Complex Regulation of Membrane-Type Matrix Metalloproteinase Expression and Matrix Metalloproteinase-2 Activation by Concanavalin A in MDA-MB-231 Human Breast Cancer Cells

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Abstract

Matrix Metalloproteinase-2 (MMP-2) is secreted as a zymogen, the activation of which has been associated with metastatic progression in human breast cancer (HBC). Concanavalin A (Con A) has been found to induce activation of MMP-2 in invasive HBC cell lines. Con A effects on the expression of mRNA for membrane-type matrix metalloproteinase (MT-MMP), a newly described cell surface-associated MMP, showed a close temporal correlation with induction of MMP-2 activation. It is surprising that MT-MMP mRNA is constitutively present in the uninjured MDA-MB-231 cell, despite a lack of MMP-2 activation. We have used actinomycin D to demonstrate a partial requirement for de novo gene expression in the induction of MMP-2 activation by Con A in MDA-MB-231 HBC cells. Furthermore, this transcriptional response to Con A appeared to require the continued presence of Con A for its manifestation. The nontranscriptional component of the Con A induction manifests rapidly, is quite substantial, and persists strongly despite actinomycin D abrogation of both constitutive and Con A-induced MT-MMP. Cycloheximide analyses suggest that protein synthesis may be involved in this rapid transcription-independent response. These studies suggest that Con A induces MMP-2-activation in part by up-regulation of MT-MMP expression but has a more complicated mode of action, involving additional nontranscriptional effects, which apparently require protein synthesis.

Introduction

Degradation and penetration of extracellular matrices, particularly basement membranes, are critical for cancer cell metastasis (1). MMPs complement serinyl, cysteinyl, and aspartyl proteinases in mediating this process (2). In the MMP family, the gelatinases (MMP-2 or gelatinase A and MMP-9 or gelatinase B) are unique in their ability to cleave the helical domains of type IV collagen, a principle structural component of the basement membrane (3). These enzymes have thus been implicated in tumor cell invasion and metastasis, as well as angiogenesis and other normal cellular scenarios involving basement membrane remodeling.

Increased MMP-2 expression has been associated with increased metastatic potential in a variety of systems (3). Specific demonstrations of MMP-2 involvement in basement membrane invasiveness include inhibition studies using either polyoncral (4) or monoclonal (5) aMMP-2 antibodies. In HBCs, elevated MMP-2 levels have been detected immunohistochemically (6, 7) and at the RNA level (6) compared to premalignant disease or normal breast. Some studies have associated increased MMP-2 immunoreactivity with the invasive regions of HBCs. Quantitative zymography has been used to demonstrate a preponderance of activated MMP-2 in HBC extracts and increased ratios of activated to latent MMP-2 in breast carcinomas of increasing grade (9). In situ hybridization analysis has shown that MMP-2 mRNA in human breast carcinoma is predominately localized to the stroma (8), suggesting that stromal-derived MMP-2 may manifest on the tumor component through a paracrine mechanism.

Recent observations (12), along with similar findings of increased MMP-2 immunoreactivity with the invasive regions of HBCs, as well as the concentration of MMP-2 immunoreactivity at the leading tumor edge (7), support our model for metastatic progression of HBC (12), which we have observed a correlation between potential for membrane activation of MMP-2 and metastatic capacity of HBC cultures in vitro and in nude mice (13, 14). These observations both in vivo and with the cell lines suggest an important role for cell surface activation/localization of MMP-2 for HBC invasion and metastasis.

The activation of MMP-2 by HBC cell lines was initially shown to be induced by type I collagen (13, 14) but can be induced more potently in the same subset of cell lines with Con A, and in some cases TPA (15). Others have reported induction of MMP-2-activation in various systems by Con A, TPA, and transforming growth factor β (reviewed in Ref. 15, see also Ref. 16). Induction by collagen (13, 14) was shown to be inhibited by Chx, consistent with earlier reports of Chx inhibition of Con A-induced activation of MMP-2 in fibroblasts. We found a coordinate regulation of MT-MMP that was found to mediate activation of MMP-2 after transfection into Cos-1 cells (17). We detected both transcriptional and nontranscriptional responses to Con A with respect to MMP-2 activation, and found a coordinate regulation of MT-MMP with the induced activation.

Materials and Methods

Cells and Chemicals. The MDA-MB-231 cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured in IMEM/FCS [Richter’s IMEM (Biofluids, Rockville, MD) supplemented with 10% FCS (GIBCO-BRL, Gaithersburg, MD)] in a 95% air, 5% CO2 atmosphere at 37°C. Cells were confirmed as free using the GenProbe kit (Gen-Probe, San Diego, CA) and maintained by weekly passage at 1:10 or 1:40. Con A (tissue culture grade), ActD, and Chx were purchased from Sigma Chemical Co. (St. Louis, MO).

Treatment of Cells with Con A, ActD, and Chx. MDA-MB-231 cells were plated (1 × 105 cells/0.5 ml IMEM/FCS) in Costar 24-well clusters overnight and the wells were washed in unsupplemented IMEM twice before treatment and further incubation in MMP-2-supplemented SFM (MMP-2/SFM) as de-
Total RNA was prepared from the cells with 4 M guanidinium thiocyanate and densitometry analysis using the Model DNA 35/Sparc Station 2 (protein + DNA with 2 mg/ml gelatin were used for electrophoresis to resolve the latent and activated species of MMP-2. The intensity of the bands were quantified by densitometry analysis using the Model DNA 35/Sparc Station 2 (protein + DNA imageWare systems, Huntington Station, NY). Northern Analysis. Cells were plated in 15-cm dishes and treated as described above, except for the omission of exogenous MMP-2 from the SFM. Total RNA was prepared from the cells with 4 M guanidinium thiocyanate and phenol-chloroform. RNA was resolved by 1% formaldehyde denatured agarose gel electrophoresis and transferred to Hybond-N+ nucleic acid transfer membranes (Amersham, Arlington Heights, IL). The filter was prehybridized for 1 h with 50% formamide at 42°C and then hybridized overnight at 42°C with a random prime-labeled 32P-labeled MT-MMP cDNA probe (16). Filters were washed with 0.1x SSC (150 mM NaCl, 15 mm sodium citrate) and 1% SDS at 37°C, and the bands were visualized by autoradiography. MT-MMP mRNA levels were normalized by methylene blue staining of the blot before probing, as well as glyceraldehyde 3-phosphate dehydrogenase probing of the same blot. RNA markers (Boehringer Mannheim, Indianapolis, IL) were used.

Western Analysis. Replicate cultures in 10-cm dishes were plated and treated as described above for Northern analysis. Monolayers were rinsed with ice-cold Tris-buffered saline (1 ml/plate), and sonicated on ice (20 s, maximal setting; Microson Ultrasonic Cell Disruptor; Heat Systems Ultrasonics, Inc., Farmingdale, NY) in the presence of protease inhibitors (1 M phenylmethane-sulfonyl fluoride, 0.1 M Aprotinin, 1 M leupeptin, 1 mM EDTA, and 1 M pepstatin). Extracts were centrifuged (15 min, Eppendorf), and pellets were stored frozen (—20°C). For analysis, pellets were dissolved in 50 µl reducing SDS-PAGE sample buffer, electrophoresed (10 µl/lane) on 12% SDS-PAGE gels copolymerized in 4% stacking gel and 8% running gel, and electrophoresed for 3 h at 150 V with 50% formamide at 42°C and then hybridized overnight at 42°C with a random prime-labeled MT-MMP cDNA probe (16). Filters were washed with 0.1x SSC (150 mM NaCl, 15 mm sodium citrate) and 1% SDS at 37°C, and the bands were visualized by autoradiography. MT-MMP mRNA levels were normalized by methylene blue staining of the blot before probing, as well as glyceraldehyde 3-phosphate dehydrogenase probing of the same blot. RNA markers (Boehringer Mannheim, Indianapolis, IL) were used.

Results and Discussion

Con A-induced MMP-2 Activation by MDA-MB-231 Cells Involves de Novo Gene Expression but Includes a Nontranscriptional Component. We have demonstrated previously that the induction of MMP-2 activation by collagen I in HBC cells is inhibitable by Chx (14). Here, we examined the potential requirement for de novo gene expression and the potential role of the newly described MT-MMP in HBC activation of MMP-2. We chose the more rapid and potent induction by Con A (15) and the MDA-MB-231 HBC cell line for these studies. The MDA-MB-231 cell line is vimentin positive and representative of the subset of HBC cell lines that are highly invasive in vitro, metastatic in nude mice, and activate exogenous MMP-2 upon induction by collagen and Con A, and in some cases, TPA (14, 15).

We first titrated the Con A dosage and found that maximal induction of activation could be achieved with doses ranging from 17 to 50 µg/ml over a 24-h period (not shown). In contrast, collagen-induced activation required 48—96-h incubation (13—15). We conducted dose-response studies with ActD and Chx, inhibitors for RNA and protein synthesis, respectively, on MMP-2 activation induced by 20 µg/ml Con A in MDA-MB-231 cells. We used the range of 0.1 ng/ml to 1 µg/ml of ActD and 1.25 ng/ml to 50 µg/ml of Chx. Both ActD (Fig. 1A) and Chx (Fig. 1B) inhibited Con A-induced MMP-2 activation by MDA-MB-231 in a dose-dependent manner. The inhibitory effect was seen with doses of 1 ng/ml ActD (Fig. 1A) and 1.25 ng/ml Chx (Fig. 1B). Maximum inhibition was achieved with 100 ng/ml for ActD and 6.25 µg/ml for Chx. In general, a higher degree of inhibition was seen with Chx than ActD.

We then tested the time frame of effectiveness for these inhibitors.

![Fig. 1. Dose-response analysis for ActD and Chx inhibition of MMP-2 activation by MDA-MB-231 cells. A. ActD: Zymographic analysis is shown for SFM control (no cells, Ctrl M) and MDA-MB-231 cells either in SFM (Ctrl C) or SFM with 0.1% ethanol (Ctrl CE), all of which contain MMP-2 in the Mr 72,000 latent form without spontaneous activation. In contrast, MDA-MB-231 cells treated with 20 µg/ml Con A alone (Con A C), or in the presence of 0.1% ethanol (Con A E) predominately contain the Mr 62,000 and 59,000 activated MMP-2 forms. Treatment with Con A in the presence of ActD at concentrations of 1, 10, 30, and 100 ng/ml and 1 µg/ml are indicated. Significant inhibition can be seen at the dose of 100 ng/ml ActD, but complete inhibition is not seen even with the highest dose of ActD. B. Chx: Cells were cultured and treated with Chx as described previously (17). Bands were localized with the enhanced chemiluminescence system (ECL kit; Amersham).](cancerres.aacrjournals.org)
Fig. 2. Time course analysis of ActD and Chx inhibition of Con A-induced MMP-2 activation. A, gelatin zymography. MDA-MB-231 cells were treated with Con A in SFM in the absence of MMP-2 for 0, 1, 2, 4, 6, 8, 12, and 24 h as indicated. The cells were then washed and incubated with SFM containing exogenous MMP-2 either alone (C), in the presence of 20 μg/ml Con A only (○), 1 μg/ml ActD only ( ), Con A plus ActD ( ), 20 μg/ml Chx only (■), or Con A plus Chx ( ) for 24 h after these treatments. MMP-2 activation was detected by gelatin zymography. B and C, densitometric analysis of zymography. The zymography gels demonstrating the effects of ActD ( ) and Chx (■) were analyzed by densitometry and plotted here. Lanes, proportion of activated MMP-2 (percentage of total active plus latent). In each case, the removal of Con A (O) and continued presence of Con A (D) lines are shown. In B, these are compared to removal of Con A but addition of ActD ( ) or readdition of Con A and addition of ActD ( ). Similarly, in C, these are compared to removal of Con A but addition of Chx ( ) or readdition of Con A and addition of Chx (■). D, Northern analysis of MT-MMP. Cells were plated overnight in 10% FCS/IMEM and washed three times in 10-cm diameter plastic dishes in SFM with 20 μg/ml Con A. Total RNA was isolated at times 0, 1, 2, 4, 6, 8, 12, 24, and 48 h after the addition of the Con A treatment, separated on a 1% denatured agarose gel, transferred to nylon membrane, and blotted with the MT-MMP cDNA probe. Lanes S, marker. The MT-MMP signal migrates to the 3.9-kb mark as indicated. Methylene blue staining of the 28S rRNA on the blot is shown for loading control. The intensity of each band was determined densitometrically, and the MT-MMP:28S ratio is plotted for each time point.
MT-MMP and MMP-2 Activation in HBC Cells

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B: Northern

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C: Western

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MT-MMP expression and MMP-2 activation in HBC cell lines. MT-MMP is a membrane-associated metalloproteinase recently discovered by PCR homology screening of a placental cDNA library (17, 18). The properties of MT-MMP are consistent with the membrane localization and inhibition profiles attributed previously to the cell surface-associated MMP-2 activator (see Refs. 13-17). Northern analysis of Con A-induced MDA-MB-231 cells revealed that MT-MMP mRNA is constitutively expressed under basal conditions and increased 2-3-fold upon Con A treatment (Fig. 2D). The induction of MT-MMP mRNA expression reached its maximum level after 8 h of Con A exposure, consistent with the 8-h window of efficacy for ActD and Chx inhibition of MMP-2 activation as shown in Fig. 2. It is interesting that the baseline expression of MT-MMP mRNA appears not to mediate extracellular activation of MMP-2 in the absence of Con A, although it may be involved in the early nontranscriptional response. Because protein synthesis appears to be required for this, Con A may induce translation of the constitutive MT-MMP mRNA.

We further compared the effects of Con A on MT-MMP mRNA expression and MMP-2 activation in MDA-MB-231 cells. Zymographic analysis of MMP-2 activation in duplicate cultures (Fig. 3A) revealed no detectable MMP-2 activation in untreated MDA-MB-231 control cells or cells treated with ActD only and only partial inhibition of Con A-induced MMP-2 activation by ActD. Northern analysis of MT-MMP expression (Fig. 3B) again showed a constitutive expression of MT-MMP mRNA in nontreated MDA-MB-231 cells and a 2-3-fold increase of MT-MMP expression after Con A treatment. Addition of ActD abrogated both constitutive and Con A-induced MT-MMP expression in MDA-MB-231 cells over the 24-h period. These data demonstrate the efficacy of the ActD treatment under the conditions used throughout the study and substantiate the nontranscriptional component described above.

Two inconsistencies were seen between the levels of steady-state MT-MMP mRNA and MMP-2 activation potential. A low level of constitutive MT-MMP mRNA expression is seen in untreated control cells in the absence of MMP-2 activation, and ActD completely...
abrogated *de novo* MT-MMP expression in Con A-treated MDA-MB-231 cells but only partially inhibited Con A-induced MMP-2-activation in these cells. Constitutively expressed mRNA encoding MT-MMP may not be translated into protein or may encode MT-MMP protein in an inactive form, consistent with the presence of a highly conserved MMP propeptide autoinhibitor domain. The MMP-2 activation seen with Con A treatment after ActD abrogation of steady-state MT-MMP levels suggests the involvement of either previously synthesized MT-MMP and/or some other component in Con A-induced MMP-2 activation in these cells. The turnover kinetics of MT-MMP is currently unknown.

Consistent with MT-MMP expression and MMP-2 activation data shown above, we have detected the induction of MT-MMP protein by Con A-treated MDA-MB-231 cells using Western analysis (Fig. 3C). We were unable to detect any MT-MMP protein in the untreated cells nor in the cells treated with ActD or Chx, even in the presence of Con A by Western analysis (Fig. 3C). Lack of MT-MMP in untreated MDA-MB-231 cells correlates well with lack of MMP-2 activation in these cells and may be due to a lack of protein translation from the constitutively expressed MT-MMP mRNA. Alternatively, MT-MMP protein may be present but below the sensitivity limit of our Western system. The status of MT-MMP in the uninduced cell remains to be resolved.

Strongin *et al.* (19) have demonstrated a two-stage loss of MMP-2 profragment during cellular activation, the first step resulting in cleavage before Leu^33^ and TIMP-2 inhibitable and the second step apparently resulting from autocatalytic cleavage at Tyr^81^ and being inhibited by both TIMP-1 and TIMP-2. Recent indications that MT-MMP can directly bind TIMP-2 with high affinity (19) suggest that the dynamics of TIMP-2 may be instrumental in mediating MT-MMP activation of MMP-2. Con A may also induce certain mechanism(s) already resident in MDA-MB-231 cells, which converts the inactive MT-MMP/MMP-2 activator into an active form, in addition to causing increased *de novo* gene expression of MT-MMP/MMP-2 activator. Our data showed that Con A-induced MMP-2 activation does appear to involve protein synthesis. It is important to note that although we have focused here on transcriptional induction of MT-MMP with Con A, Con A effects on TIMP-2 or other proteins may also occur in the same time frame. Given its broad binding capacity, Con A may also serve to physically concentrate MMP-2 and MT-MMP at the cell surface, facilitating both MT-MMP/MMP-2 interactions and autocatalytic processing of MMP-2. Furthermore, the generation of this "complex" at the cell surface is consistent with the observed requirement for membrane anchoring of the MT-MMP for MMP-2 activational activity (20).

Fig. 4 shows our hypothesized model for the mechanisms involved in Con A-induced MMP-2 activation in MDA-MB-231 cells. In this model, Con A triggers two distinguishable mechanisms for activating MMP-2. Con A, acting through some cell surface receptor(s), induces MT-MMP transcription, leading to increased steady-state levels of MT-MMP mRNA and protein. Con A also rapidly induces another pathway that may either directly or indirectly activate MMP-2, possibly by influencing MT-MMP. Induction of this pathway apparently requires protein synthesis. The transcriptional response to Con A, which includes MT-MMP up-regulation, does not cause increased MMP-2-activation unless Con A is present, suggesting some convergence of the transcriptional and nontranscriptional pathways. It seems quite possible that MT-MMP is involved in both these processes, although other molecules undoubtedly participate.

The MDA-MB-231 cell line represents the invasive and metastatic subset of HBC cell lines that display vimentin expression and lack E-cadherin (12). Con A induces MDA-MB-231 cells, as well as other vimentin-positive HBC cell lines, to activate MMP-2, and the presence and activation of MMP-2 has been associated with metastatic progression in HBC (6–9). Thus, Con A induction may provide a model for physiological or pathophysiological events occurring *in situ*. An understanding of these molecular events may be useful in directing diagnosis and therapy for metastatic breast cancer and possibly other metastatic diseases. The results of our study have associated MT-MMP with the HBC activation of MMP-2 and demonstrated
a complex interplay between MT-MMP, MMP-2, and possibly other components at the cell surface.

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References

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