

# Limited Processing of Pro-Matrix Metalloprotease-2 (Gelatinase A) Overexpressed by Transfection in PC-3 Human Prostate Tumor Cells: Association With Restricted Cell Surface Localization of Membrane-Type Matrix Metalloproteinase-1

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**ABSTRACT:** The expression and activation of matrix metalloproteinases (MMPs) by tumor cells is correlated with progression to invasive and metastatic status. The purpose of this study was to examine the role of increased MMP-2 (gelatinase A) expression in prostate cancer progression utilizing human prostate PC-3 cancer cells that overexpress MMP-2 using gene transfection. PC-3 cells were transfected with pCR-3 vector only and pCR-3 MMP-2 plasmids employing the LipofectAMINE method, and stable transfectants were selected with G418. The expression of MMP-2, tissue inhibitor of metalloproteinase-2 (TIMP-2), and membrane-type MMP 1 (MT1-MMP) in PC-3 parental and transfected cells under serum-free conditions was determined by zymography, immunoblotting, immunofluorescent microscopy, Northern blotting, and/or reverse transcriptase-polymerase chain reaction (RT-PCR). MMP-2 transfected cells produced primarily the proenzyme form of MMP-2; the parental and vector control transfected PC-3 cells did not express any MMP-2 that was detectable by the methods we employed. Treatment of PC-3 MMP-2 transfected cells with Concanavalin A (Con A), in contrast

to HT-1080 cells, processed only a small amount of the secreted 72-kd proenzyme to a 62-kd intermediate and a cell-associated 59-kd active form. The low level of secreted pro-MMP-2 processing induced by Con A was inhibited by serine protease inhibitors and was unaffected by cyclic adenosine monophosphate (cAMP). Immunoblotting showed that these cells produced abundant TIMP-2 and lower amounts of MT1-MMP in comparison with Con A-responding HT-1080 cells. HT-1080 cells respond to Con A by translocating MT1-MMP from intracellular localization sites to the plasma membrane, an effect not observed in PC-3 cells. The molecular basis for the low level of processing of pro-MMP-2 by PC-3 cells may be due to an overabundance of TIMP-2 and/or a low level of cell surface active MT1-MMP.

Key words: Tissue inhibitor of metalloproteinase-2, prostate cancer, Concanavalin A, reverse transcriptase-polymerase chain reaction.

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A critical phase in cancer cell progression is attainment of the capability to invade adjacent tissues and metastasize to distant organs. The expression and function of matrix metalloproteinases (MMPs), a large family of 25 or more structurally related enzymes that degrade extracellular matrix proteins, has been correlated with the development of invasive and metastatic behavior in tumor cells (Basbaum and Werb, 1996; Nagase et al, 1996; Pei, 1999a,b,c; Stetler-Stevenson, 1999; Park et al, 2000; Lohi

et al, 2001). These proteinases can also release growth factors, especially angiogenic factors, from an extracellular matrix and the cell surface and can thus affect tumor and host cells in the microenvironment (Stetler-Stevenson, 1999; McCawley and Matrisian, 2001). The activities of MMPs are regulated at the levels of transcription, translation, proenzyme activation, subcellular localization, and inhibition by endogenous tissue inhibitors of MMPs (TIMPs) (Birkedal-Hansen et al, 1993; Basbaum and Werb, 1996; Nagase et al, 1996; Stetler-Stevenson, 1999; Lohi et al, 2001). Substances that activate 2 MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which are type IV collagenases (gelatinases), are found in malignant tumors from a variety of tissue sources. These MMPs can degrade the basement membrane constituents such as type IV and V collagens and laminin, which im-

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plicates them in the early stages of tumor invasion (Birkedal-Hansen et al, 1993; Massova et al, 1998).

The expression of MMPs is found in both normal and pathological tissue changes in the prostate, and important roles for MMPs have been implicated in the progression of carcinoma of the prostate (Wilson, 1995). Primary human prostatic cancers express higher levels of MMP-2 protein and messenger RNA (mRNA) than normal prostate tissues (Stearns and Wang, 1993); and normal, hyperplastic, or neoplastic prostatic tissues secrete MMP-2 and MMP-9 and TIMP-1 and TIMP-2 *in vivo* (Wilson et al, 1993) and *in organ culture* (Lokeshwar et al, 1993). The balance of MMPs to TIMPs favors MMPs, since neoplastic prostate tumors secrete only trace amounts of TIMPs (Lokeshwar et al, 1993); and there is an increased ratio of MMP activities to immunoreactive TIMP-1 (Jung et al, 1998) and mRNA for MMP-2 to TIMP-2 (Still et al, 2000) in prostate cancer vs normal prostate tissues. Using immunohistochemical techniques, MMP-2 has been shown to be localized to basal and, to a lesser extent, secretory epithelial cells, but not to stromal cells, of normal prostate and benign prostatic hyperplasia (BPH) (Boag and Young, 1994; Montironi et al, 1996). mRNA for MMP-2 is weakly expressed in the secretory epithelium of the normal and benign hyperplastic prostate (Boag and Young, 1994) but is also localized to basal cells (Still et al, 2000). The number of transcripts for MMP-2 increases (Kuniyasu et al, 2000; Still et al, 2000), as does its protein, in prostatic intraepithelial neoplasia (PIN) and with progression to invasive cancer (Stearns and Wang, 1993; Boag and Young, 1994; Montironi et al, 1995, 1996). Malignant progression of prostate cancers and metastasis to lymph nodes are associated with an increase in the active, compared with the proenzyme, form of MMP-2 (Stearns and Stearns, 1996).

We have found that the progression to metastasis in established human prostate tumor cell lines is associated with the production of MMP-2 and MMP-9 (Wilson and Sinha, 1993) and that tumor growth factor beta (TGF- $\beta$ ) induces the proenzyme but not the active forms of MMP-2 and MMP-9 in primary cultures of prostatic epithelial cells from normal, BPH, or prostatic cancer tissues (Wilson et al, 2002). The involvement of MMP-2 in tumor cell invasion and metastasis is more directly implicated by transfection studies in which MMP-2 was overexpressed in C127 breast cancer cells (Cockett et al, 1998) or MYU3L bladder cancer cells (nontransfected cells produce MMP-9 but are not metastatic) (Kawamata et al, 1995), creating an invasive and metastatic phenotype. The aggressive phenotype of cancer cells is associated with cell surface localization of MMP-2, the activation of which is thought to occur with binding of pro-MMP-2 to MT1-MMP complexed with TIMP-2 in plasma membranes (Emmert-Buck et al, 1995; Strongin et al, 1995;

Lohi et al, 1996; Butler et al, 1998; Zucker et al, 1998). The activation of pro-MMP-2 in fibroblasts and some tumor cells is stimulated by a number of agents that increase the expression of MT1-MMP; these agents include Concanavalin A (Con A) (Overall and Sodek, 1990), monensin (Li et al, 1997), orthovanadate (Li et al, 1998), trifluoperazine (Ito et al, 1998), TGF- $\beta$ 1 (Brown et al, 1990), soluble kappa elastin peptides (Brassart et al, 1998), and cytochalasin D (Aillenberg and Silverman, 1996). Pro-MMP-2 can be activated in cells treated with plasmin (Baramova et al, 1997) and thrombin (Zucker et al, 1995), but there is disagreement as to whether thrombin-induced MMP-2 activation is mediated through MT1-MMP (Nguyen et al, 1999; Lafleur et al, 2001). MT1-MMP is immunolocalized to secretory cells in human high-grade PIN and prostatic cancer cells (Upadhyay et al, 1999), and it is expressed in the androgen-independent human prostate cancer cell lines PC-3 and DU-145 but not in androgen-responsive LNCaP cells (Nagakawa et al, 2000; Jung et al, 2003). Thus, the control of cell surface localization and activation of MMP-2 is expected to be critical in prostatic neoplasia. The purpose of this study was to determine whether overexpression of MMP-2 in PC-3 human prostate cancer cells by transfection would result in increased secreted and/or cell-associated active MMP-2.

## **Materials and Methods**

### *Growth of Prostate Tumor Cells*

The human prostate tumor cell line PC-3 and HT-1080 fibrosarcoma cells were obtained from the American Type Culture Collection. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin antibiotics (Sigma Chemical Co, St Louis, Mo). Tumor cells that were grown to about 70% confluence were collected by treatment of culture flasks with 0.5% trypsin and 1 mM EDTA (Gibco) and washed 3 times by resuspension in phosphate-buffered saline (PBS) (pH 7.4) and centrifugation.

### *Transfection of PC-3 Cells With MMP-2*

We chose PC-3 prostate tumor cells for these studies, since they are the more aggressive of the 3 established human prostate cancer cell lines with respect to *in vitro* and *in vivo* (nude mice) invasion (see Wilson, 1995). In addition, PC-3 cells express more than 10-fold higher levels of MT1-MMP than DU-145 cells, and MT1-MMP is nearly undetectable in LNCaP cells (Jung et al, 2003; Wilson et al, unpublished data). PC-3 cells have also been characterized as having no (Nakagawa et al, 2000) or low (Stearns and Wang, 1991; Greene et al, 1997; Festuccia et al, 2000; Dong et al, 2001) levels of MMP-2 expression. The studies to establish stable transfectants of PC-3 cells for MMP-2 were undertaken using 2 different plasmids: pCR-3 vector only and pCR-MMP-2 employing the LipofectAMINE

method (Gibco BRL Life Technologies, Gaithersburg, Md). Stable transfectants were selected with G418 (Pei and Weiss, 1996). Parental PC-3 cells, vector control cells, and MMP-2 stably transfected cells from 10 independently derived clones were examined for the level of MMP-2 expression through an analysis of its mRNA by employing Northern blotting and for its protein by employing MMP zymography. Cell morphology and proliferation rates were also closely monitored to examine for possible artifacts in the clonal selection process.

#### *Cell Proliferation and Wound Healing Migration Assays*

The rates of proliferation of the parental cells, vector control cells, and MMP-2 transfected PC-3 cells were monitored by the MTT assay (Romijn et al, 1988). The cells were seeded at approximately  $5 \times 10^3$  cells per well in a 96-well tissue culture plate and grown in RPMI 1640 media plus 5% heat-inactivated FCS. The number of cells was measured every 24 hours for 4 days.

Cell migratory behavior of the control and MMP-2 transfected PC-3 cells and of the HT-1080 cells was monitored to determine if the transfection of the protease changed this property of the tumor cells. Confluent monolayers of cells in 24-well plates were wounded with a pipette tip. After washing to remove free cells and cellular debris, the cells were cultured with RPMI 1640 media plus 5% FCS. The morphology of cells at the edge of the wound in the monolayer and the rate of closure of the void between the 2 edges of the cell layer were monitored by digital image analysis at 0, 8, and 24 hours.

#### *In Vivo Lung Metastases of PC-3 Cells*

Male athymic nude mice (BALB/c, nu/nu) 4–5 weeks of age were obtained from Simonsen Laboratories (Gilroy, Calif) and were housed on a constant photoperiod (light-dark, 12:12) under barrier conditions. These mice were used for the intravenous and subcutaneous injection of tumor cells under protocols approved by the Animal Studies Committee of the Minneapolis VA Medical Center.

Tumor cells were collected by the gentle scraping of culture flasks to suspend cells in PBS, pH 7.2. After washing in PBS, tumor cells were suspended in PBS ( $1 \times 10^6$  in 0.10 mL) for intravenous and subcutaneous injection. Mice (5–6 weeks old) in groups of 6–8 were injected via the tail vein or subcutaneously (3 per group) with 0.2 mL of a cell suspension of 4 different MMP-2 transfectant PC-3 cell sublines (MMP-2 production was verified in conditioned media via zymography), one vector only transfectant PC-3 subline, and with parental PC-3 cells. All mice were sacrificed 48 days after injection. The lungs of mice injected via the tail vein were removed and fixed in Bouin fluid. After washing in 70% ethanol, the surfaces of the lungs were examined for tumor nodules, after which they were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histology. The subcutaneous tumors were removed, and portions were frozen on dry ice for RNA isolation or tissue homogenization; other portions were fixed in Bouin fluid and examined histologically.

#### *Treatment of Cells In Vitro*

HT-1080, PC-3 parental, PC-3 vector control, and MMP-2 transfected PC-3 cells were resuspended in culture media with serum

before plating ( $1 \times 10^5$  cells/well) in 24-well plates for experimental protocols. After 72 hours, the cells were washed twice in serum-free medium and cultured in serum-free media containing 0.2% lactalbumin hydrolysate for 24 hours. After this, serum-free culture medium was used containing 0.2% lactalbumin hydrolysate (200  $\mu$ L/well) and the agent to be tested, and the cells were cultured for an additional 24–48 hours (20–50  $\mu$ g/mL Con A or succinylated Con A [sCon A]; 2.5, 25, and 250 ng/mL phorbolmyristate acetate [PMA]). The conditioned media were collected and centrifuged at  $960 \times g$  for 10 minutes at 4°C, and the supernatant was saved. The cell layer was washed with PBS and was lysed with addition of sodium dodecyl sulfate (SDS) sample buffer without 2-mercaptoethanol.

#### *Preparation of Membranes*

Membrane fractions were prepared as described by Ward et al (1991) with some modifications. The control and Con A–stimulated cells were scraped from culture flasks into serum-free media and pelleted by centrifugation ( $960 \times g$ , 5 minutes, 4°C). The cells were resuspended in cold 5 mM Tris HCl (pH 7.8) at  $2 \times 10^7$ /mL, maintained on ice for 10 minutes, and then passed 30 times through a 26-gauge needle. Crude membranes were prepared by centrifugation of the cell lysate at  $10000 \times g$  for 15 minutes at 4°C. The supernatant was centrifuged at  $105000 \times g$  for 1 hour at 4°C; then, the supernatant was removed and saved, and the membrane fraction was resuspended in 20 mM Tris HCl (pH 7.8), 10 mM  $\text{CaCl}_2$ , and 0.05% Brij 35.

#### *Immunofluorescent Microscopy*

HT-1080 and PC-3 parental cells were grown on coverslips in RPMI 1640 media with 5% FCS. The media were changed to RPMI-1640 without FCS but containing 50  $\mu$ g/mL of Con A and 5  $\mu$ M of BB-94 (gift of British Biotech Pharmaceuticals, Oxford, United Kingdom), and the cells were incubated for 24 hours. In brief, the cells were incubated with a fluorescein isothiocyanate–labeled mouse antihuman MT1-MMP monoclonal antibody (R & D Systems, Minneapolis, Minn), washed, and examined by confocal microscopy (Model 1024 confocal microscope, BioRad, Richmond, Calif).

#### *Zymography of Proteases*

Aliquots of the conditioned media and cell extracts prepared in SDS lysis buffer without 2-mercaptoethanol were subjected to electrophoresis in gelatin-containing polyacrylamide (9% acrylamide) gels in the presence of SDS under nonreducing conditions (Heussen and Dowdle, 1980; Wilson et al, 1993; Wilson and Sinha, 1993). The gels (0.75 mm thick) were electrophoresed for about 35 minutes at 200 V in a BioRad Mini-Protean II system. Following electrophoresis, the gels were rinsed with distilled water and then washed with gentle shaking at room temperature with 2.5% Triton X-100 (2 changes) for 1 hour. The gels were again rinsed with distilled water and incubated in 50 mM Tris HCl (pH 8.4) containing 5 mM  $\text{CaCl}_2$ . Incubation was overnight (18–20 hours) at 37°C. Following the incubation, they were stained with Coomassie blue (Pharmacia, Piscataway, NJ). Areas of proteolysis appear as clear zones against a blue background. Molecular mass determinations were made by referencing prestained protein standards (BioRad) that were coelectro-

phoresed in these gels. The dried zymograms were digitally scanned into a Dell computer and processed using the Photoshop program.

### Western Blot Analysis

Immunoblotting was performed as described previously (Pei and Weiss, 1996). The conditioned media from PC-3 parental, PC-3 vector control, and PC-3 MMP-2 transfected cells grown in serum-free media, or in media with Con A or sCon A, for 48 hours were concentrated 20-fold with a speed vac. The conditioned media from HT-1080 cells were used without concentration. These media were separated by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose paper electrophoretically, and the nitrocellulose blot was probed with a mouse monoclonal anti-TIMP-2 immunoglobulin G (IgG) (0.5 µg/mL) (R & D Systems) and an alkaline phosphatase-conjugated secondary antibody (Invitrogen, San Diego, Calif). For the immunoblot detection of MT1-MMP, cell extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, and 0.1% Nonidet P-40) with protease inhibitors (10 mM leupeptin, 0.1 mM *p*-APMSF, and 1 mM aprotinin) were electrophoresed and blotted to nitrocellulose, which was probed with a mouse monoclonal antibody to MT1-MMP (0.5 mg/mL) (Chemicon, Temecula, Calif) and an alkaline phosphatase-conjugated secondary antibody.

### Northern Blot Analysis

Isolation of RNA was performed using TRIzol (Gibco BRL Life Technologies) with a modified protocol provided by the supplier. The total RNA concentration was determined by absorbency measurement at 260 nm, and the integrity of the RNA was checked by electrophoresis in a 1.0% agarose gel and stained with ethidium bromide. The expression of MT1-MMP was examined using a complementary DNA (cDNA) probe for MT1-MMP that was an *EcoRI-HindII* fragment (1.6 kb) isolated from the pCR3.1 MT1-MMP vector and radiolabeled using a Redi-Prime (Amersham, Arlington Heights, Ill) kit and 32p-dCTP as recommended by the supplier. The probe (10<sup>9</sup> cpm) was used for Northern blotting with a Rapid Hyb Solution. For Northern blotting analysis of MMP-2, the blot was probed with a cDNA fragment containing the entire coding region that was isolated from a pCR3.1-Gel A vector (Pei, 1999a).

### Reverse Transcriptase-Polymerase Chain Reaction Analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was carried out using a StrataGene ProStar HF Single-Tube RT-PCR System (HF) (La Jolla, Calif) and an MJ Research PTC-150HB MiniCycler thermocycler (Watertown, Mass). The RNA to be analyzed was stored in Formazol (Molecular Research Center, Cincinnati, Ohio). At analysis, the RNA was diluted in water so that approximately 0.1 µg of RNA (0.67% Formazol final concentration) was added to each reaction mixture. RT-PCR was performed according to the manufacturer's instructions with some modifications. The reaction mixture included 1 × HF buffer (StrataGene proprietary buffer), 100 ng of upstream primers, 100 ng of downstream primers, 25 mM of deoxynucleotidetriphos-

phates, 3 U of RT, 2.5 U of *Taq* polymerase, and 100 ng of RNA. The reaction mixture for TIMP-2 also included 15 mM of ammonium sulfate. The RT incubation was carried out for 15 minutes at 37°C for MMP-2 and TIMP-1 and at 42°C for TIMP-2. The PCR program consisted of 2 minutes at 95°C to denature nucleic acids and 30 cycles of PCR (95°C for 30 seconds for denaturing, 60°C for 30 seconds for annealing, and 2 minutes at 68°C for elongation) and a final 10-minute extension at 68°C. Sense and antisense primers for PCR were designed according to published cDNA sequences and were synthesized and purchased from Gibco BRL Life Technologies. These included 1) human MMP-2, sense CTGACATTGACCTTGGCACC; antisense TAGCCAGTCGGATTTGATGC, producing a 630-bp band size (Brassart et al, 1998); 2) human TIMP-1, sense TTCGTGGGGACACCAGAAGTCAAC; antisense TGGACA-CTGTGCAGGCTTCAGTTC, producing a 527-bp band size (Wick et al, 1994); 3) human TIMP-2, sense CTCGGCAG-TGTGTGGGGTC; antisense CGAGAAACTCCTGCTTGGGG, producing a 364-bp band size (Brassart et al, 1998); and 4) GAPDH (glyceraldehyde 6 phosphate dehydrogenase) sense AC-CACAGTCCATGCCATCAC; antisense TCCACCACCCTGTT-GCTGTA, producing a 443-bp band size (Brassart et al, 1998). Following amplification, the samples were electrophoresed in a 1.2% agarose gel containing ethidium bromide, 40 mM Tris-acetate (pH 8.5), and 2 mM EDTA (TAE) buffer. The gels were photographed using a Fotodyne MP4 Instant Image Camera with Foto UV System (Fotodyne Inc, New Berlin, Wis) and Polaroid 667 film, and the images were digitally scanned into the Photoshop program.

## Results

### Expression of MMP-2 by PC-3 Cells

The expression of MMP-2 was analyzed by zymography and Northern blotting to detect its activity and mRNA transcripts, respectively. No MMP-2 activity in the conditioned media (Figure 1A) or the transcripts (Figure 1B) was detected for the PC-3 cells used for the transfection of MMP-2. The vector control transfected PC-3 cells did not demonstrate MMP-2 activity (Figure 1A) or MMP-2 mRNA (Figure 1B). Varying levels of MMP-2 activity were found in zymograms of the conditioned media (Figure 1A) of different strains of PC-3 cells created by transfection with MMP-2; these transfectants also demonstrated MMP-2 mRNA expression (Figure 1B). The MMP-2 secreted by the transfected cells was predominantly in the proenzyme form (72 kd), with only a small portion of processed MMP-2 (62 kd). The parental, vector control, and MMP-2 transfected PC-3 cells all expressed mRNA for MT1-MMP (Figure 1C). There was no difference in cell morphology, rate of proliferation, or cell migration in an in vitro wound healing assay for the parental, vector control, and MMP-2 transfected PC-3 cells (data not shown). There were low numbers of tumor nodules (0–5 nodules) visible on the surface of lungs of nude mice

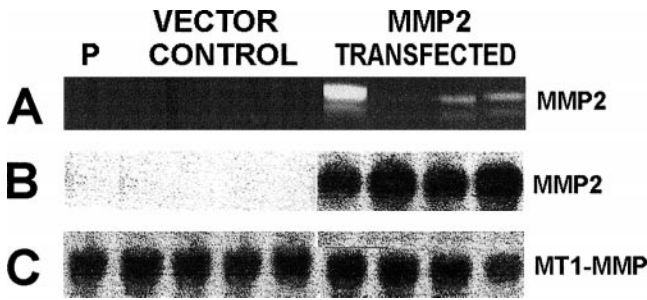


Figure 1. Expression of matrix metalloproteinase (gelatinase A) (MMP-2) and membrane type MMP 1 (MT1-MMP) by PC-3, PC-3 vector control, and PC-3 MMP-2 transfected cells. **(A)** Zymogram of MMP-2 activities of representative PC-3 parental cells (P), PC-3 vector control cells, and PC-3 MMP-2 transfected cell lines. MMP-2 activities of different intensities were found in the conditioned media of isolated MMP-2 transfected PC-3 cell strains but not in the conditioned media of parental and vector control transfected PC-3 cells. **(B)** Northern blot analysis of MMP-2 messenger RNA (mRNA) expression in control and MMP-2 transfected cells. Transcripts for MMP-2 were not found in PC-3 parental and vector control transfected PC-3 cells, but they were expressed in the PC-3 cells transfected with MMP-2. **(C)** Northern blot analysis of MT1-MMP mRNA expression in the control and MMP-2 transfected cells. Transcripts for MT1-MMP were detected in the parental, vector control, and MMP-2 transfected PC-3 cells.

injected intravenously with PC-3 parental, PC-3 vector control, or PC-3 MMP-2 transfected cells. There was no difference in the number of nodules found or in the extent of histologic tumor growth within the lungs in parental, vector control, and MMP-2 expressing PC-3 cells. The subcutaneous tumors of PC-3 MMP-2 transfected cells expressed MMP-2 mRNA as determined by RT-PCR, whereas the parental PC-3 tumors did not (30 cycles), indicating that the transfected cells continued to produce MMP-2 *in vivo* (data not shown).

#### *Effects of Con A and PMA Treatment on MMP-2 Processing*

We examined the effects of Con A and PMA treatment on MMP-2 expression and molecular processing in the MMP-2 transfected PC-3 cells. Treatment of other cell types (primarily fibroblasts) with either of these agents has been reported to stimulate activation of the proenzyme form of MMP-2 (Shofuda et al, 1998). Control PC-3 MMP-2 transfected cells secreted primarily the proenzyme and only a small level of the 62-kd form of MMP-2 (Figure 2). Treatment of these cells with 50  $\mu\text{g}/\text{mL}$  of Con A for 48 hours resulted in a small increased proportion of secreted 62-kd MMP-2 and the appearance of a minor band of 59 kd. However, in cell extracts of the Con A-treated MMP-2 transfected cells, the lower-molecular-weight form of MMP was primarily the active 59-kd form. The level of molecular processing of pro-MMP-2 as found in the conditioned media of PC-3 MMP-2 transfected cells stimulated by Con A is low compared with the response of HT-1080 cells to Con A, in which most pro-MMP-2 was processed to the 62- and 59-kd molec-

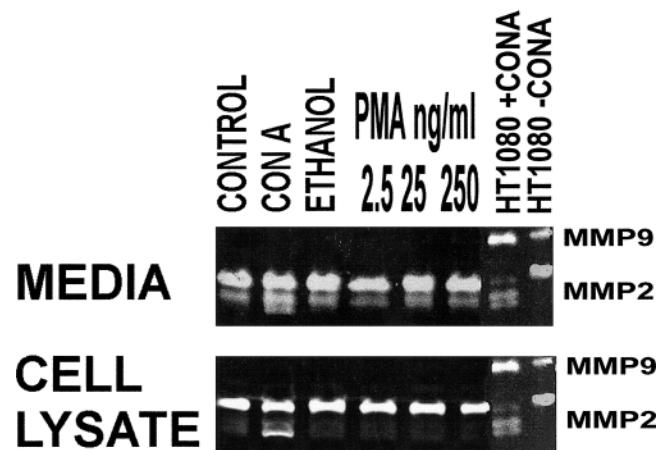


Figure 2. The effects of Concanavalin A (Con A) or phorbolmyristate acetate (PMA) on matrix metalloproteinase (gelatinase A) (MMP-2) activities in the conditioned media and cell extracts of a PC-3 MMP-2 transfected cell line. PC-3 MMP-2 transfected cells were treated with Con A (50  $\mu\text{g}/\text{mL}$ ) or PMA (2.5, 25, or 250 ng/mL) or vehicle controls for 48 hours in serum-free media. Treatment with Con A resulted in an increase in the lower-molecular-weight forms of MMP-2 in both the conditioned media and cell extracts, whereas there was no effect of PMA on the molecular forms of MMP-2, even at 250 ng/mL. Conditioned media from HT-1080 cells, incubated with or without Con A, were included in the gels as controls. In HT-1080 cells, there was a marked reduction in the proenzyme form of MMP-2 with an increase in the 2 lower-molecular-weight forms.

ular forms. Treatment of PC-3 MMP-2 transfected cells with orthovanadate (Li et al, 1998), monensin (Li et al, 1997), or elastin peptides (Brassart et al, 1998) (data not shown) also did not produce pro-MMP-2 processing. There was no effect of treatment with PMA, even at 250 ng/mL, or its ethanol control on the molecular forms of MMP-2 in the conditioned media or cell extracts of PC-3 MMP-2 transfected cells. In addition, there was no effect for the PMA or Con A treatment of PC-3 MMP-2 transfected cells on MMP-9 induction.

#### *The Effect of Con A and sCon A on Processing of Pro-MMP-2*

Many of the effects of Con A on cells are mediated through the clustering of cell surface molecules. This mediation is not seen with sCon A (Reeke et al, 1974; Yu et al, 1997). The response of PC-3 and PC-3 MMP-2 transfected cells to Con A and sCon A with respect to the processing of pro-MMP-2 was examined (Figure 3). PC-3 cells did not produce or secrete zymogram-detectable MMP activities, whereas the PC-3 MMP-2 transfected cells secreted predominantly the proenzyme form of MMP-2 (Figure 3). The low level of MMP-2 activity of 62 kd in the conditioned media did not change substantially upon incubation with Con A or sCon A. However, in cell lysates of Con A-stimulated PC-3 MMP-2 transfected cells, there was an increase in the 62-kd form of MMP-2, an effect not produced by sCon A. HT-1080 se-

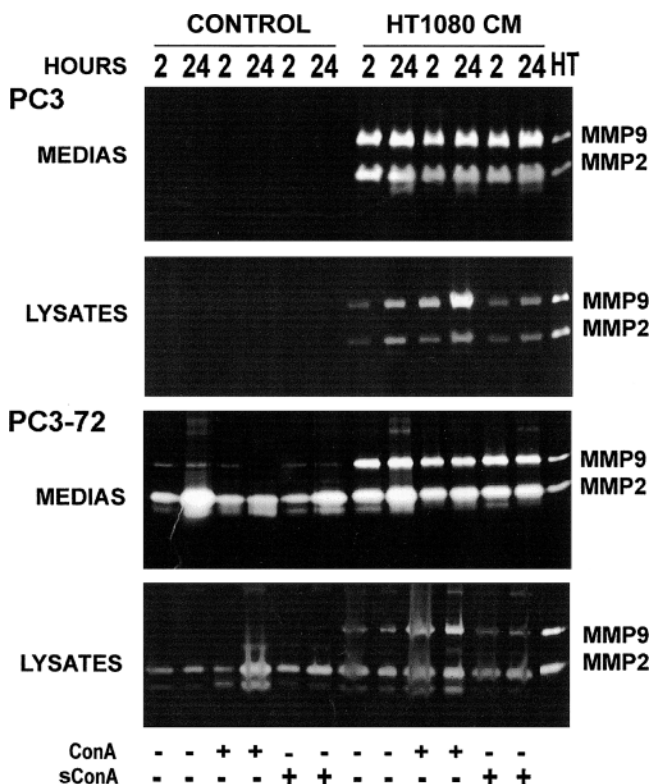


Figure 3. The effect of Concanavalin A (Con A) and succinylated Con A (sCon A) on the secreted and cellular molecular forms of matrix metalloproteinase (gelatinase A) (MMP-2) in PC-3 parental and PC-3 MMP-2 transfected cell lines and on the processing of added pro-MMP-2 of conditioned media (CM) from HT-1080 cells. Cells were incubated for 2 or 24 hours with control, Con A, or sCon A additions to serum-free media. No MMP-2 activities were found in the CM or cell lysates of PC-3 cells under control conditions or following treatment with Con A or sCon A. In a second part of this experiment, HT-1080-conditioned media were added to PC-3 cells that were incubated for 2 or 24 hours. There was no change in the molecular form of MMP-2 at 2 hours, but a small portion of a lower-molecular-weight form was found after 24 hours of incubation. The relative amount of the lower-molecular-weight form was not influenced by Con A or sCon A treatment. PC-3 cells bound pro-MMP-2 and pro-MMP-9 from the HT-1080-conditioned media. This was because these activities appeared in the zymograms of the PC-3 cell extracts. Treatment with Con A increased the binding of MMP-9 to PC-3 cells, an effect not elicited by sCon A. The PC-3 MMP-2 transfected cells secreted predominantly the proenzyme (72 kd) and low levels of 62 kd MMP-2 into the media. The relative amount of processed pro-MMP-2 in the media was not influenced by Con A or sCon A. In the lysates of transfected cells, there was a higher proportion of the activated form (lower molecular weight) in the Con A-treated than in the control or sCon A-treated cells. When the transfected cells were incubated with HT-1080 CM, there was no effect on the molecular forms of MMP-2 in the media. However, there was a higher level of the active form of MMP-2 in cell lysates of Con A-treated cells incubated with HT-1080 CM. There was also a greater binding of MMP-9 to Con A-treated transfected cells.

rum-free conditioned media that contained pro-MMP-2 and pro-MMP-9 were added to PC-3 parental and MMP-2 transfected cells. There was a small conversion of pro-MMP-2 to the 62-kd form in the media at 24 hours of incubation for these cell groups, an observation that was not changed by Con A or sCon A treatment. However, there was a notable Con A-stimulated binding of pro-

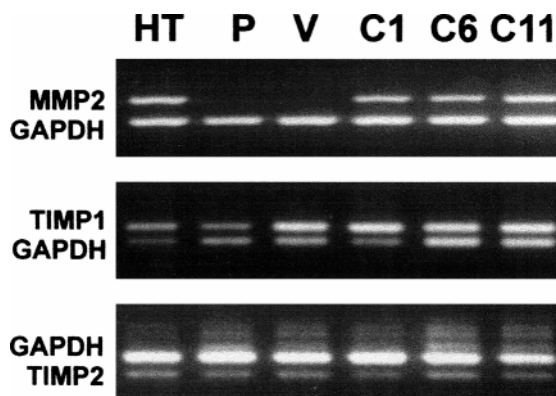


Figure 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of expression of matrix metalloproteinase (gelatinase A) (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), and tissue inhibitor of metalloproteinase-2 (TIMP-2) in HT-1080 and PC-3 parental, PC-3 vector control, and PC-3 MMP-2 transfected cells. The expression of MMP-2, TIMP-1, and TIMP-2 transcripts in the RNA of the transfected PC-3 cells was examined by RT-PCR. The HT-1080 (HT) and PC-3 parental (P), vector control (V), and 3 MMP-2 transfected PC-3 cell sublines (C1, C6, and C11) express TIMP-1 (527 bp) and TIMP-2 (364 bp); RT-PCR for GAPDH (glyceraldehyde 6 phosphate dehydrogenase) (443 bp) was performed in each RNA preparation as a control for RNA loading. mRNA for MMP-2 (630 bp) was detected in the HT-1080 cells and in the PC-3 MMP-2 transfected cells but not in the PC-3 parental or vector control cells.

MMP-9 to PC-3 and PC-3 MMP-2 transfected cells, as evidenced by an increased pro-MMP-9 activity in cell lysates. The binding of pro-MMP-9 to PC-3 and PC-3 MMP-2 transfected cells was not induced by sCon A.

#### *TIMP-1, TIMP-2, and MT1-MMP Expression*

The activation of pro-MMP-2 at the cell surface is postulated to involve the binding of pro-MMP-2 to an MT1-MMP/TIMP-2 complex followed by the cleavage of the propeptide from MMP-2 (Strongin et al, 1995). HT-1080 and PC-3 control and MMP-2 transfected cells expressed TIMP-1 and TIMP-2 transcripts (Figure 4). However, MMP-2 mRNA is seen only in HT-1080 cells and PC-3 cells transfected with MMP-2. Since the PC-3 MMP-2 transfected cells also express MT1-MMP mRNA (Figure 1), the transcripts for all the participants in pro-MMP-2 activation are present. Since our data showed a limited amount of processing of pro-MMP-2 by PC-3 MMP-2 transfected cells (Figures 1 through 3), we examined PC-3 cells for the expression of TIMP-2 and MT1-MMP proteins. Western blot analysis of the conditioned media showed the secretion of TIMP-2 by PC-3 parental, vector control, and MMP-2 transfected cells (Figure 5A). The amount of secreted TIMP-2 was not affected by treatment of cells with Con A or sCon A. MT1-MMP proteins of 60 and 65 kd were detected in PC-3 parental and MMP-2 transfected PC-3 cells (Figure 5B). The level of MT1-MMP protein expressed, however, is substantially lower than that found in HT-1080 cells. The low level of acti-

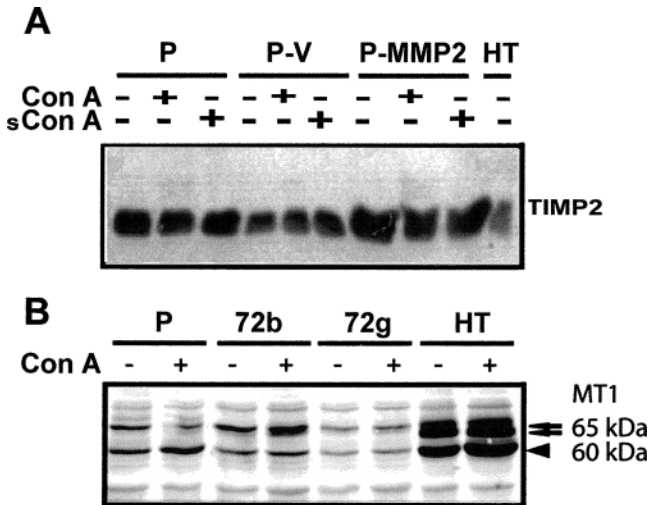


Figure 5. Western blot for tissue inhibitor of metalloproteinase-2 (TIMP-2) in conditioned media and membrane-type matrix metalloproteinase 1 (MT1-MMP) in cell extracts from PC-3 parental and MMP-2 transfected cells. (A) The conditioned media of PC-3 control and transfected cells were concentrated 20-fold using a speed vac. The HT-1080 control media were used unconcentrated. The nitrocellulose blot of these electrophoresed samples was probed with a mouse monoclonal anti-tissue inhibitor of metalloproteinase-2 (anti-TIMP-2) antibody. All the PC-3 control and transfected cells expressed TIMP-2 protein, and there was no effect for the treatment of Concanavalin A (Con A) or succinylated Con A (sCon A) on the level of TIMP-2 protein expressed. (B) Cell extracts prepared in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors were electrophoresed and blotted to nitrocellulose, which was probed with a mouse monoclonal antibody to MT1-MMP. The levels of expression of MT1-MMP proenzyme (65 kDa) and processed active form (60 kDa) were much lower in PC-3 and PC-3 MMP-2 transfected cells than in HT-1080 cells.

vation of MMP-2 by PC-3 cells with Con A treatment may be due to an overabundance of the TIMP-2 inhibitor and/or a low level of cell surface-active MT1-MMP.

#### Effects of Serine Protease Inhibitors and Cyclic Adenosine Monophosphate on Pro-MMP-2 Processing

The processing of pro-MMP-2 to lower-molecular-weight forms by PC-3 transfected cells is stimulated to a limited extent by Con A, especially when compared to control

HT-1080 cells (Figure 6). In view of reports of plasmin and thrombin cell-mediated effects on MMP-2 activation (Zucker et al, 1995; Baramova et al, 1997), the effects of serine protease inhibitors on the low level of pro-MMP-2 processing were examined. Incubation of PC-3 MMP-2 transfected cells with the serine protease inhibitors  $\epsilon$ -aminocaproic acid and amiloride was able to block the processing of pro-MMP-2 to lower-molecular-weight forms, as was incubation with dexamethasone. These data indicate that a serine protease may be involved in some step of pro-MMP-2 processing. Urokinase may be involved at some stage in this process, since amiloride selectively inhibits urokinase (Vassali and Belin, 1987), and dexamethasone stimulates an increase in plasminogen activator inhibitor-1 (Coleman et al, 1986). The Con A stimulation of MMP-2 activation in cells such as HT-1080 is associated with a transcriptional induction of MT1-MMP, and this induction can be repressed by elevation of intracellular cyclic adenosine monophosphate (cAMP) levels (Yu et al, 1998). The addition of dibutyl-cAMP directly, or of isoproterenol, which increases intracellular cAMP, did not alter the Con A response in the PC-3 MMP-2 transfected cells. This may indicate that the Con A effect in PC-3 cells is not mediated through the induction of MT1-MMP.

#### Subcellular Distribution of MMP-2

The subcellular distribution of MMP-2 in HT-1080 and PC-3 MMP-2 transfected cells was examined by differential centrifugation. The activities of MMP-2 were higher in the heavy membrane/mitochondrial (Figure 7B) and cytosol (Figure 7D) fractions of HT-1080 cells. The activities of the combined molecular forms of MMP-2 in these 2 fractions were higher in the HT-1080 cells stimulated by Con A. For the PC-3 MMP-2 transfected cells, the MMP-2 activities were higher in the light membrane, plasma membrane-enriched fraction (Figure 7C). There was no increased processing of pro-MMP-2 in the cell extract or any subcellular fraction of Con A-incubated

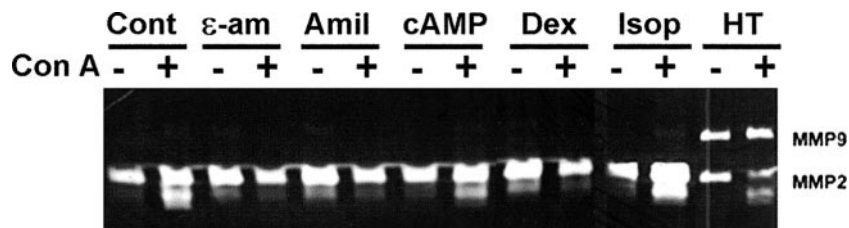


Figure 6. The effects of different agents on the Concanavalin A (Con A)-induced processing in pro-matrix metalloproteinase 2 (gelatinase A) (pro-MMP-2) of PC-3 MMP-2 transfected cells. MMP-2 transfected PC-3 cells were incubated with different agents in the presence or absence of Con A for 48 hours in serum-free media. The conditioned media from these incubations were examined by zymography for MMP activities. A limited processing of pro-MMP-2 to a lower-molecular-weight form was stimulated by Con A. The inclusion of 2 serine protease inhibitors,  $\epsilon$ -aminocaproic acid ( $\epsilon$ -am, 10 mM) or amiloride (Amil, 0.2 mM), in these incubations blocked the Con A effect. Treatment with dexamethasone (Dex,  $10^{-7}$  M) also blocked the Con A effect on pro-MMP-2 processing. Addition of dibutyl-cyclic adenosine monophosphate (cAMP) (cAMP,  $10^{-5}$  M) directly or isoproterenol (Isop,  $10^{-6}$  M), which increases intracellular cAMP, did not alter the Con A response in the PC-3 MMP-2 transfected cells. The conditioned media of HT-1080 (HT) cells were included as a control.

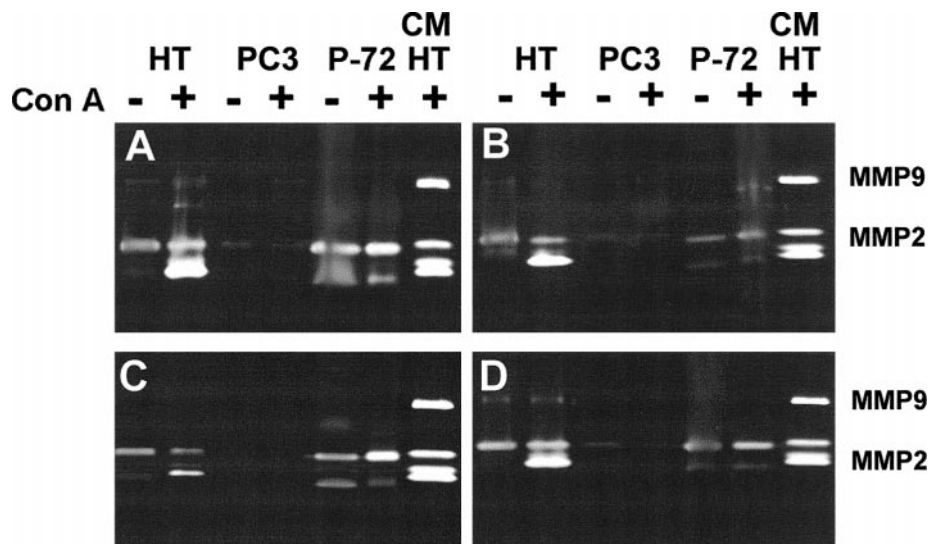


Figure 7. The effect of Concanavalin A (Con A) on the subcellular distribution of matrix metalloproteinase (gelatinase A) (MMP-2) in HT-1080, PC-3, and MMP-2 transfected PC-3 cells. PC-3 parental cells (PC-3), MMP-2 transfected PC-3 cells (P-72), and HT-1080 (HT) cells were grown in the presence or absence of Con A for 48 hours, after which cell extracts were prepared that were subfractionated by differential centrifugation. MMP-2 activities in the (A) cell extracts (36  $\mu\text{g}$  protein/lane), (B) 10000  $\times$  g pellet (36  $\mu\text{g}$  protein/lane), (C) 105000  $\times$  g pellet (20  $\mu\text{g}$  protein/lane), and (D) 105000  $\times$  g supernatant (12  $\mu\text{g}$  protein/lane) were examined using zymography. The MMP-2 activities of HT-1080 cells were higher in the heavy membrane-mitochondrial and cytosol fractions, whereas in the MMP-2 transfected PC-3 cells, the activity was predominantly in the light membrane fraction that is considered to have a higher proportion of the plasma membrane.

PC-3 transfected cells, but there was an indication of increased pro-MMP-2 in the light membrane fraction of Con A-treated cells.

#### Effect of Con A on Subcellular Distribution of MT1-MMP

MT1-MMP was localized to intracellular sites by immunofluorescent microscopy in both HT-1080 and PC-3 cells (Figure 8A and C). When these cells were exposed to 50  $\mu\text{g}/\text{mL}$  Con A, HT-1080 cells showed a strong localization of MT1-MMP on the cell surface (Figure 8B), whereas PC-3 did not show any demonstrable change in subcellular MT1-MMP localization (Figure 8D). This change of MT1-MMP subcellular localization was accompanied by the molecular processing of pro-MMP-2 in HT-1080-conditioned media to the active form by HT-1080 cells (Figure 8E). There was no processing of pro-MMP-2 from HT-1080 conditioned by PC-3 cells (Figure 8F).

## Discussion

We successfully transfected MMP-2 into human prostate PC-3 cancer cells as was shown by the expression of MMP-2 activity using zymography as well as by the presence of MMP-2 transcripts using Northern blotting and RT-PCR. Overexpression of MMP-2 in PC-3 cells did not alter the *in vitro* morphology or growth rate of the cells. The PC-3 parental cells we transfected did not express MMP-2 at the protein (activity) or mRNA level (RT-PCR for 30 cycles). These PC-3 cells may have expressed low

levels of MMP-2 and MMP-9 that were not detectable by the methods we employed. There are differing reports in the literature as to the basal, constitutive expression of MMP-2 by PC-3 cells. In addition to this study, an absence of MMP-2 activities in PC-3 cells has been reported by Nagakawa et al (2000), whereas low levels of MMP-2 expression have also been reported (Stearns and Wang, 1991; Greene et al, 1997; Festuccia et al, 2000; Dong et al, 2001).

The molecular processing of pro-MMP-2 in MMP-2 transfected PC-3 cells is very low, which may account for the low level of lung colonization that was similar to that of the PC-3 parental cells. The lack of pliancy of PC-3 cells in activating pro-MMP-2 is evidenced by the absence of an effect of PMA and only marginal processing of MMP-2 in response to Con A treatment. Unlike some other cell lines, PC-3 cells do not up-regulate MT1-MMP or translocate existing MT1-MMP to the cell surface in response to Con A. Con A and 12-*o*-tetradecanoylphorbol 13-acetate (TPA) enhances MT1-MMP expression in several cell lines, but only Con A stimulates pro-MMP-2 activation (Shofuda et al, 1998). However, both phorbol esters and Con A induce pericellular gelatinolytic activity of HT-1080 cells (Lohi and Keski-Oja, 1995). The limited activation observed in PC-3 MMP-2 transfected cells was blocked by incubation with the serine protease inhibitors  $\epsilon$ -aminocaproic acid and amiloride. Although serine proteases such as plasmin do not activate purified pro-MMP-2 (Murphy et al, 1989) but may convert the intermediate 62-kd form to the 58-kd active form (Baramova et al,

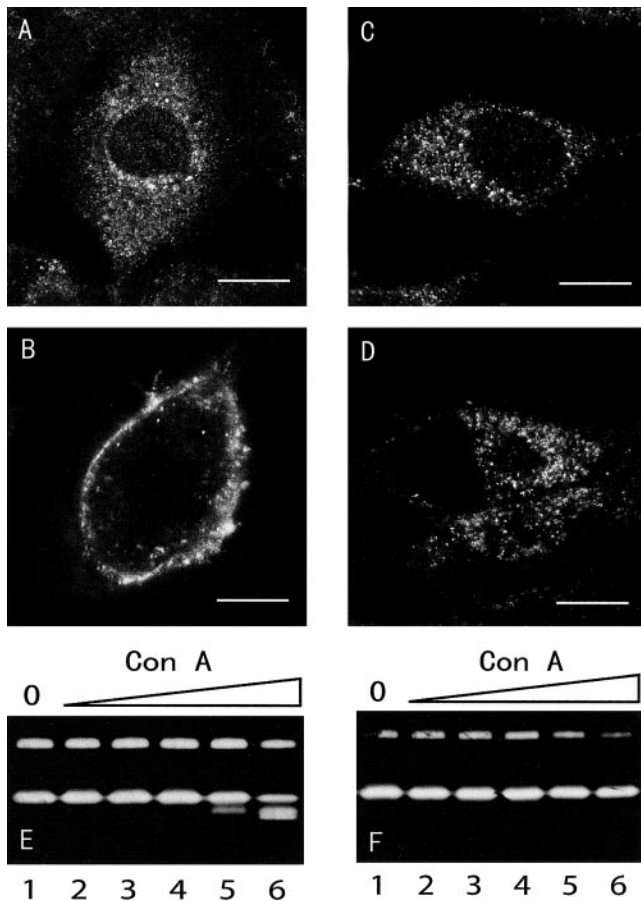


Figure 8. The effect of Concanavalin A (Con A) on the subcellular localization of membrane-type matrix metalloproteinase 1 (MT1-MMP) and processing of pro-MMP-2 in HT-1080 and PC-3 cells. HT-1080 and PC-3 cells were grown for 24 hours in serum-free media containing 5  $\mu\text{M}$  of BB-94 (matrix metalloproteinase inhibitor) (**A and C, respectively**) and 50  $\mu\text{g/mL}$  of Con A (**B and D, respectively**), and MT1-MMP localization was detected by the use of a fluorescein isothiocyanate (FITC)-labeled anti-MT1-MMP antibody and immunofluorescent microscopy. HT-1080 (**E**) and PC-3 (**F**) cells were also grown in HT-1080-conditioned media with varying concentrations of Con A (0, 0.6, 1.9, 5.6, 16.6, and 50  $\mu\text{g/mL}$ ; lanes 1 through 6). Activation of pro-MMP-2 in the HT-1080-conditioned media was examined by gelatin zymography. Both HT-1080 and PC-3 cells showed intracellular localization of MT1-MMP (**A, C**). HT-1080 cells responded to Con A with translocation of MT1-MMP from these intracellular sites to the cell surface (**B**) and also with molecular processing of pro-MMP-2 to lower-molecular-weight forms (**E**). PC-3 cells, on the other hand, showed no change of subcellular localization of MT1-MMP (**D**) or any processing of pro-MMP-2 (**F**).

1997), these serine protease inhibitors may block a proteolytic step leading to the processing of the activator protease for pro-MMP-2. The cell surface activation of exogenously added pro-MMP-2 to TPA-activated HT-1080 cells is not blocked by  $\epsilon$ -aminocaproic acid or aprotinin (Brown et al, 1990). However, aprotinin and  $\epsilon$ -aminocaproic acid can block the cell-mediated activation of TGF- $\beta$ 1 (Sato and Rifkin, 1989; Cao et al, 1996), a growth factor that can induce MMP-2 in primary cultures of prostate epithelial cells (Wilson et al, 2002). There are

varied responses of MMP-2 activation in other cell types in response to agents that affect MT1-MMP expression. For example, activation of MMP-2 occurs in HT-1080 cells but not in CCL-137 human embryonic lung fibroblasts in response to Con A or PMA, even though there is an up-regulation of the MT1-MMP mRNA by both of these cell types. The fibroblasts did not process MT1-MMP to the 43-kd form that was associated with MMP-2 processing in HT-1080 cells (Lohi et al, 1996). Since there was no activation of pro-MMP-2 in the PC-3 MMP-2 transfected cells exposed to sCon A, which does not stimulate cell surface protein clustering, the marginal activation in response to Con A may reflect the nontranscriptional component of a Con A-mediated response (Yu et al, 1997). Since elevation of cAMP levels in MDA-MB231 human breast cancer cells inhibits Con A transcriptional effects on MT1-MMP expression and thus MMP-2 activation (Yu et al, 1998), the absence of inhibitory effects of dibutyryl-cAMP or isoproterenol treatment (raises cAMP levels) on the low level of pro-MMP-2 processing by PC-3 MMP-2 transfected cells also suggests that the effect of Con A that was observed is not through transcriptional effects on MT1-MMP.

Strongin et al (1995) proposed that the binding of TIMP-2 to the cell surface activated MT1-MMP complexes with pro-MMP-2, resulting in the cleavage and activation of MMP-2. The level of TIMP-2 secreted in 11 human cancer cell lines was inversely correlated with pro-MMP-2 activation; for example, HT-1080 cells that secrete a high level of TIMP-2 activate little pro-MMP-2 without stimulation, even though they have a high level of MT1-MMP mRNA expression (Shofuda et al, 1998). Pro-MMP-2 is activated in TIMP-2 transfected COS-1 cells with a low level of TIMP-2 expression but not in those with a higher level of TIMP-2 production (Cao et al, 1996). Con A or PMA treatment of HT-1080 cells increases MT1-MMP mRNA and MMP-2 activation, but it does not change the level of TIMP-2 expression (Lohi et al, 1996). The effect of Con A treatment may be to stabilize the complex of MT1-MMP with TIMP-2 on cell membranes, reducing the internalization and degradation of the complex (Shofuda et al, 1998). Thus, the low level of pro-MMP-2 activation in PC-3 MMP-2 transfected cells may be due, in part, to the inhibitory effects of an abundance of TIMP-2.

The low-level response of the MMP-2 transfected PC-3 cells to Con A would appear to be due to changes in protein organization on the cell surface. This might involve the binding of MMP-2 to PC-3 cells through an alternative mechanism to MT1-MMP. Activation of MMP-2 by fibroblasts grown in type I collagen lattices is mediated by the  $\alpha$ 2 $\beta$ 1 integrin receptor (Seltzer et al, 1994) and occurs intracellularly in the Golgi membranes (Lee et al, 1997). The cell surface localization of MMP-

2 in fibroblasts is mediated via the collagen-binding domain of the enzyme that binds pericellular type I collagen, which is anchored to cell membrane  $\beta 1$ -integrins (Steffensen et al, 1998). Another mechanism to localize MMP-2 on the cell surface is the binding of the pro and active forms of the enzyme to integrin  $\alpha v \beta 3$  (Brooks et al, 1996). However, this mechanism appears to involve MT1-MMP, since the transfection of  $\alpha v \beta 3$  into human melanoma cells that express MT1-MMP, MMP-2, and TIMP-2, but do not activate MMP-2, does result in the activation of MMP-2. Activated MT1-MMP and  $\alpha v \beta 3$  colocalize on the cell surface of the  $\alpha v \beta 3$  expressing MMP-2-activating melanoma cells (Hoffman et al, 2000). PC-3 prostate cancer cells and primary human prostate cancer cells, but not normal prostatic epithelial cells or the noninvasive LNCaP human prostate cells, express  $\alpha v \beta 3$  (Zheng et al, 1999). This suggests that the lack of MMP-2 activation in PC-3 cells may reside in the expression or processing of the MT1-MMP protein. It is clear that PC-3 cells, unlike HT-1080 cells, do not respond to Con A with translocation of MT1-MMP to the cell surface. The low level of pro-MMP-2 activation by PC-3 MMP-2 transfected cells that was observed may be due to the autocatalytic processing of cell surface MT1-MMP with the release of a 20-kd soluble fragment with the catalytic center of the enzyme (Lehti et al, 2000). Alternatively, it has been proposed by Itoh et al (2001) that pro-MMP-2 activation at the cell surface involves formation of homophilic complexes of MT1-MMP. The limited pro-MMP-2 processing that was observed in the PC-3 MMP-2 transfected cells may be due to the low level of MT1-MMP protein expression and hence dimer formation in these cells. However, the lack of pro-MMP-2 processing in sCon A-treated cells may result from an absence of cell surface mobility of MT1-MMP, which leads to the formation of functional MT1-MMP complexes better able to activate pro-MMP-2.

The control of pericellular proteolysis in prostate tumors may be mediated by a number of proteases working in concert. Up-regulation of MT1-MMP stimulated by Con A in SW1353 chondrosarcoma cells is accompanied by a coordinated activation of not only MMP-2 but also of MMP-9 and procollagenase 3 (Cowell et al, 1998). Thus, mechanisms that regulate activation of pro-MMP-2 and zymogen forms of other MMPs may lead to activation of multiple proteinases enhancing the invasive properties of these cells.

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