

## Shedding of Membrane Type 1 Matrix Metalloproteinase in a Human Breast Carcinoma Cell Line

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Membrane type 1 matrix metalloproteinase (MT1-MMP) with a transmembrane domain is a new member of the MMP gene family and is expressed on the cell surfaces of many carcinoma cells to activate the zymogen of MMP-2 (gelatinase A). We have previously reported that MT1-MMP is released into culture media in a complex form with tissue inhibitor of metalloproteinases 2 (TIMP-2) from a human breast carcinoma cell line, MDA-MB-231, treated with concanavalin A (Con A). In the present study, we further studied the release mechanism of MT1-MMP. Immunoblot analysis indicated that the amounts of MT1-MMP in culture media increase with the time of exposure and the concentration of Con A, and those in cell lysates conversely decrease in a similar way. Time- and dose-dependent release of MT1-MMP into the media was confirmed by a sandwich enzyme immunoassay specific to MT1-MMP. The molecular weight of the immunoreactive MT1-MMP in the media was  $M_r$  56,000, which was 4,000- $M_r$  smaller than that in the cell lysates. Northern blot analysis demonstrated that the mRNA expression level of MT1-MMP is about 3-fold enhanced after a 24 h-exposure to Con A and this is maintained up to 72-h exposure. The release of MT1-MMP from the Con A-treated cells was inhibited by metalloproteinase inhibitors such as EDTA and *o*-phenanthroline, but not by MMP inhibitors including TIMP-1, TIMP-2 and BB94 or other proteinase inhibitors of serine, cysteine and aspartic proteinases. During the Con A treatment of the cells, cell viability decreased time- and dose-dependently and dead cells reacted positively in the TdT-mediated dUTP Nick-End Labeling (TUNEL) method. Con A-treated MDA cells showed apoptotic morphology when stained with Hoechst dye and hematoxylin and eosin. DNA ladder formation was detected by electrophoresis of the DNA from Con A-treated MDA cells. These results suggest that MT1-MMP release from Con A-treated cells is due to shedding mediated by metalloproteinase(s) other than MMPs, and is associated with apoptosis.

Key words: Membrane type matrix metalloproteinase — Shedding — Invasion and metastasis — Apoptosis — Concanavalin A

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases consisting of at least 18 different members. They are classified into 5 subgroups according to their structures and substrate specificities: interstitial collagenases, gelatinases, stromelysins, membrane type-MMPs (MT-MMPs) and others.<sup>1,2</sup> Since they are synthesized as inactive enzymes (proMMPs), their activation is a rate-limiting step for the catalytic function.<sup>3</sup> Among the MMPs, active MMP-2 (gelatinase A) is considered to be closely associated with invasion and metastasis of cancer cells.<sup>1</sup> Thus, the activation mechanism of the zymogen of MMP-2 (proMMP-2) was intensively studied. Differently

from other MMPs, proMMP-2 is activated on the cell surface,<sup>3</sup> and we have identified a new MMP with a transmembrane domain, i.e. membrane type 1-MMP (MT1-MMP), which can activate proMMP-2 on the cell membranes.<sup>4</sup> The expression of MT1-MMP in lung,<sup>5</sup> breast,<sup>6</sup> stomach<sup>7</sup> and thyroid<sup>8</sup> carcinomas correlates well with the activation of proMMP-2, suggesting that MT1-MMP is a key activator of proMMP-2 in the carcinoma tissues. Besides the activator function of MT1-MMP, the MMP can also digest extracellular matrix (ECM) macromolecules including interstitial collagens.<sup>9</sup> Thus, MT1-MMP is thought to play an important role in cancer invasion and metastasis by degrading pericellular ECM through both activation of proMMP-2 and direct ECM cleavage.<sup>9</sup>

As with MMPs, MT1-MMP expression is controlled by many factors in various cells: concanavalin A (Con A),

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phorbol myristate acetate, basic fibroblast growth factor and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) up-regulate the expression, while glucocorticoids down-regulate it.<sup>10-15</sup> However, another important regulation mechanism of the MT1-MMP activity may be shedding from the membranes, as reported for other membrane-bound proteins.<sup>16-20</sup> Recently, we have demonstrated that MT1-MMP is released into culture media from a human breast carcinoma cell line, MDA-MB-231 (MDA) cells, after treatment with Con A.<sup>9,21</sup> The released MT1-MMP was found to be complexed with tissue inhibitor of metalloproteinases 2 (TIMP-2), and had no enzymic activity without dissociation of the inhibitor.<sup>9,21</sup> Thus, the rate of turnover of MT1-MMP by being released from the cells may affect the pericellular ECM degradation in cancer tissues, suggesting the possibility that acceleration of MT1-MMP release contributes to the suppression of cancer invasion and metastasis. Although previous studies<sup>9,21</sup> showed that Con A treatment induces the release of MT1-MMP, the mechanism involved remains unknown.

In the present study, we examined the release mechanism of MT1-MMP from MDA cells after Con A treatment. The data suggest that the release involves proteolysis on the cell membranes mediated by metalloproteinase(s) other than MMPs, and that apoptosis is associated with the shedding.

## MATERIALS AND METHODS

**Materials** Materials were obtained as follows: bovine serum albumin (BSA), Con A, ethylenediaminetetraacetic acid (EDTA), glycine, guanidine thiocyanate, 2-mercaptoethanol, *o*-phenylenediamine, sodium dodecyl sulfate (SDS), sodium lauroyl sarcosinate, trichloroacetic acid and trypan blue from Wako Chem. (Tokyo); actinomycin D, diamminobenzidine tetrahydrochloride, epoxysuccinyl leucylamido (E64), Hoechst 33342 dye, leupeptin, pepstatin A, *o*-phenanthroline, phenylmethanesulfonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane and Triton X-100 from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum, lactalbumin hydrolysate and trypsin from Gibco Laboratories (Grand Island, NY); Dulbecco's modified Eagle's medium (DMEM) and Hanks' solution from Nissui Pharm. (Tokyo); nitrocellulose filter for immunoblotting from Atto Co. (Tokyo); biotinylated horse anti-(mouse IgG) IgG from Funakoshi Co. (Tokyo); hydrogen peroxide solution from Mitsubishi Gas Chemical Co. (Tokyo); BB94 from British Biotech (Oxford, England). Human recombinant TIMP-1 and TIMP-2 were purified as previously described,<sup>22</sup> and provided by Fuji Chemical Industries, Ltd. (Takaoka); <sup>32</sup>P-nucleotides from Amersham Co. (Tokyo); acrylamide from Daiichi Pure Chemicals Co. (Tokyo), proteinase K, RNase A from Boehringer Mannheim Co. (Tokyo). MDA-MB-231 (MDA) human

breast carcinoma cell line was a gift from Dr. M. Toi (Komagome Hospital, Tokyo).

**Cell cultures** MDA cells were maintained in monolayer cultures in DMEM with 10% (v/v) fetal bovine serum. After having been rinsed with Hanks' solution, they were treated with Con A (0, 5, 10, 20, 50, 100, 200  $\mu$ g/ml) in serum-free DMEM containing 0.2% lactalbumin hydrolysate for up to 72 h. For inhibitor studies, the cells were first cultured in the presence of 10  $\mu$ g/ml Con A for 24 h and then treated for 72 h with 200  $\mu$ g/ml Con A in the medium containing 1 mM EDTA, 200  $\mu$ M *o*-phenanthroline, 1 or 5  $\mu$ g/ml TIMP-1, 1 or 5  $\mu$ g/ml TIMP-2, 10 or 100  $\mu$ M BB94, 1 mM PMSF, 100  $\mu$ M E64, 100  $\mu$ M leupeptin or 100  $\mu$ M pepstatin A. The culture media were harvested, and the cells were lysed in SDS-sample buffer containing 3.8% 2-mercaptoethanol (500  $\mu$ l/dish) and sonicated. They were stored at -20°C until used.

**Immunoblotting** The conditioned media (1,000  $\mu$ l) concentrated by precipitation with 3.3% trichloroacetic acid and cell lysates of MDA cells dissolved in SDS-reduction buffer (approximately  $3.6 \times 10^5$  cells/lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% total acrylamide) under reduction and electrotransferred onto nitrocellulose filters. After blocking nonspecific binding of IgG with 3% bovine serum albumin, the filters were reacted for 12 h at room temperature with a monoclonal antibody specific to the catalytic domain of human MT1-MMP (clone 114-1F2).<sup>4</sup> The antibody bound to the antigen was complexed with biotinylated horse anti-(mouse IgG) IgG and the protein bands were visualized by the avidin-biotin-peroxidase complex method.<sup>7</sup> The densities of the immunoreactive bands were measured by computer-assisted image analysis according to the method of Davies *et al.*<sup>23</sup>

**Sandwich enzyme immunoassay for MT1-MMP** Concentrations of MT1-MMP secreted in the culture media after treatment with Con A (0, 10, 50 and 200  $\mu$ g/ml) for 24, 48 or 72 h were determined by a sandwich enzyme immunoassay (EIA). The EIA system was developed by utilizing a pair of monoclonal antibodies (clones 114-1F2 and 113-15E7) which specifically recognize different epitopes of MT1-MMP.<sup>4,24</sup> The assay was carried out as previously described.<sup>25</sup> Microtitration plates were first coated with IgG of clone 113-15E7 (5  $\mu$ g/well). A 50  $\mu$ l aliquot of culture medium which had been incubated with 10 mM EDTA for 24 h at 4°C was mixed with the same volume of 1  $\mu$ g/ml IgG of clone 114-1F2-horseradish peroxidase conjugate in 10 mM sodium phosphate buffer, pH 7.0, 0.1 M NaCl, 1% BSA, 10%  $\alpha$ -Minimum Essential Medium (Gibco BRL, Gaithersburg, MD), transferred to each previously coated microplate well, and allowed to stand for 16 h at 4°C without shaking. After rinsing, the reaction was carried out by incubation at room temperature for 60 min with 100  $\mu$ l of 0.5 mg/ml *o*-phenylenedi-

amine in 0.1 M citric acid-sodium phosphate buffer, pH 5.0 containing 0.02% hydrogen peroxide. The reaction was stopped by addition of 100  $\mu$ l of 2 N sulfuric acid and the absorbance at 492 nm was measured by a microplate reader.

**Northern blotting** MDA cells were cultured in the presence and absence of 10  $\mu$ g/ml Con A in the serum-free DMEM containing 0.2% lactalbumin hydrolysate for 0, 2, 4, 8, 12, 24 and 72 h, and then total RNA was isolated from the cells by the acid guanidium-phenol-chloroform method.<sup>4)</sup> The RNA samples (10  $\mu$ g/lane) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde and transferred onto Hybond N<sup>+</sup> membranes (Amersham International, Tokyo). The membranes were hybridized with <sup>32</sup>P-labeled probes for MT1-MMP (3.5-kb cDNA fragment corresponding to nucleotides 1647–2889)<sup>4)</sup> and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously.<sup>4)</sup> The blotted membranes were scanned by a Bioimage Analyzer BAS 1000 (Fuji Film Co., Tokyo)

**Measurement of cell viability** MDA cells were treated with 0, 5, 10, 20, 50, 100 and 200  $\mu$ g/ml Con A in the serum-free DMEM containing 0.2% lactalbumin hydrolysate for 24, 48 or 72 h. Since the cells could not be released from the dishes by trypsin after the Con A treatment, the viability of the cells was determined by counting the cells on the dishes stained with 0.3% trypan blue. Each measurement was carried out in triplicate.

**TdT-mediated dUTP Nick-End Labeling (TUNEL)** Apoptotic cells were detected by TUNEL according to the kit manufacturer's instructions (Promega Co., Tokyo). Briefly, MDA cells were cultured on Lab-Tek slides (Miles Laboratories, Naperville, IL) and treated with Con A (0, 10, 50 and 200  $\mu$ g/ml) for 24, 48 and 72 h in the serum-free DMEM. They were then fixed with 4% formaldehyde for 25 min at 4°C, permeabilized with 0.2% Triton X-100 for 5 min at 4°C and allowed to react with TdT enzyme and fluorescein-12-dUTP for 60 min at 37°C. The reaction was terminated by immersing the slides in standard saline citrate for 15 min at room temperature. Photographs were taken on an Olympus Vanox X AH2-FL microscope fitted with standard fluorescein isothiocyanate filters on Kodak Ektachrome 400 films upgraded to 1,600 ASA.

**Detection of apoptotic morphology** MDA cells were cultured and treated with 0, 10, 50 and 200  $\mu$ g/ml Con A for 24, 48 and 72 h as described above. The cells were fixed with methanol, then stained with hematoxylin and eosin to observe the cell morphology under a light microscope. The nuclei were also stained with 10  $\mu$ g/ml Hoechst 33342 dye to detect apoptotic bodies and nuclear condensations under an Olympus Vanox X AH2-FL microscope.

**Detection of ladder formation** After treatment with 0,

10, 50 and 200  $\mu$ g/ml Con A for 24 h, MDA cells (approximately  $5 \times 10^6$ ) were collected and lysed in 20  $\mu$ l of 50 mM Tris-HCl buffer, pH 7.8 containing 10 mM EDTA and 0.5% sodium lauroyl sarcosinate. The lysates were incubated for 30 min at 50°C with 500  $\mu$ g/ml RNase A, and then digested with 1 mg/ml proteinase K for 60 min at 50°C. Electrophoresis of prepared DNA ( $2.3 \times 10^6$  cells/lane) was carried out on 2% agarose gels and bands were stained with ethidium bromide. The DNA fragments were detected by UV transillumination, and photographed. As a control, MDA cells were treated with 0.1  $\mu$ g/ml actinomycin D for 24 h and the DNA extracted from the cells was analyzed as described above.

**RESULTS**

**Release of MT1-MMP from Con A-treated MDA cells**

To monitor the amounts of MT1-MMP in culture media (CM) and cell lysates (CL), immunoblotting was performed with samples of CM and CL prepared from MDA

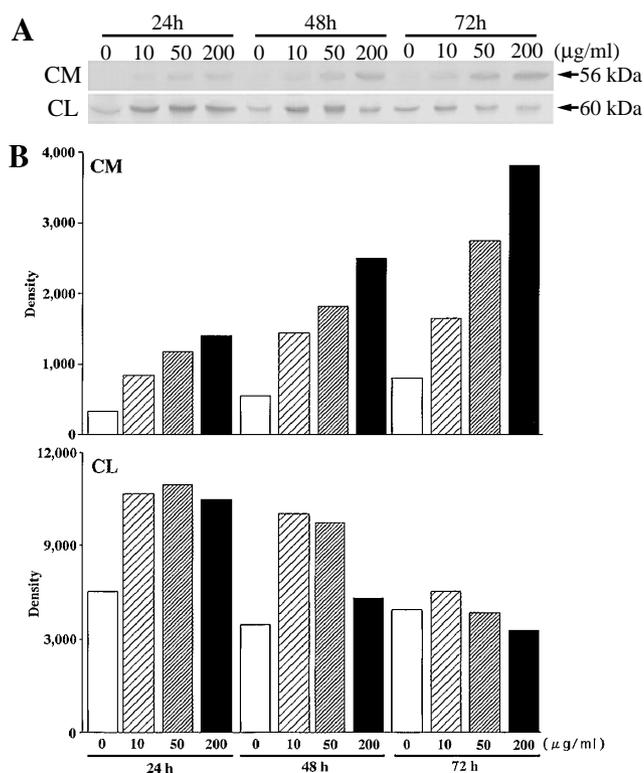


Fig. 1. Immunoblotting of MT1-MMP in CM and CL after Con A treatment. CM and CL were prepared from MDA cells stimulated with 0, 10, 50 and 200  $\mu$ g/ml Con A for 24, 48 and 72 h. The immunoblotting for MT1-MMP (A) and its densitometric analysis (B) were performed as described in "Materials and Methods."

cells treated with various concentrations of Con A (0, 10, 50 and 200  $\mu\text{g/ml}$ ) for 24, 48 or 72 h. As shown in Fig. 1A, the density of the MT1-MMP immunoreactive band in

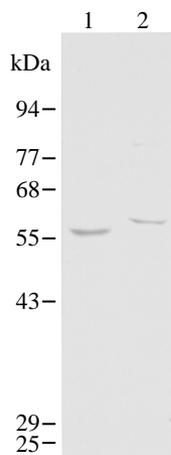


Fig. 2. Molecular weight of the MT1-MMP species in CM and CL. CM (lane 1) and CL (lane 2) were prepared from MDA cells stimulated with 200  $\mu\text{g/ml}$  Con A for 72 h and 10  $\mu\text{g/ml}$  Con A for 24 h, respectively. They were subjected to immunoblotting as described in "Materials and Methods."

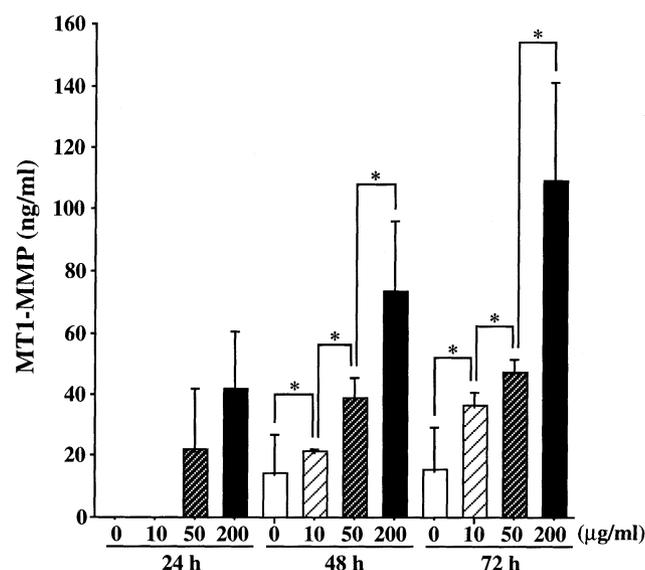


Fig. 3. Amounts of MT1-MMP in CM assayed by the EIA system. The CM were prepared by culturing the MDA cells treated with 0, 10, 50 and 200  $\mu\text{g/ml}$  Con A for 24, 48 and 72 h. MT1-MMP in the CM was measured by the EIA system as described in "Materials and Methods." The experiments were performed 3 times and values are mean  $\pm$  SD. \*  $P < 0.05$  (Mann-Whitney  $U$  test).

the CM appeared to increase time- and concentration-dependently. Conversely, the density in the CL seemed to decrease dependently on the time of exposure and the concentration of Con A (Fig. 1A). Densitometric analysis of immunoreactive bands demonstrated that MT1-MMP released into the CM increases with incubation time and concentration used, and MT1-MMP in the CL decreases in a similar way (Fig. 1B), showing the time- and concentration-dependent release of MT1-MMP.

The molecular weight of MT1-MMP recognized in the CM with the antibody was  $M_r$  56,000, and that in the CL was  $M_r$  60,000 (Fig. 2). As we have previously reported,<sup>9,21</sup> these two MT1-MMP species correspond to the  $\text{NH}_2$ - and  $\text{COOH}$ -terminally truncated form of MT1-MMP and the  $\text{NH}_2$ -terminal-deleted active species, respectively.

The amounts of MT1-MMP in the CM were further measured by the EIA system for MT1-MMP. Although

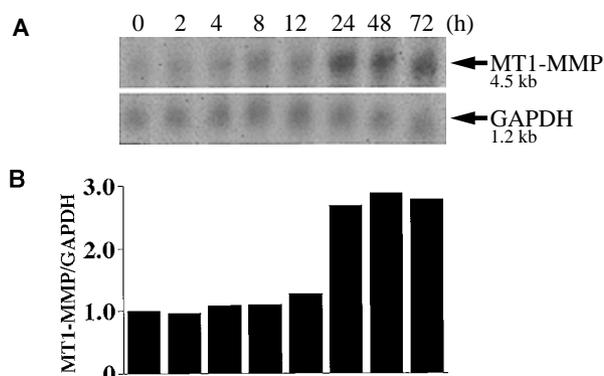


Fig. 4. Expression of MT1-MMP mRNA in MDA cells treated with Con A. Total RNA was isolated from MDA cells treated with 10  $\mu\text{g/ml}$  Con A at the time indicated. A, RNA (10  $\mu\text{g}$ ) was subjected to northern blotting using the probes for MT1-MMP and GAPDH. B, The intensity of the MT1-MMP band was normalized with respect to that of GAPDH.

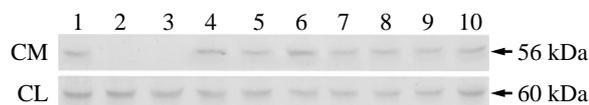


Fig. 5. Effect of proteinase inhibitors on the release of MT1-MMP. MDA cells were first treated with 10  $\mu\text{g/ml}$  Con A for 24 h to enhance the expression of MT1-MMP, and then stimulated with 200  $\mu\text{g/ml}$  Con A for 72 h in the presence of 1 mM EDTA (lane 2), 200  $\mu\text{M}$  *o*-phenanthroline (lane 3), 1  $\mu\text{g/ml}$  TIMP-1 (lane 4), 1  $\mu\text{g/ml}$  TIMP-2 (lane 5), 10  $\mu\text{M}$  BB94 (lane 6), 1 mM PMSF (lane 7), 100  $\mu\text{M}$  E64 (lane 8), 100  $\mu\text{M}$  leupeptin (lane 9) and 100  $\mu\text{M}$  pepstatin A (lane 10), or in the absence of inhibitors (lane 1). Both CM and CL were subjected to immunoblotting for MT1-MMP as described in "Materials and Methods."

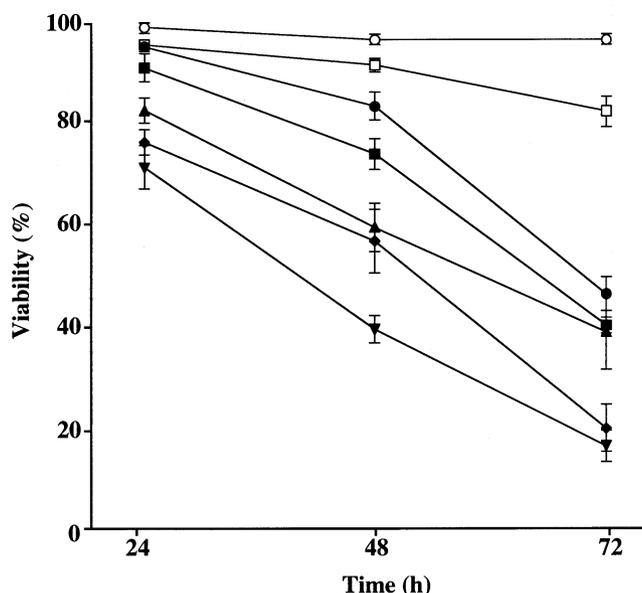


Fig. 6. Viability of Con A-treated MDA cells. MDA cells were treated with 0 (○), 5 (□), 10 (●), 20 (■), 50 (▲), 100 (◆) and 200 µg/ml (▼) Con A for 24, 48 and 72 h as described in "Materials and Methods." The viability of the cells was measured by the trypan blue dye exclusion method. Values are the means of three independent determinations. Bars, SD.

MT1-MMP in the samples prepared from the cells treated with 0 and 10 µg/ml Con A for 24 h was undetectable, other samples showed measurable amounts of MT1-MMP in the CM (Fig. 3). MT1-MMP in the CM from the cells treated with Con A for 48 and 72 h significantly increased dose- and time-dependently (Fig. 3).

**Northern blotting for MT1-MMP** The time-course of expression of MT1-MMP mRNA in MDA cells treated with 10 µg/ml Con A was analyzed by northern blotting. As shown in Fig. 4A, the signal of the MT1-MMP transcript was enhanced after a 24-h incubation with Con A. The ratios of MT1-MMP to GAPDH obtained by computer-assisted densitometric analysis demonstrated that the level of MT1-MMP mRNA expression after a 24-h exposure is approximately 3-fold enhanced compared with that in the untreated cells and remains constant for at least 72 h (Fig. 4B).

**Inhibition of MT1-MMP release by proteinase inhibitors** To test the effects of various inhibitors on the release of MT1-MMP in the CM, immunoblot analysis for MT1-MMP was performed with the samples of CM and CL obtained from Con A-treated MDA cells in the presence of inhibitors of metallo-, serine, cysteine and aspartic proteinases. As shown in Fig. 5, EDTA and *o*-phenanthroline, metalloproteinase inhibitors, completely inhibited the secretion of MT1-MMP in the CM. There was no inhibi-

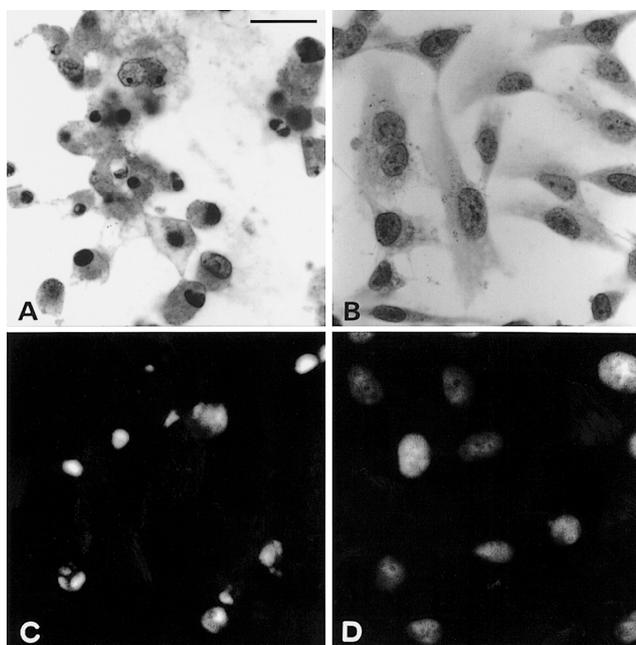


Fig. 7. Detection of apoptotic bodies and nuclear condensations in MDA cells treated with (A and C) or without (B and D) 50 µg/ml Con A for 72 h. Cells were stained with hematoxylin and eosin (A and B) or Hoechst 33342 dye (C and D) as described in "Materials and Methods." After the staining, the cells were observed under a light microscope (A and B) or a fluorescence microscope with a WU cube (C and D). Bar, 20 µm.

tion of MT1-MMP synthesis with the inhibitors, since MT1-MMP was detectable in the CL (Fig. 5). Interestingly, metalloproteinase inhibitors specific to MMPs including TIMP-1, TIMP-2 and BB94 (a synthetic hydroxamate MMP inhibitor) showed no inhibition of the release (Fig. 5). Similar data were obtained even if the cells were treated with higher concentrations of the inhibitors (5 µg/ml TIMP-1 and TIMP-2 or 100 µM BB94) (data not shown). No inhibitor effects were observed with other inhibitors of serine, cysteine and aspartic proteinases (Fig. 5).

**Viability of MDA cells after Con A treatment** The time-course of viability of Con A-treated MDA cells was measured by observation of the cells stained with trypan blue. Although the cells survived well in the serum-free medium, Con A treatment decreased the cell viability time- and dose-dependently (Fig. 6). The decrease in the cell viability was especially remarkable after a 48-h treatment and ~80% of the cells were killed by 100 and 200 µg/ml Con A after 72 h. The viability of the cells treated with 200 µg/ml Con A was also examined in the presence of proteinase inhibitors including 1 mM EDTA and 200 µM *o*-phenanthroline, but no effect of the inhibitors on the viability was observed (data not shown).

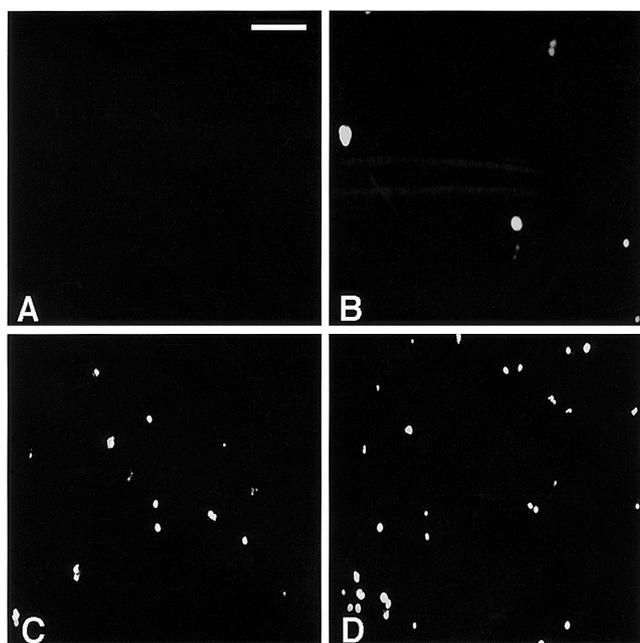


Fig. 8. Detection of apoptotic cells by TUNEL after treatment with Con A. MDA cells were treated with 0 (A), 10 (B), 50 (C) and 200 (D)  $\mu\text{g}/\text{ml}$  Con A for 24 h, and the nuclei were labeled as described in "Materials and Methods." After the reaction, the cells were observed under a fluorescence microscope with a NIBA cube. Bar, 100  $\mu\text{m}$ .

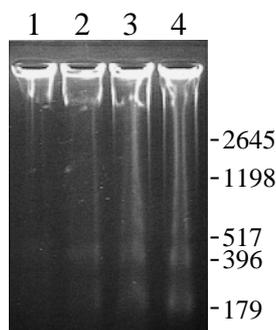


Fig. 9. Electrophoresis of DNA from Con A-treated MDA cells. Total DNA was isolated from MDA cells treated with 0, 10, 50 and 200  $\mu\text{g}/\text{ml}$  Con A (lanes 1–4, respectively) and electrophoresed on a 2% agarose gel as described in "Materials and Methods."

**Apoptosis in Con A-treated MDA cells** We could detect many cells with apoptotic bodies by observation of the Con A-treated cells stained with hematoxylin and eosin under a light microscope (Fig. 7, A and B). Apoptotic bodies were identified by staining with Hoechst dye and

the results are shown in Fig. 7, C and D. Since these data suggest that apoptosis of the cells was induced by the Con A treatment, we performed TUNEL of the Con A-treated cells. The TUNEL signal increased dependently on the concentration of Con A (Fig. 8). The values of the ratio (%) of TUNEL-positive cells to total cells treated with 0, 10, 50 and 200  $\mu\text{g}/\text{ml}$  Con A for 24 h were  $1.2 \pm 1.0$ ,  $4.6 \pm 0.3$ ,  $7.6 \pm 1.7$ , and  $21.0 \pm 4.5\%$  (mean  $\pm$  SD,  $n=3$ ), respectively. The ratios also increased with time of treatment (data not shown). The omission of TdT in the reaction gave a negative result, and the signal was detectable in nuclei of all the cells pretreated with DNase I (data not shown).

In order to examine DNA ladder formation, the DNA was extracted from MDA cells treated with Con A and analyzed by electrophoresis. As shown in Fig. 9, the Con A-treated MDA cells exhibited fragmentation of DNA into oligonucleosome-sized units characteristic of apoptotic cell death.

## DISCUSSION

The present study has demonstrated that MT1-MMP is released from MDA cells after treatment with Con A. Since the molecular weight of the species present in CM is 4,000- $M_r$  smaller than that in CL, the soluble species of MT1-MMP in the CM is thought to be generated either by direct processing by the cells or extracellular proteolysis of the leaked enzyme. However, the finding that the CM contained only the processed form favors the former possibility. The finding that time-dependent release of MT1-MMP in CM is associated with a corresponding decrease in the amount of MT1-MMP in CL further supports the hypothesis. In addition, northern blot analysis indicated that there is no change in the expression level of MT1-MMP mRNA up to 72 h after Con A-treatment, suggesting constant levels of production and release of MT1-MMP from the cells. These data suggest that the release of MT1-MMP in CM is a shedding process mediated by proteolysis, but not simply release due to cell bursting.

Many membrane proteins are known to be shed after proteolysis by cell membrane-associated proteinases, named secretases or sheddases.<sup>26–28)</sup> These sheddases belong to classes of metalloproteinases or serine proteinases as judged from inhibitor studies.<sup>26)</sup> In the present studies, MT1-MMP release was inhibited by EDTA and *o*-phenanthroline, but not by other inhibitors of serine, cysteine and aspartic proteinases, indicating the involvement of metalloproteinase(s) in the processing. However, since the shedding was not affected by TIMPs or BB94, the sheddase is considered to be different from MMPs including MT-MMPs. Sheddases for a wide variety of membrane proteins, including the TNF- $\alpha$  ligand/receptor superfamily,<sup>16, 29, 30)</sup>  $\beta$ -amyloid precursor protein,<sup>17)</sup> angio-

tensin converting enzyme,<sup>18)</sup> transforming growth factor- $\alpha$ <sup>19, 31)</sup> and interleukin 6 receptor<sup>20)</sup> are reported to be metalloproteinases. Among them, only TNF- $\alpha$  converting enzyme has been cloned and shown to be a member of the ADAM (a disintegrin and metalloproteinase) family.<sup>32, 33)</sup> Previous studies have demonstrated that the activity of TNF- $\alpha$  converting enzyme is inhibited by synthetic hydroxamic acid-based metalloproteinase inhibitors.<sup>16, 29)</sup> However, since the release of MT1-MMP is not affected at all by BB94, the secretase for MT1-MMP is thought to be different from TNF- $\alpha$  converting enzyme.

The viability of MDA cells decreased remarkably after the Con A treatment. Interestingly, this treatment induced apoptosis of the cells. This was demonstrated by the presence of apoptotic morphology of the cells and their TUNEL reactivity. DNA ladder formation was also demonstrated in these cells. A similar apoptosis-inducing effect of Con A has been reported with fibroblasts in culture,<sup>34)</sup> although the study did not show ladder formation.<sup>34)</sup> The expression of various genes, such as *c-fos*, *c-jun* and *c-myc*, is dramatically up-regulated during apoptosis,<sup>35)</sup> and an increase of intracellular  $Ca^{2+}$  concentration is required to activate DNases to cause DNA fragmentation into oligonucleosome size in apoptosis.<sup>36-38)</sup> Since Con A treatment of Jurkat T-cells is known to increase c-Fos and c-Jun protein levels and intracellular  $Ca^{2+}$  concentration,<sup>39)</sup> such a condition generated by Con A treatment may be responsible for the induction of apoptosis in the MDA cells.

Our preliminary studies (Harayama *et al.*, unpublished data) showed that among phorbol myristate acetate, TNF- $\alpha$ , interleukin 1 and Con A, all of which stimulate cells to up-regulate the *MT1-MMP* gene,<sup>13, 15)</sup> only Con A treatment caused shedding of MT1-MMP from MDA cells. Thus, the shedding appears to be specific to Con A treatment. It is possible that gene expression of a sheddase for MT1-MMP is simply induced by the Con A treatment. However, another possibility is that Con A may cause clustering of MT1-MMP molecules close to the sheddase to facilitate the cleavage, since Con A is reported to activate proMMP-2 by concentrating proMMP-2 and MT1-MMP molecules on the cell membranes.<sup>40)</sup> Although MT1-MMP release was associated with apoptosis, the relationship between the two phenomena is not clear. However, the MT1-MMP shedding does not seem to be a direct cause of apoptosis, because blocking of release of MT1-MMP with EDTA or *o*-phenanthroline failed to stop the apoptosis. Since prevention of apoptosis was difficult once MDA cells were treated with Con A, the effects of apoptosis on the MT1-MMP shedding could not be determined in

our experiments. Recent studies have demonstrated that membrane proteins such as aminopeptidase N (CD13), CD33 and  $\beta$  amyloid protein are shed from the cells undergoing apoptosis.<sup>41, 42)</sup> Thus, we speculate that the MT1-MMP release results from apoptosis induced by Con A. However, further work is necessary to elucidate the precise mechanisms of the MT1-MMP shedding during apoptosis and to identify the factors that induce the shedding under pathophysiological conditions.

MT1-MMP shed from Con A-treated MDA cells is a complex form with TIMP-2 as we have previously reported,<sup>9, 21)</sup> and the species is not active in the complex form. However, since neutrophil elastase can re-activate MT1-MMP/TIMP-2 complex by degrading TIMP-2 in the complex (Ohuchi *et al.*, unpublished data), released MT1-MMP may become involved in the degradation of the ECM macromolecules after reactivation by other proteinases under certain conditions. Another possibility is that the release of MT1-MMP/TIMP-2 complex from the cell membranes is a regulation mechanism of proMMP-2 activation by MT1-MMP, since our recent studies have demonstrated that a trimolecular complex of proMMP-2/TIMP-2/MT1-MMP is essential to the pericellular activation of proMMP-2 by MT1-MMP.<sup>43)</sup> Enhanced or decreased release of MT1-MMP/TIMP-2 is expected to affect the efficiency of the activation. It is, therefore, possible that control of the MT1-MMP sheddase may contribute to the regulation of the actions of MMP-2 as well as MT1-MMP. Previous studies have demonstrated that MT1-MMP is highly expressed in many human carcinoma tissues such as lung,<sup>5)</sup> breast,<sup>6)</sup> stomach<sup>7)</sup> and thyroid<sup>8)</sup> carcinomas. In these carcinomas MT1-MMP may be shed to the extracellular milieu and be drained into the body fluids such as blood and lymph. Thus, measurement of the levels of soluble MT1-MMP in the body fluids may be a useful method to monitor human carcinomas.

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## REFERENCES

- 1) Stetler-Stevenson, W. A. G., Aznavoorian, S. and Liotta, L. A. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.*, **9**, 541–573 (1993).
- 2) Okada, Y. Proteinases and matrix degradation. In “Textbook of Rheumatology,” 6th Ed., ed. W. N. Kelley, E. D. Harris, Jr., S. Ruddy and C. B. Sledge (1999). W. B. Saunders Co., Philadelphia, in press.
- 3) Nagase, H. Activation mechanisms of matrix metalloproteinases. *Biol. Chem.*, **378**, 151–160 (1997).
- 4) Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. and Seiki, M. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature*, **370**, 61–65 (1994).
- 5) Tokuraku, M., Sato, H., Murakami, S., Okada, Y., Watanabe, Y. and Seiki, M. 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 (1995).
- 6) Ueno, H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M. and Okada, Y. Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in human invasive breast carcinomas. *Cancer Res.*, **57**, 2055–2060 (1997).
- 7) Nomura, H., Sato, H., Seiki, M., Mai, M. and Okada, Y. Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. *Cancer Res.*, **55**, 3263–3266 (1995).
- 8) Nakamura, H., Ueno, H., Yamashita, K., Shimada, T., Yamamoto, E., Noguchi, M., Fujimoto, N., Sato, H., Seiki, M. and Okada, Y. Enhanced production and activation of progelatinase A mediated membrane-type 1 matrix metalloproteinase in human papillary thyroid carcinomas. *Cancer Res.*, **59**, 467–473 (1999).
- 9) Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.*, **272**, 2446–2451 (1997).
- 10) Atkinson, S. J., Crabbe, T., Cowell, S., Ward, R. V., Butler, M. J., Sato, H., Seiki, M., Reynolds, J. J. and Murphy, G. Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membrane. *J. Biol. Chem.*, **270**, 30479–30485 (1995).
- 11) Lewalle, J. M., Munaut, C., Pichot, B., Cataldo, D., Baramova, E. and Foidart, J. M. Plasma membrane-dependent activation of gelatinase A in human vascular endothelial cells. *J. Cell. Physiol.*, **165**, 475–483 (1995).
- 12) Lohi, J. and Keski-Oja, J. Calcium ionophores decrease pericellular gelatinolytic activity via inhibition of 92-kDa gelatinase expression and decrease of 72-kDa gelatinase activation. *J. Biol. Chem.*, **270**, 17602–17609 (1995).
- 13) Lohi, J., Lehti, K., Westermarck, J., Kähäri, V. M. and Keski-Oja, J. Regulation of membrane-type matrix metalloproteinase-1 expression by growth factors and phorbol 12-myristate 13-acetate. *Eur. J. Biochem.*, **239**, 239–247 (1996).
- 14) Okada, A., Bellocq, J. P., Rouyer, N., Chenars, M. P., Rio, M. C., Chanbon, P. and Basset, P. Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. *Proc. Natl. Acad. Sci. USA*, **92**, 2730–2734 (1995).
- 15) Imai, K., Ohta, S., Matsumoto, T., Fujimoto, N., Sato, H., Seiki, M. and Okada, Y. Expression of membrane-type 1 matrix metalloproteinase and activation of progelatinase A in human osteoarthritic cartilage. *Am. J. Pathol.*, **151**, 245–256 (1997).
- 16) Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J. and Black, R. A. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature*, **370**, 218–220 (1994).
- 17) Roberts, S. B., Ripellino, J. A., Ingalls, K. M., Robakis, N. K. and Felsenstein, K. M. Non-amyloidogenic cleavage of the  $\beta$ -amyloid precursor protein by integral membrane metalloendopeptidase. *J. Biol. Chem.*, **269**, 3111–3116 (1994).
- 18) Ramchandran, R. and Sen, I. Cleavage processing of angiotensin-converting enzyme by a membrane-associated metalloprotease. *Biochemistry*, **34**, 12645–12652 (1995).
- 19) Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S. and Massague, J. Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. *J. Biol. Chem.*, **271**, 11376–11382 (1996).
- 20) Mullberg, J., Durie, F. H., Otten-Evans, C., Alderson, M. R., Rose-John, S., Cosman, D., Black, R. A. and Mohler, K. M. A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the p60 TNF receptor. *J. Immunol.*, **155**, 5198–5205 (1995).
- 21) Imai, K., Ohuchi, E., Aoki, T., Nomura, H., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. Membrane-type matrix metalloproteinase 1 is a gelatinolytic enzyme and is secreted in a complex with tissue inhibitor of metalloproteinases 2. *Cancer Res.*, **56**, 2707–2710 (1996).
- 22) Hayakawa, T., Yamashita, K., Ohuchi, E. and Shinagawa, A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J. Cell Sci.*, **107**, 2373–2379 (1994).
- 23) Davies, B., Miles, D. W., Happerfield, D. C., Naylor, M. S., Bobrow, L. G., Rubens, R. D. and Baldwill, F. R. Activity of type IV collagenases in benign and malignant breast disease. *Br. J. Cancer*, **67**, 1126–1131 (1993).
- 24) Tanaka, M., Sato, H., Takino, T., Iwata, K., Inoue, M. and Seiki, M. Identification of mouse MT2-MMP gene from a lung cDNA library and identification of its product. *FEBS Lett.*, **402**, 219–222 (1997).
- 25) Ohuchi, E., Azumano, I., Yoshida, S., Iwata, K. and Okada,

- Y. A one-step sandwich enzyme immunoassay for human matrix metalloproteinase 7 (matrilysin) using monoclonal antibodies. *Clin. Chim. Acta*, **244**, 181–198 (1996).
- 26) Hooper, N. M., Karran, E. H. and Turner, A. J. Membrane protein secretases. *Biochem. J.*, **321**, 265–279 (1997).
- 27) Ehlers, M. R. W. and Riordan, J. F. Membrane proteins with soluble counterparts: role of proteolysis in the release of transmembrane proteins. *Biochemistry*, **30**, 10065–10074 (1991).
- 28) Rose-John, S. and Heinrich, P. C. Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem. J.*, **300**, 281–290 (1994).
- 29) Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L., Leber, T. M., Mangan, M., Miller, K., Nayee, P., Owen, K., Patel, S., Thomas, W., Wells, G., Wood, L. M. and Woolley, K. Processing of tumour necrosis factor- $\alpha$  precursor by metalloproteinases. *Nature*, **370**, 555–557 (1994).
- 30) McGeehan, K. G. M., Becherer, J. D., Bast, R. C., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S., McElroy, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Sekut, L., Truesdale, A., Verghese, M., Warner, J. and Ways, J. P. Regulation of tumour necrosis factor- $\alpha$  processing by a metalloproteinase inhibitor. *Nature*, **370**, 558–561 (1994).
- 31) Pandiella, A. and Massague, J. Cleavage of the membrane precursor for transforming growth factor  $\alpha$  is a regulated process. *Proc. Natl. Acad. Sci. USA*, **88**, 1726–1730 (1991).
- 32) Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, C. J., March, C. J. and Cerretti, D. P. A metalloproteinase disintegrin that releases tumour-necrosis factor- $\alpha$  from cells. *Nature*, **385**, 729–733 (1997).
- 33) Moss, M. L., Jin, S.-L. C., Milla, M. E., Burkhart, W., Carter, H. L., Chen, Wen-Ji, Clay, W. C., Didsbury, J. R., Hassler, D., Hoffman, C. R., Kost, T. A., Lambert, M. H., Leesnitzer, M. A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J., Warner, J., Willard, D. and Becherer, J. D. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor- $\alpha$ . *Nature*, **385**, 733–736 (1997).
- 34) Kulkarni, G. V. and McCulloch, C. A. G. Concanavalin A induced apoptosis in fibroblasts: the role of cell surface carbohydrates in lectin mediated cytotoxicity. *J. Cell. Physiol.*, **165**, 119–133 (1995).
- 35) Schwartz, L. M. and Osborne, B. A. Programmed cell death, apoptosis and killer genes. *Immunol. Today*, **14**, 582–590 (1993).
- 36) McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H. and Orrenius, S. Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic  $Ca^{2+}$  concentration. *Arch. Biochem. Biophys.*, **269**, 365–370 (1989).
- 37) Bellomo, G., Perotti, M., Taddei, F., Mirabelli, F., Finardi, G., Nicotera, P. and Orrenius, S. Tumour necrosis factor  $\alpha$  induces apoptosis in mammary adenocarcinoma cells by an increase in intranuclear free  $Ca^{2+}$  concentration and DNA fragmentation. *Cancer Res.*, **52**, 1342–1346 (1992).
- 38) Kelley, L. L., Koury, M. J. and Bondurant, M. C. Regulation of programmed cell death in erythroid progenitor cells by erythropoietin: effects of calcium and of protein and RNA synthesis. *J. Cell. Physiol.*, **151**, 487–496 (1992).
- 39) Kvanta, A., Kontny, E., Jondal, M., Okret, S. and Fredholm, B. B. Mitogen stimulation of T-cells increases c-Fos and c-Jun protein levels, AP-1 binding and AP-1 transcriptional activity. *Cell. Signal.*, **4**, 275–286 (1992).
- 40) Yu, M., Sato, H., Seiki, M. and Thompson, E. W. Complex regulation of membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation by concanavalin A in MDA-MB-231 human breast cancer cells. *Cancer Res.*, **55**, 3272–3277 (1995).
- 41) Brown, S. B., Kluck, R. M. and Ellem, K. A. O. Loss and shedding of surface markers from the leukemic myeloid monocytic line THP-1 induced to undergo apoptosis. *J. Cell. Biochem.*, **60**, 246–259 (1996).
- 42) Galli, C., Piccini, A., Ciotti, M. T., Castellani, L., Calissano, P., Zaccheo, D. and Tabaton, M. Increased amyloidogenic secretion in cerebellar granule cells undergoing apoptosis. *Proc. Natl. Acad. Sci. USA*, **95**, 1247–1252 (1998).
- 43) Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y. and Seiki, M. TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. *J. Biol. Chem.*, **273**, 16098–16103 (1998).