 72˚C for 7 min.

For the estimation of the efficiency of cDNA synthesis from each mRNA, β_2-microglobulin cDNA dilutions of 2, 4 and 8 ng which are within the linear range of amplification were amplified independendly. PCR amplification for β_2-microglobulin was carried out over 20 cycles with 2.5 mM $MgCl_2$ and primer annealing step at 57˚C using otherwise same reaction conditions as for MT1-MMP. The sequences for the PCR primers are shown in table 1 and these primers yield 632 bp product for MT1-MMP and 141 base pairs product for β_2-microglobulin. The sequences for RT-PCR products of MT1-MMP and β_2-microglobulin were verified by direct sequencing using T7 DNA Sequencing kit of PCR products (data not shown).

Detection and quantitative analysis of amplification products: For the detection and quantitative analysis of PCR products, Phospho-Image analysis after Southern blotting (Sambrook *et al*. 1989) were performed. For Southern blotting, PCR products were diluted to 1:10 and after gel separation of 2 μl aliquots, DNA was transferred on to a nylon membrane (Hybond N^+) by means of capillary method and cross linked by UV exposure. The membranes were then prehybridized and hybridized to alkali-denatured cDNA probes labeled with [α-^{32}P]dCTP (3000 Ci/mmol) the use of the rediprime random primer labelling kit. Specific DNA probes for MT1-MMP and β2-microglobulin were obtained by Gene Clean purification of PCR products. The blots were washed to a final stringency of 0.1 × SSPE at 65˚C. The membranes were autoradiographed by exposure to Hyper

Table 1. Primer sequences for RT-PCR

	MT1-MMP	β_2-microglobulin
Sense	CGGCCGGATGAGGGGACT GA (1648-1667 bp)	TTAGCTGTGCTCGCGCTACT CTCTC (901-925 bp)
Antisense	GCCTCCTGAGGTCAGAGTT C (2280-2299 bp)	GTCGGATTGATGAAACCCAG ACACA (1410-1434 bp)
Product size	632 bp	141 bp
Reference	Sato *et al*., 1994	Gussow *et al*., 1987

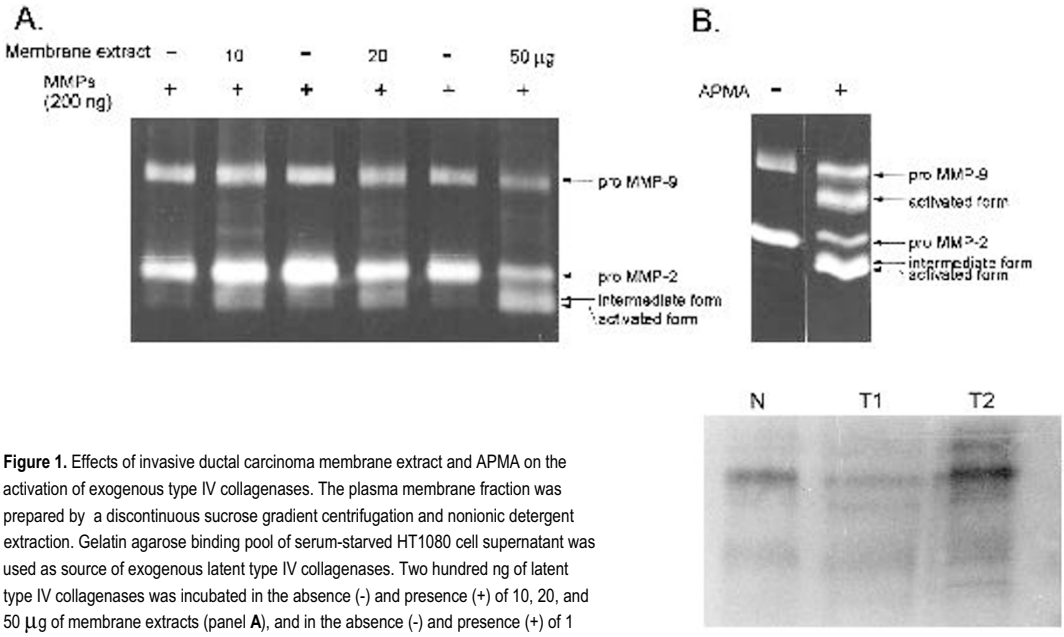

Figure 1. Effects of invasive ductal carcinoma membrane extract and APMA on the activation of exogenous type IV collagenases. The plasma membrane fraction was prepared by a discontinuous sucrose gradient centrifugation and nonionic detergent extraction. Gelatin agarose binding pool of serum-starved HT1080 cell supernatant was used as source of exogenous latent type IV collagenases. Two hundred ng of latent type IV collagenases was incubated in the absence (-) and presence (+) of 10, 20, and 50 μg of membrane extracts (panel **A**), and in the absence (-) and presence (+) of 1 mM APMA (panel **B**) for 2 h at 37˚C and subjected to zymogram gel electrophoresis. MMP-2 specific activation by membrane extract is shown in a dose dependent manner while APMA converts both 72-kDa MMP-2 and 92-kDa MMP-9 to their activated forms. MMPs denote exogenous latent type IV collagenases.

Figure 2. Western blot analysis of MT1-MMP protein in invasive ductal carcinoma and normal breast tissue using anti-MT1-MMP monoclonal antibody. Fifty μg of tumor (T) and normal (N) plasma membrane extracts were subjected to 12 % SDS-PAGE under reducing conditions. The blot was incubated with anti-MT1-MMP monoclonal antibody (3 μg/ml, 113-15E7) and visualized by ECL Western blotting detection system. Two separate preparations of tumor membrane extracts (T1 and T2) were run for comparison with normal tissue.

film for 2 h at -70˚C.

Quantitative analysis of each band was done by counting the incorporated radioactivity using Phospho-Image analyzer. For the linear range finding-experiments, the amounts of PCR products (measured as incorporated radioactivity) were plotted against input cDNA dilutions. For the quantitation of MT1-MMP mRNA, the amounts of PCR products amplified from all 3 points cDNA-dilutions for each sample were subjected to Phospho-Image analysis and normalized on the basis of the corresponding signals derived from 3 doses of cDNA-dilutions of the control gene, β_2-microglobulin. In each case, relative hybridization signal for invasive carcinoma was presented as ratio of the value between tumor and paired normal tissue (T/N). Each T/N ratio is presented as the mean and relative standard deviation (SD) from 3 doses of cDNA dilutions.

Statistical data analysis

The Wilcoxon signed-ranks test was used to compare the levels of MT1-MMP mRNA examined by RT-PCR between carcinomas and the counterpart normal tissues.

Results

Invasive ductal carcinoma tissue of human breast contains specific membrane activator of 72 kDa proMMP-2 which is likely to be MT1-MMP

Gelatin zymography (Figure 1) showed that 200 ng of gelatin-agarose binding pool of serum-starved HT-1080 cell supernatant containing both 72- and 92-kDa latent type IV collagenases was processed to the corresponding intermediates (64 and 84 kDa, respectively) and activated forms (62 and 82 kDa respectively) by incubation with 1 mM APMA for 2 h (Figure 1B). However, the membrane extracts of carcinoma tissues (T2) clearly could not convert 92-kDa proenzyme to the lower molecular weight products but only 72-kDa proMMP-2 was processed to intermediate and 62-kDa activated form in a dose-dependent manner (Figure 1A). These results demonstrate that plasma membrane fraction of invasive ductal carcinoma of breast contains specific activator of proMMP-2. This activator is likely to be MT1-MMP since the specificity of processing by the membrane activator corresponds to the known specificity of MT1-MMP (Sato *et al*., 1994; Kinoshita *et al*., 1996).

MT1-MMP protein is expressed in both carcinoma and normal tissues

Western blot analysis of plasma membrane preparations isolated from pooled samples of normal and

invasive carcinoma tissues showed that 63-kDa MT1-MMP protein presents in the membrane fractions of both tissues (Figure 2). Two separate preparations of carcinoma tissue membrane (T1 and T2) showed high expression of MT1-MMP protein but the protein bands shown by carcinoma membrane preparations were not significantly higher than that in normal tissue membrane (N).

MT1-MMP mRNA is expressed in both carcinoma and normal tissues

Northern blot analysis: Two cases evaluated by Northern blot analysis revealed that 4.5 kb MT1-MMP mRNA was expressed both in paired normal tissue and invasive ductal carcinoma of the breast (Figure 3). This is in agreement with the result from Western blot analysis showing the appearance of 63-kDa MT1-MMP protein in both carcinoma and paired normal tissue (Figure 2). However, in case 1, actin-corrected MT1-MMP mRNA level for tumor was much stronger than that for the paired normal tissue, whereas in case 2, a higher signal was seen in paired normal tissue than in tumor tissue. The T/N ratio for case 1 and 2 were 6 and 0.25, respectively.

RT-PCR analysis: Nine cases of invasive carcinoma and paired normal tissues were analyzed for MT1-MMP mRNA expression by semi-quantitative RT-PCR. Figure 4 shows the result from initial linear range-finding experiments for MT1-MMP and β_2-microglobulin. For MT1-MMP, 3 cDNA dilutions, 20, 40 and 80 ng are found to be within the linear range of amplification and for β_2-microglobulin, 2, 4 and 8 ng are found to be best within the linear range. Before carrying out RT, contamination of genomic DNA was ruled out by performing the PCR using β_2-microglobulin primers, with mRNA preparations as PCR template (Data not shown). Fig. 5 presents 3 representative results, showing autoradiograms of 642 bp, MT1-MMP and 141 bp, β_2-microglobulin. In all cases, the chosen primers for MT1-MMP and β_2-microglobulin generated specific single amplicons with no additional bands and amplicon products increased as the input cDNA increased. Figure 6 shows the result of Phospho

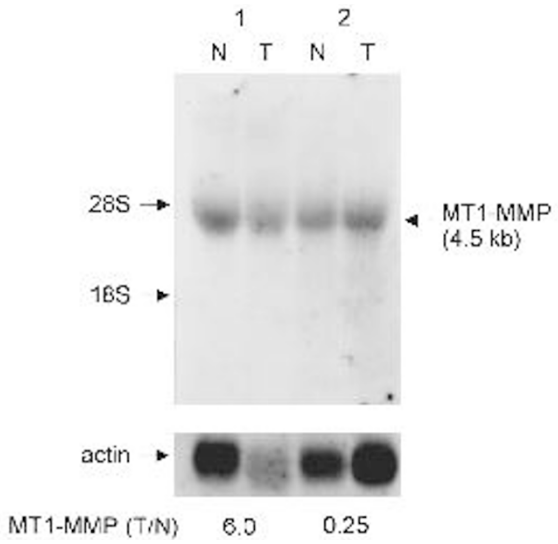

Figure 3. Northern blot analysis of MT1-MMP mRNA in invasive ductal carcinoma and paired normal breast tissue. Twenty μg of total RNA was applied to 2.2 M formaldehyde/1% (W/V) agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized with 1.2 kb human MT1-MMP cDNA labeled with [α-^{32}P] dCTP. Equalities in loadings were checked by reprobing the blot with 0.9 kb human actin cDNA. The incorporated radioactivities were counted by Phospho-Image analyzer and the ratio of the value between the tumor and paired normal tissue (T/N) is indicated at the bottom of the figure. Patient numbers are indicated as 1 and 2.

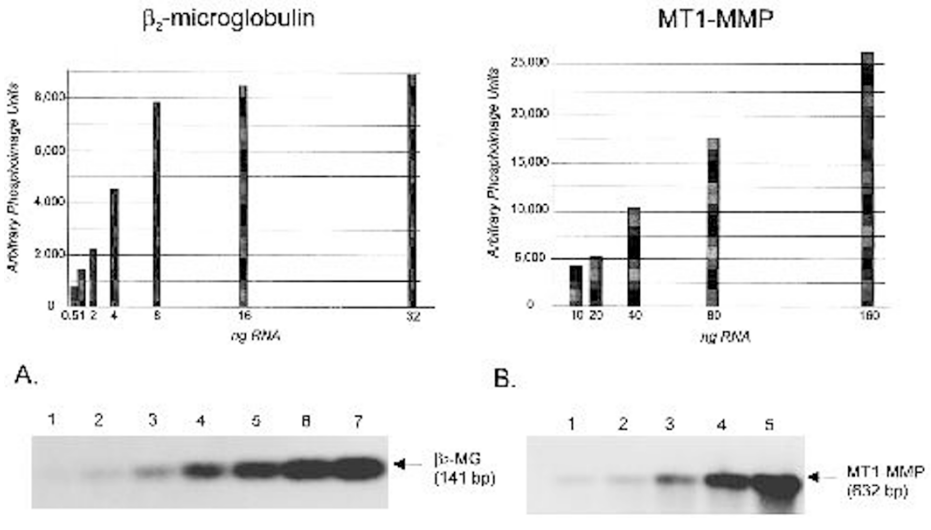

Figure 4. Autoradiogram (**A, B**) showing PCR products of serially diluted cDNA from a sample of invasive ductal carcinoma of breast. Lane 1-7 in panel **A** show autoradiogram of β_2-microglobulin amplicons (141 bp) produced by RT-PCR on 0.5-32 ng RNA. Lane 1-5 in panel **B** show autoradiogram of MT1-MMP amplicons (632 bp) produced by RT-PCR on 10-160 ng RNA. Upper panels show graphs of corresponding Phospho-Image analysis values. Linear range of amplification only was chosen to use for subsequent quantitative analyses.

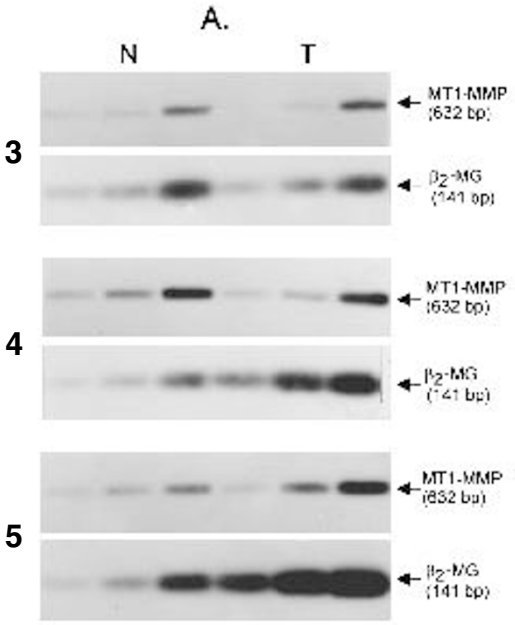

Figure 5. Examples of RT-PCR analysis of MT1-MMP mRNA. Autoradiograms of 632 bp and 141 bp RT-PCR products of MT1-MMP and β_2-microglobulin RNA are shown. Three fixed cDNA dilutions used for the amplification of MT1-MMP and β_2-microglobulin correspond to 20, 40 and 80 ng and 2, 4 and 8 ng respectively. T and N denote tumor and paired normal tissues respectively. Patient numbers are indicated as 3–5.

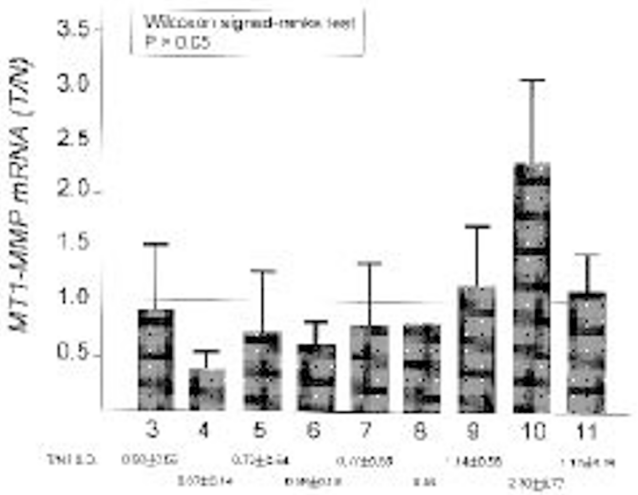

Figure 6. MT1-MMP mRNA expression in invasive ductal carcinoma tissues. Incorporated radioactivities of the amplicons produced by RT-PCR were counted by Phospho-Image analyzer and shown as the ratio of the value between the tumor and paired normal tissue (T/N). Means of T/N ratio and SD are indicated at the bottom of the figure. Patient numbers are indicated as 3–11. The difference between the tumor and normal tissues is statistically insignificant on the Wilcoxon signed-ranks test ($P > 0.05$).

Image analysis for MT1-MMP mRNA in 9 cases. Each bar presents mean of T/N and SD, for each case. The difference of MT1-MMP mRNA between 9 carcinomas and paired normal tissues was insignificant on Wilcoxon signed-ranks test ($P > 0.05$) though case 9 shows T/N ratio considerably higher than the remaining cases. The value of SD, for case 8 is not available.

Discussion

A significant statistical association between the expression level of activated MMP-2 in tumor tissue and tumor spread or grade has been reported (Brown *et al*., 1993b), particularly in breast cancer tissue (Azzam *et al*., 1993; Davies *et al*., 1993), suggesting proMMP-2 activator to be an important substance triggering breast cancer cell invasion and metastasis. Our interest is to investigate a hypothesis that physiological activator for proMMP-2 in invasive ductal carcinoma of human breast is recently cloned MT1-MMP protein (Sato *et al*., 1994) and therefore MT1-MMP expression is elevated in tumor tissue compared from the normal tissue.

It is well known that activation of MMP-2 proenzyme occurs in association with the plasma membrane (Kleiner and Stetler-Stevenson., 1993; Monsky *et al*., 1993; Strongin *et al*., 1993). We therefore isolated a fraction of plasma membrane protein from invasive ductal carcinoma tissues and examined proMMP-2 activating function of this fraction with the use of gelatin agarose binding pool of HT 1080 cell supernatant as exogenous latent type IV collagenases. Gelatin zymography demonstrated that membrane fraction of ductal carcinoma has potential of 72 kDa proMMP-2 specific processing in a dose-dependent manner and has no effect on proMMP-9 (Figure 1A), whereas APMA (1 mM) processed both proMMP-2 and proMMP-9 to their corresponding intermediates and activated forms (Figure 1B) (Stetler-Stevenson *et al*., 1989; Goldberg *et al*., 1992). These results indicate that plasma membrane fraction of invasive ductal carcinoma tissue of breast contains specific activator for proMMP-2. This activator is likely to be MT1-MMP since the specificity of processing by the membrane activator corresponds to that of recently reported MT1-MMP protein which has a function of proMMP-2 specific activator *in vitro* (Sato *et al*., 1994; Kinoshita *et al*., 1996). We did not determine whether cleavage is solely due to the function of MT1-MMP and not by other contaminating proteases in the membrane preparation. However, *in vitro* study by Kinoshita *et al*.,

(1996) using recombinant MT1-MMP-GST fusion protein, strongly suggested that soluble MT1-MMP activity without involvement of other proteases was sufficient for proMMP-2 specific cleavage.

In order to prove MT1-MMP-associated activation of proMMP-2 in invasive ductal carcinoma tissue, it is reasonable to examine whether tumor tissues express more MT1-MMP at transcriptional or translational level than normal tissues. In lung (Tokuraku *et al*., 1995) and gastric carcinoma (Nomura *et al*., 1995), the overexpression of MT1-MMP is well corrrelated with the activation of proMMP-2. Northern blot analysis for MT1-MMP mRNA in these tissues showed that the level of MT1-MMP expression in carcinoma tissue was significantly and consistently higher than in the surrounding normal control tissues and therefore, it was suggested that proMMP-2 activation by MT1-MMP in these tissues is a key step for the extracellular matrix degradation in cancer cell invasion and metastasis. Similar data in breast cancer tissues are not available though malignant breast cancer is a tumor together with other tumors including lung (Brown *et al*., 1993b), and stomach and colon (Yamagata *et al*., 1991) in which activated MMP-2 has been detected in surgically excised specimens but is low or undetectable in normal tissues (Brown *et al*., 1993a; Davies *et al*., 1993). Our result from Western blot analysis showed that 63-kDa MT1-MMP protein presents in membrane fractions of both carcinoma and normal tissues and interestingly, protein bands appearing in 2 separately prepared carcinoma membrane fractions were not significantly stronger than that in normal group (Figure 2). We further examined the expression of MT1-MMP mRNA in carcinoma and paired normal tissue by Northern blot and semi-quantitative RT-PCR analysis. Data analyzed by Northern blot analysis are available only for a small number of samples. However, the result seems to indicate that MT1-MMP transcripts are expressed at variable levels among the tumor tissues. In case 1, the level of MT1-MMP mRNA in tumor tissue was 6 fold greater than that in counterpart normal tissue whereas in case 2, paired normal tissue express 4 fold increased amount of MT1-MMP mRNA than tumor tissue (Figure 3). The mechanism causing the difference of MT1-MMP expression observed in 2 sample cases can not be explained in this study. The level could be variable depending on the phenotypic heterogeneity of breast carcinoma.The fact that in one case out of two cases, counterpart normal tissue expresses more MT1-MMP mRNA than carcinoma tissue supports the result from Western blot analysis showing constitutive level of MT1-MMP protein in normal group. In agreement with the result of Western blot analysis, semi-quantitative RT-PCR analysis for MT1-MMP mRNA in tissue samples of 9 cases (Figure 5 and 6) showed that the levels of MT1-MMP mRNA expression in paired normal tissues are comparable to those in carcinoma tissues in all cases except one case of which T/N ratio showed significantly higher than the remaining cases. The difference of MT1-MMP mRNA between 9 carcinoma and paired normal tissues is statistically insignificant on Wilcoxon signed-ranks test ($P > 0.05$). Thus, our results of Western blot , Northern blot and semi-quantitative RT-PCR analysis are consistent with the suggestion that MT1-MMP gene expression occurs in adjacent normal tissue at levels not lower than carcinoma tissues although certain tumors can express more MT1-MMP gene than normal tissues. Okada et al. (1995) reported that by *in situ* hybridization, MT1-MMP gene expression in human tumor including colon, breast and head and neck occurred in stroma of tumor and it was thought that the expressions of MT1-MMP transcript in any normal tissues at a distance from cancerous tissues are lower than corresponding carcinoma tissues. Although the number of patients studied in the present work is relatively low, our results on the expression of MT1-MMP gene are contradicting to the suggestion described above. The important question then arises of why adjacent normal tissues of breast carcinoma produce low or undetectable levels of activated MMP-2 despite the presence of a constitutive or comparable level of MT1-MMP expression. Similarly, an *in vitro* study by Yu *et al*. (1995) using invasive breast carcinoma cell line MDA-MB-231, reported that constitutive level of MT1-MMP mRNA was expressed in unstimulated cells despite a lack of MMP-2 activation. They suggested that MMP-2 activation occurring in Con A-stimulated MDA-MB-231 cell line involves additional effects in addition to up-regulation of MT1-MMP expression. Our results can be best explained in the same context, that the mechanism of pro-MMP-2 activation in breast tissue involves a complicated process rather than just consequence of up-regulation of MT1-MMP expression. In fact, Strongin *et al*. (1995) demonstrated in *in vitro* system that MT1-MMP binds TIMP-2 and that the resulting complex acts as a receptor for the C-terminus of gelatinase A, following which activation of MMP-2 occurs. Thus, the ability to form a heterotrimeric complex with MT1-MMP on cell membrane by unknown mechanism may be equally as important as up-regulation of MT1-MMP expression for efficient activation of proMMP-2. We also can not rule out the possibility that there is another activator for MMP-2 proenzyme in addition to MT1-MMP in breast tissue.

Acknowledgement

This work was supported by Genetic Engineering Fund, Ministry of Education, Korea for 1995

References

Adams, M. D., Kelley, J. M., Gocayne, J. D. and Dubnick, M. (1991) Complementary DNA sequencing: Expressed sequence taqs and human genome project. *Science* 252: 1651-1656

Azzam, H. S., Arand, G., Lippman, M. E. and Thompson, E. W. (1993) Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. *J. Natl. Cancer. Inst.* 85: 1758-1764

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-264

Brown, P. D., Levy, A. T., Marguiles, L. M. K., Liotta, L. A. and Stetler-Stevenson, W. G. (1990) Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumourigenic cell lines. *Cancer Res.* 50: 6184-6191

Brown, P. D., Bloxidge, R. E., Anderson, E. and Howell, A. (1993a) Expression of activated gelatinase in human invasive breast carcinoma. *Clin. Exp. Metastasis* 11: 183-189

Brown, P. D., Bloxidge, R. E., Stuart, N. S. A., Gatter, K. C. and Carmichael, J. (1993b) Association between expression of activated 72-kilodalton gelatinase and tumor spread in non-small-cell lung carcinoma. *J. Nat. Cancer Inst.* 85: 574-578

Chai, K. J., Lee, S. J. and Lee, K. S. (1994) Type IV collagenases in normal and malignant breast tissues: No correlation with c-erbB2/neu gene amplification. *Korean J. Biochem.* 26: 221-228

Davies, B., Miles, D. W., Happerfield, L. C., Naylor, .M. S, Bobrow, L. G., Rubens, R. D. and Balkwill, F. R. (1993) Activity of type IV collage-nases in benign and malignant breast disease. *Br. J. Cancer.* 67: 1126-1131

Goldberg, G. I., Collier, I. E., Eisen, A. Z., Grant, G. A., Marmer, B. L. and Wilhelm, S. M. (1992) Mosaic structure of the secreted ECM metalloproteinases and interaction of the type IV collagenase with inhibitors. *Matrix Suppl.* 1: 25-30

Gussow, D., Rein, R., Ginjaar, I., Hochstenbach, F., Seemann, G., Kottman, A. and Ploegh, H. L. (1987) The human β_2-microglobulin gene primary structure and definition of the transcriptional unit. *J. Immunol.* 139: 3132-3138

He, C., Wilhelm, S. M., Pentland, A. P., Marmer, B. L., Grant, G. A., Eisen, A. Z. and Goldberg, G. I. (1989) Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. *Proc. Natl. Acad. Sci. USA* 86: 2632-2636

Kinoshita, T., Sato, H., Takino, T., Itoh, M., Akizawa, T. and Seiki, M. (1996) Processing of a precursor of 72-kilodalton type IV collagenase/gelatinase A by a recombinant membrane-type matrix metalloproteinase. *Cancer Res.* 56: 2535-2538

Kleiner, D. E .J. and Stetler-Stevenson, W.G. (1993) Structural biochemistry and activation of matrix metalloproteinase. *Curr. Opin. Cell Biol.* 5: 891-897

Liotta, L. A., Steeg, P. S. and Stetler-Stevenson, W. G. (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 64: 327-336

Monsky, W. L., Kelly, T., Lin, C.-Y., Yeh, Y., Stetler-Stevenson, W. G., Mueller, S. G. and Chen, W. T. (1993) Binding and location of MW 72000 matrix metalloproteinase at cell surface invadopodia. *Cancer Res.* 53: 3159-3164

Monteagudo, C., Merino, M. J., San-Juam, J., Liotta, L. A. and Stetler-Stevenson, W. G. (1990) Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. *Am. J. Pathol.* 136: 585-592

Nagase, H., Suzuki, K., Morodomi, T., Englhild, J. J. and Salvesen, G. (1992) Activation mechanisms of the precursors of matrix metalloprotei-nases 1, 2 and 3. *Matrix suppl.* 1: 237-244

Nomura, H., Sato, H., Seiki, M., Mai, M. and Okada, Y. (1995) Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. *Cancer Res.* 55: 3263-3266

Okada, Y., Harris, E.D. Jr. and Nagase, H. (1988) The precursor of a metalloendopeptidase from human rheumatoid synovial fibroblasts. *Biochem. J.* 254: 731-741

Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K. and Hayakawa, T. (1992) Matrix metalloproteinase 9 (92-kDa gelatinase type IV collagenase) from HT1080 human fibrosarcoma cells. *J. Biol. Chem.* 267: 21712-21719

Okada, A., Bellocq, J.-P., Rouyer, N., Chenard, M.-P., Rio, M.-C., Chambon, P. and Basset P. (1995) Membrane-type matrix metallopro-teinase (MT-MMP) gene is expressed in stromal cells of human colon, breast and head and neck carcinomas. *Proc. Natl. Acad. USA* 92: 2730-2734

Onisto, M., Riccio, M. P., Scannapieco, P., Caenazzo, C., Griggio, L., Spina, M., Stetler-Stevenson, W. G. and Garbisa, S. (1995) Gelatinase A/Timp-2 imbalance in lymph-node-positive breast carcinomas as measured by RT-PCR. *Int. J. Cancer* 63: 621-626

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular cloning*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. and Seiki, M. (1994) A matrix metalloproteinase expressed on the surface of invasive tumor cells. *Nature* 370: 61-65

Stetler-Stevenson, W. G., Krutzsch, H. C., Wachner, M. P., Marguiles, I. M. K. and Liotta, L. A. (1989) The activation of human type IV collagena-se proenzyme. Sequence identification of the major conversion product following organomercurial activation. *J. Biol. Chem.* 264: 1353-1356

Stetler-Stevenson, W. G., Aznavoorian, S. and Liotta, L. A. (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.* 9: 541-573

Strongin, A. Y., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1993) Plasma membrane dependent activation of the 72 kDa type IV collagenase is prevented by complex formation with TIMP-2. *J. Biol. Chem.* 268: 14033-14039

Strongin, A. Y., Collier, I. E., Bannikov, G. A., Marmer, B. L., Grant, G. A. and Goldberg, G. I. (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase: Isolation of the activated form of the membrane metalloprotease. *J. Biol. Chem.* 270: 5331-5338

Suzuki, K., Enghild, J. J., Morodomi, T., Salvesen, G. and Nagase, H. (1992) Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). *Biochemistry* 29: 10261-10270

Tokuraku, M., Sato, H., Murakami, S., Okada, Y., Watanabe, Y. and Seiki, M. (1995) Activation of the precursor of gelatinase A/72 kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. *Int. J. Cancer* 64: 355-359

Tryggvason, K. (1993) Type IV collagenase in invasive tumors. *Breast Cancer Res. Treat.* 24: 209-218

Yamagata, S., Yoshii, Y., Suh, J. G., Tanaka, R. and Shimizu, S. (1991) Occurrence of an active form of gelatinase in human gastric and colorectal carcinoma tissues. *Cancer Lett.* 59: 51-55

Yu, M., Sato, H., Seiki, M. and Thompson, E. W. (1995) Complex regulation of membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation by concanavalin in MDA-MB-231 human breast cancer cells. *Cancer Res.* 55: 3272-3277

Vassalli, J. D. and Pepper, M. S. (1994) Tumor biology. Membrane proteases in focus. *Nature* 370: 14-15