Oxidative Stress and Prostate Cancer Progression Are Elicited by Membrane-Type 1 Matrix Metalloproteinase

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Abstract

Oxidative stress caused by high levels of reactive oxygen species (ROS) has been correlated with prostate cancer aggressiveness. Expression of membrane-type 1 matrix metalloproteinase (MT1-MMP), which has been implicated in cancer invasion and metastasis, is associated with advanced prostate cancer. We show here that MT1-MMP plays a key role in eliciting oxidative stress in prostate cancer cells. Stable MT1-MMP expression in less invasive LNCaP prostate cancer cells with low endogenous MT1-MMP increased activity of ROS, whereas MT1-MMP knockdown in DU145 cells with high endogenous MT1-MMP decreased activity of ROS. Expression of MT1-MMP increased oxidative DNA damage in LNCaP and in DU145 cells, indicating that MT1-MMP-mediated induction of ROS caused oxidative stress. MT1-MMP expression promoted a more aggressive phenotype in LNCaP cells that was dependent on elaboration of ROS. Blocking ROS activity using the ROS scavenger N-acetylcysteine abrogated MT1-MMP-mediated increase in cell migration and invasion. MT1-MMP-expressing LNCaP cells displayed an enhanced ability to grow in soft agar that required increased ROS. Using cells expressing MT1-MMP mutant cDNAs, we showed that ROS activation entails cell surface MT1-MMP proteolytic activity. Induction of ROS in prostate cancer cells expressing MT1-MMP required adhesion to extracellular matrix proteins and was impeded by anti-β1 integrin antibodies. These results highlight a novel mechanism of malignant progression in prostate cancer cells that involves β1 integrin-mediated adhesion, in concert with MT1-MMP proteolytic activity, to elicit oxidative stress and induction of a more invasive phenotype.

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Introduction

Prostate cancer, a disease which currently accounts for more than 27,000 deaths per year in the United States (1), is most often a disease effectively treated by surgery or radiation. However, in many cases, prostate cancer progresses from an indolent to a more aggressive state that is refractory to conventional therapy. The factors underlying prostate cancer transition to the more advanced state remain poorly understood and present a significant challenge for improving treatment and survival of prostate cancer patients.

Oxidative stress, a state in which cellular reactive oxygen species (ROS) level exceeds the ability to detoxify the ROS, plays a key role in eliciting oxidative stress in prostate cancer cells. Stable MT1-MMP expression increased oxidative DNA damage in LNCaP and DU145 cells, indicating that MT1-MMP-mediated induction of ROS caused oxidative stress. MT1-MMP expression promoted a more aggressive phenotype in LNCaP cells that was dependent on elaboration of ROS. Blocking ROS activity using the ROS scavenger N-acetylcysteine abrogated MT1-MMP-mediated increase in cell migration and invasion. MT1-MMP-expressing LNCaP cells displayed an enhanced ability to grow in soft agar that required increased ROS. Using cells expressing MT1-MMP mutant cDNAs, we showed that ROS activation entails cell surface MT1-MMP proteolytic activity. Induction of ROS in prostate cancer cells expressing MT1-MMP required adhesion to extracellular matrix proteins and was impeded by anti-β1 integrin antibodies. These results highlight a novel mechanism of malignant progression in prostate cancer cells that involves β1 integrin-mediated adhesion, in concert with MT1-MMP proteolytic activity, to elicit oxidative stress and induction of a more invasive phenotype.

A sizable body of literature has accumulated implicating membrane-type 1 matrix metalloproteinase (MT1-MMP), a member of a family of 24 zinc-dependent endopeptidases that mediates extracellular matrix (ECM) degradation and remodeling (7), in invasion and metastasis of many different cancer types including prostate cancer (8, 9). The ability of MT1-MMP to enhance cell migration is believed to be one of the key factors fostering cancer invasion and metastasis (10). As a member of the MMP family, MT1-MMP is able to degrade ECM components, including collagens, laminins, fibronectin, and vitronectin, which can clear a path to facilitate cell migration and invasion (10). In prostate cancer cell lines, increased MT1-MMP has been associated with the transition from androgen-dependent to -independent growth (11, 12). Higher MT1-MMP mRNA levels were reported in human precancerous prostatic intraepithelial neoplasia and in prostate cancer tissue than in benign epithelial tissue (13). Despite a body of

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evidence implicating MT1-MMP in prostate cancer invasion and in promoting prostate cancer aggressiveness, the underlying mechanism by which this occurs is poorly understood.

A link between a matrix metalloproteinase and oxidative stress had been previously described, in which MMP-3 expression in breast cancer cells caused oxidative stress via upregulation of Rac1b (14), prompting us to ask whether there is also an association between MT1-MMP and enhanced ROS production. We report herein that MT1-MMP expression triggers oxidative stress in prostate cancer cells. The presence of MT1-MMP produced a more aggressive phenotype in prostate cancer cells, as illustrated by enhanced cell migration and invasion and anchorage-independent cell growth, all of which required increased ROS production. We found that MT1-MMP-mediated elaboration of ROS requires MT1-MMP proteolytic activity and its localization at the cell surface. Because degradation and remodeling of the ECM by MT1-MMP play an integral role in promoting cell migration and invasion, we asked whether the interaction of MT1-MMP-expressing cells with the ECM can elicit oxidative stress. By determining ROS production on different substrates, we noted that MT1-MMP induction of ROS was influenced by β1 integrin–mediated adhesion to specific ECM substrates including collagen, laminin, and fibronectin. Our results allow us to propose a novel pathway of prostate cancer progression that entails integrin-mediated cell adhesion to the ECM, together with MT1-MMP proteolytic activity, which collectively generates oxidative stress and enhanced prostate cancer aggressiveness.

Materials and Methods

Cell culture

LNCaP cells stably transfected with green fluorescent protein (GFP) or with MT1-MMP-GFP were described previously (15). DU145 cells were from American Type Culture Collection. MT1-MMP small hairpin RNA (shRNA) constructs and retrovirus preparation were previously described (15). DU145 cells infected with retrovirus-encoding GFP or MT1-MMP shRNAs were cultured for 2 weeks with 4 μg/mL puromycin.

Detection and quantitative determination of ROS

Cells were stained with 25 μmol/L dihydroethidium 6G (DHR), 1 μmol/L PF-H₂TMRos, 5 μmol/L dihydroethidium (DHE), or 25 μmol/L 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluoresceindiacetate (CM-H₂DCFDA) and viewed with 510 to 560 nm excitation and 575 to 590 nm emission for DHR and PF-H₂TMRos and 450 to 490 nm (for CM-H₂DCFDA) and 585 nm (for DHE) bandpass filters.

Western blotting

Cells were lysed using SDS sample buffer and protein concentrations were determined using the bicinchoninic acid method (Thermo Fisher Scientific). Equal amounts of protein were electrophoresed through a NuPAGE 4% to 12% Bis–Tris acrylamide and transferred according to manufacturer’s instructions (Invitrogen Corp.). Western blotting was conducted as previously described (15).

8-Hydroxydeoxyguanosine

Genomic DNA was extracted using the DNEasy Blood and Tissue Kit (Qiagen Inc.) according to manufacturer’s instructions. Ten micrograms of each DNA sample was denatured with heat, digested with S1 nuclease, and dephosphorylated with shrimp alkaline phosphatase. 8-Hydroxydeoxyguanosine (8-OHdG) measurement was conducted by ELISA (Cell Biolabs, Inc.) according to manufacturer’s instructions.

Immunohistochemistry staining of 8-OHdG was conducted on rehydrated 5-μm paraffin sections from mouse xenograft tumors, using goat anti-8-OHdG (Abd Serotech) diluted in Tris-buffered saline with 1% normal rabbit serum. Secondary antibody staining was conducted using the Vector ABC system for anti-goat primary antibodies using biotin-labeled rabbit anti-goat horseradish peroxidase–conjugated streptavidin. Detection was conducted with 3,3′-diaminobenzidine reagents. Secondary antibody and detection reagents were from Vector Laboratories, Inc. and were used according to manufacturer’s instructions.

Cell migration and cell scattering

Cell migration was conducted using the BD Falcon FluoroBlok 96 System (BD Biosciences). Cells labeled with 5 nmol/L of DiIC₁₈ for 30 minutes were seeded in 4 replicates wells with 27,000 cells in serum-free, phenol red–free RPMI-1640 medium. Migrated cells through the opaque polyethylene terephthalate (PET) membrane after 8 hours were detected relative to control wells lacking cells using the SpectraMax M2 Microplate Reader (Molecular Devices, Inc.) at excitation/emission wavelengths of 549/565 nm.

To assay scattering, cells were seeded at 20,000 cells per mL in 0.2% rat tail Type I collagen (BD Biosciences) in RPMI-1640 medium at pH 7.5 and analyzed as previously described (15). Cells were imaged using Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System coupled to a Zeiss Inverted Axiovert
200 M Microscope using fluorescein isothiocyanate filter sets, and color-coded depth was rendered on Zeiss LSM Image Browser version 4.2.0.121 software.

**Xenograft prostate tumor growth**

A total of 1.5 × 10⁶ LNCaP/MT1-MMP and LNCaP/GFP cells were suspended in 50 μL of PBS, mixed with 50 μL Matrigel (BD Biosciences), and injected subcutaneously in the right flank of 6-week-old athymic (nu/nu) male mice (Taconic Farms). Animals were weighed, tumor dimension was measured with calipers, and volume was determined by the formula: $\text{length} \times \text{width}^{2}/2$. Animals were euthanized when the tumor diameter reached 1.5 cm, if the tumor was ulcerated, or if the animal lost 10% or more of its body weight. At euthanasia, tumor tissue was collected in 4% paraformaldehyde and embedded in paraffin.

**Soft agar clonogenic assay**

Cells were cultured in 96-well plates at 500 cells per well in 50 μL of 0.35% agar over a base of 50 μL of 0.5% agar in complete medium. Live colonies in each well was monitored by adding alamarBlue (Invitrogen Corp.) solution to cells per manufacturer instructions for 24 hours before measuring fluorescence at 570-nm excitation and 585-nm emission.

**MT1-MMP mutants and gelatin zymography**

COS-1 cells were transiently transfected with MT1-MMP mutant constructs including MT1Δ535 (17) and MT1E240→A (17). The MTAPEX construct was prepared as previously described (18). In brief, an N-terminal MT1-MMP fragment using the MT1-MMP forward primer (5'-CAGCAATTCGGACCATGTCTCCCGCCCCAA GA-3') and reverse primer (5'-AGCGCGCTCACCG CCCACAGATGTTGGGCCCATA-3') and a C-terminal fragment using the forward primer (5'-GGGCGGTGAGCGCGCCGTCGGG-3') and reverse MT1-MMP primer (5'-ACCCTGGATGGCGTAGAAGCT-3') were generated from the MT1-MMP cDNA template. The products were used as template to generate MT1-MMP with a deleted PEX domain using cDNA template. The products were used as template to generate MT1-MMP with a deleted PEX domain using cDNA template.

**Fluorescence-labeled gelatin degradation assay**

Acid-washed cover glasses were coated with a solution of Texas Red–labeled gelatin, 1 mg/mL in 70% glycerol for 1 hour at room temperature in the dark. Coated cover glasses were viewed and photographed at excitation/emission wavelengths of 549/565 nm.

**Cell adhesion**

Cells were prelabeled with 5 μmol/L Calcein AM (Invitrogen Corp.), counted on the Cellometer Auto T4 (Nexcelom Bioscience) cell counter, and seeded at 1.5 × 10⁵ cells per cm² in triplicate wells of 96-well Millipore Millicon ECM-coated plates [collagen I, collagen IV, laminin, fibronectin, vitronectin, and bovine serum albumin (BSA)] with or without anti-integrin antibodies in serum-free media for 1 to 2 hours. ECM-coated plates were purchased from Millipore. Prelabeled cells seeded with 2 μg/mL monoclonal anti-β1 integrin (clone 4B4; Beckman-Coulter) or monoclonal anti-α6 integrin (Mab 13444-20; Millipore) antibodies were preincubated with the antibodies for 30 minutes at room temperature before seeding. Fluorescence was determined at excitation/emission wavelengths of 487/520 nm before and after washing twice with serum-free media. Adhesion was expressed for each respective well, as the percentage of fluorescence after relative to before washing.

**Aconitase assay**

All reagents and chemicals were purchased from Cayman Chemical Company and the assay was conducted according to manufacturer's instructions. Brieﬂy, cells cultured in 10-cm diameter plates were washed with cold PBS, scraped, transferred to Eppendorf tubes, and pelleted at 800 × g for 10 minutes at 4°C. The cell pellet was resuspended in 50 mmol/L Tris, pH 7.4, containing sodium citrate, sonicated to homogenize, and spun at 20,000 × g for 15 minutes at 4°C. Total protein level was determined using the bicinchoninic acid assay (Thermo Fisher Scientific) on the supernatant. Fifty micrograms of each supernatant sample, with or without oxalomalate inhibitor, was mixed with NADP⁺, isocitric dehydrogenase, and sodium citrate and incubated at 37°C for 15 minutes. A340 was determined every 30 seconds for a total of 15 minutes and the slope of A340 versus time in minutes was determined. The slope of each sample was subtracted from the slope of blank samples, and aconitate activity for each cell sample was determined by the following formula:

$$\frac{\text{Slope (Sample)} - \text{Slope (Sample – Inhibitor)}}{0.313 \times \text{Total volume}}$$

where 0.313 (μmol/L)⁻¹ is the extinction coefficient of the NADPH adjusted for the path length of samples in a 96-well plate.

**Results**

**MT1-MMP induces oxidative stress in prostate cancer cells**

Both oxidative stress (2, 3, 6) and MT1-MMP expression (12, 13, 20, 21) have been reported to be important for prostate cancer pathogenesis and aggressiveness. To test whether there is a link between MT1-MMP expression and ROS activity in prostate cancer, we assayed ROS...
activity in MT1-MMP-expressing prostate cancer cells, using a number of different dyes designed to detect ROS. These include DHE, DHR, CM-H₂DCFDA, and PF-H₂TMRos (Fig. 1).

Using androgen-dependent LNCaP cells which express an undetectable level of MT1-MMP, we measured ROS levels in LNCaP cells stably transfected with MT1-MMP-GFP cDNA or with control GFP cDNA (15). By adding DHE to adherent cells, followed by flow cytometry, we found that LNCaP/MT1-MMP-GFP cells had significantly greater DHE fluorescence than LNCaP/GFP or untransfected LNCaP cells (Fig. 1A, left). We were unable to use...
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CM-H₂DCFDA for flow cytometry in these cells because the GFP excitation/emission properties in these cells overlap with those of CM-H₂DCFDA and interfered with flow cytometric measurements. To confirm results obtained from DHE staining, we used DHR, which, like CM-H₂DCFDA, is sensitive to oxidation by hydrogen peroxide and PF-H₂TMRos, an indicator of intracellular redox potential, for ROS staining in cells. In accordance with results from flow cytometry, we noted increased intracellular fluorescence intensity of both DHR and PF-H₂TMRos by fluorescence microscopy, in MT1-MMP-GFP–expressing LNCaP cells relative to LNCaP/GFP (Fig. 1A, right). These observations were consistent with a link between overexpression of MT1-MMP and elevated ROS.

Figure 1. Expression of MT1-MMP in prostate cancer cells elicits oxidative stress. A, left, untransfected LNCaP, GFP-transfected control (LNCaP/GFP), and MT1-MMP–GFP–transfected LNCaP cells (LNCaP/MT1-MMP-GFP) were stained with the DHE for 1 hour, washed, and fluorescence intensity of 10,000 cells per group was analyzed by flow cytometry at excitation/emission wavelengths of 490/585 nm. Data represent the geometric mean of 3 experiments, each result of which was normalized to DU145/GFP shRNA (Fig. 1B, left) and by Western blotting (data not shown) compared with DU145 cells expressing an irrelevant GFP shRNA (DU145/GFP shRNA). We assessed ROS production in these cell lines using PF-H₂TMRos and CM-H₂DCFDA. Intracellular CM-H₂DCFDA fluorescence intensity, as determined by flow cytometry, showed significantly decreased mean fluorescence intensity in both DU145/MT1 shRNA1 and DU145/MT1 shRNA2 cells than in uninfected DU145 cells or DU145/GFP shRNA (Fig. 1B, middle). Decreased redox potential, as indicated by PF-H₂TMRos intensity, was also observed in DU145/MT1 shRNA than in DU145/GFP shRNA cells (Fig. 1B, right). We further confirmed importance of MMP proteolytic activity in ROS generation by comparing ROS levels of DU145/GFP and DU145/MT1 shRNA with the respective cell lines treated with 1 μmol/L of the broad-spectrum MMP inhibitor BB3103 (Fig. 1C).

Decreased ROS in DU145/GFP cells upon BB3103 treatment provides further evidence that activity of MMPs, in particular, that of MT1-MMP can induce ROS. One of the consequences of increased ROS and oxidative stress is oxidative DNA damage, measured by determining the level of 8-OHdG, a byproduct of DNA damage. Thus, 8-OHdG is a commonly used marker of oxidative stress. Moreover, oxidative damage to DNA is thought to have significant implications for prostate cancer tumorigenesis by contributing to increased mutation rates and genomic instability (22, 23). Using an ELISA to measure 8-OHdG, we found that LNCaP/MT1-MMP-GFP cells displayed significantly increased 8-OHdG levels than LNCaP/GFP and untransfected LNCaP cells (Fig. 1D, left). Conversely, DU145/MT1 shRNA1 cells had significantly decreased level of 8-OHdG relative to control DU145/GFP shRNA (Fig. 1D, left). To determine whether MT1-MMP expression can cause prostate cancer oxidative stress in vivo, LNCaP/GFP and LNCaP/MT1-MMP-GFP cells were injected into nude mice. As expected from previous studies (24), mean tumor volume was significantly greater within 25 days in mice injected with MT1-MMP-GFP–transfected LNCaP cells than that of GFP-transfected LNCaP cells (data not shown). Immunohistochemical staining of 8-OHdG in tumor tissue sections from these mice showed greater staining in LNCaP/MT1-MMP-GFP cancer cells than in LNCaP/GFP cells (Fig. 1D, right), consistent with findings by 8-OHdG ELISA in the cell lines (Fig. 1D, left).

To confirm the existence of oxidative stress, we measured the aconitase activity of LNCaP/GFP and LNCaP/MT1-MMP-GFP. Aconitase is an iron–sulfur protein that catalyzes the conversion of citrate to isocitrate (25). Exposure of aconitase to pro-oxidants has been shown to inhibit its activity (26); thus, loss of aconitase activity is a sensitive indicator of oxidative damage, and aconitase suppression is a sensitive endogenous marker of ROS. We found that LNCaP/MT1-MMP-GFP had more than 3-fold lower aconitase activity than control LNCaP/GFP cells.
little color variation. These results support the notion that resulting in all cells in the depth-colored field displaying depth coloration ranging from red to blue.

LNCaP/MT1-MMP-GFP cells, with cells in the same field displaying 3-dimensional scattering of interference contrast and confocal laser scanning microscopy and Zeiss LSM Image Browser version 4.2.0.121 software to render relative depth using color codes, with cells colored from red to blue representing closest to farthest, respectively. Thus, cells displaying 3-dimensional scattering would be expected to display a greater spectral range, indicating variety of depth. LNCaP cells are minimally invasive and are unable to migrate and scatter in a 3-dimensional collagen matrix (15), and as expected, LNCaP/GFP displayed very little color variation in the same field, indicating lack of 3-dimensional scattering. As we anticipated, expression of full-length MT1-MMP in LNCaP cells stimulated cell scattering/invasion at 4 days, in a 3-dimensional collagen matrix relative to control LNCaP/GFP cells, as shown in Figure 2A. Both differential photomicrograph images from interference contrast and confocal laser scanning microscopy with color-coded depth show 3-dimensional scattering of LNCaP/MT1-MMP-GFP cells, with cells in the same field displaying depth coloration ranging from red to blue. Treatment of LNCaP/MT1-MMP-GFP with NAC was found to inhibit MT1-MMP–mediated cell scattering, resulting in all cells in the depth-colored field displaying little color variation. These results support the notion that MT1-MMP–induced cell scattering/invasion is dependent on ROS activity.

Because ROS is required for MT1-MMP–mediated cell invasion, we asked whether MT1-MMP–induced ROS activity plays a role in cell migration. To this end, we tested relative cell migration in a modified Boyden chamber in the presence of 1 mmol/L NAC. In agreement with previous reports (10, 24, 27), MT1-MMP–promoted LNCaP cell migration as compared with controls (Fig. 2B). Interestingly, addition of NAC reduced MT1-MMP–enhanced cell migration to the level of GFP–expressing LNCaP cells (Fig. 2B), suggesting that MT1-MMP–mediated prostate cancer cell migration and invasion occurs through elevated ROS activity. These results are consistent with previously published observations (28) that repeated exposure of epithelial cells to sublethal doses of hydrogen peroxide, over as short a period as 2 days, caused an increase in invasive behavior.

MT1-MMP promotes a more invasive, aggressive phenotype via an ROS-dependent mechanism

To determine whether ROS influences the ability of MT1-MMP to promote invasion, we cultured LNCaP/GFP and LNCaP/MT1-MMP-GFP cells in 0.2% type I collagen with or without 1 mmol/L of the ROS scavenger N-acetylcysteine (NAC). We had verified independently that 1 mmol/L NAC is not cytotoxic by alamarBlue fluorescence detection of viable cells (data not shown). Presence of GFP in our LNCaP cells allowed us to use confocal laser scanning microscopy and Zeiss LSM Image Browser version 4.2.0.121 software to render relative depth using color codes, with cells colored from red to blue representing closest to farthest, respectively. Thus, cells displaying 3-dimensional scattering would be expected to display a greater spectral range, indicating variety of depth. LNCaP cells are minimally invasive and are unable to migrate and scatter in a 3-dimensional collagen matrix (15), and as expected, LNCaP/GFP displayed very little color variation in the same field, indicating lack of 3-dimensional scattering. As we anticipated, expression of full-length MT1-MMP in LNCaP cells stimulated cell scattering/invasion at 4 days, in a 3-dimensional collagen matrix relative to control LNCaP/GFP cells, as shown in Figure 2A. Both differential photomicrograph images from interference contrast and confocal laser scanning microscopy with color-coded depth show 3-dimensional scattering of LNCaP/MT1-MMP-GFP cells, with cells in the same field displaying depth coloration ranging from red to blue. Treatment of LNCaP/MT1-MMP-GFP with NAC was found to inhibit MT1-MMP–mediated cell scattering, resulting in all cells in the depth-colored field displaying little color variation. These results support the notion that MT1-MMP–induced cell scattering/invasion is dependent on ROS activity.

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ref. 15), and a tethering-terminal domain mutant that removes both the cytoplasmic and transmembrane domains and thus converts the MT1-MMP molecule into a soluble, secreted form (MT1Δ535), as described schematically in Figure 3A (left). Transfection efficiency was observed to be 40% to 60%, based on estimates from control transfections of GFP-expressing vector (data not shown). Western blot analysis of equal amounts of protein from each
transfected cell sample showed that the expression level of MT1-MMP wild-type and deletion mutants were similar (Fig. 3A, right).

Full-length MT1-MMP was able to proteolytically activate pro-MMP-2 to its fully active form, as shown by gelatin zymography, whereas MT1-MMP deletion mutants were unable to convert pro-MMP-2 to active MMP-2 (Fig. 3B). COS-1 cells transfected with full-length MT1-MMP were also able to directly degrade Texas Red–labeled gelatin substrate, as shown by a Texas Red–free cleared area surrounding some MT1-MMP–transfected cells (Fig. 3C). Significant Texas Red–labeled gelatin degradation was also observed by COS-1 cells transfected with MTΔPEX (Fig. 3C), suggesting that the PEX domain of MT1-MMP is not required for direct degradation of ECM substrates. However, cells transfected with either MT1/E240→A or MT1Δ535 were unable to effect degradation of Texas Red–labeled gelatin to a visually appreciable extent (Fig. 3C), suggesting a necessity for the catalytic domain and for cell membrane localization in direct MT1-MMP proteolysis of ECM substrates.

Transfected cells were stained with DHE, and mean intracellular fluorescence was determined by flow cytometry. Results from 3 independent experiments were normalized relative to full-length MT1-MMP–transfected cells, and the mean ROS levels are displayed graphically (Fig. 3D). Of the mutants tested, the MT1/E240→A catalytic domain mutant displayed approximately 67% reduction in ROS relative to full-length MT1-MMP–transfected cells, using DHE fluorescence of vector-transfected cells as baseline. The MT1Δ535 membrane tethering domain deletion mutants displayed a more modest, yet consistent approximately 27% reduction in ROS, relative to full-length MT1-MMP–transfected cells (Fig. 3D). These results suggest that MT1-MMP catalytic function and its ability to be localized to the cell membrane are important properties for induction of ROS. The MTΔPEX transfec-

tants did not display decreased ROS, indicating that the

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**Figure 3.** Induction of ROS requires MT1-MMP proteolytic activity and membrane anchorage. COS-1 cells were transiently transfected with expression vectors expressing full-length MT1-MMP or MT1-MMP mutants. A, MT1-MMP constructs are shown schematically (left). Relative expression of each transfectant was monitored by Western blotting using anti-MT1-MMP antibody to the hinge region to stain MT1-MMP and anti-α-tubulin antibody to stain tubulin loading control (right). B, ability of each MT1-MMP construct to activate pro-MMP-2 was determined by assessing the activity of fully active MMP-2 in the conditioned media of each COS-1–transfected sample via gelatin zymography. Latent, intermediate, and fully active MMP-2 are shown highlighted by arrows. C, transfected cells were seeded onto cover glasses coated with Texas Red–labeled gelatin in serum-free medium and incubated overnight. Cover glasses were fixed with 4% paraformaldehyde and mounted onto glass slides. The capability of each MT1-MMP construct to effect degradation of Texas Red–labeled gelatin, shown as black areas, is shown highlighted by arrows. Because transfection efficiency of COS-1 cells is approximately 50% (data not shown), only 50% of the cells in each panel would be expected to be expressing MT1-MMP constructs. Bar represents 50 μm. D, transfected cells were assayed for ROS by labeling with 5 μmol/L DHE for 2 hours and determining the mean fluorescence intensity of each sample by flow cytometry. Each data point in the graph represents the mean of 3 independent experiments, each normalized to full-length MT1-MMP–transfected controls. ± SEM. *, P < 0.05 as determined from 2-tailed Student’s t test in comparing DHE levels with full-length MT1-MMP transfectants using fluorescence of vector-transfected cells as baseline. TM, transmembrane.
PAX domain of MT1-MMP is not vital for MT1-MMP induction of ROS.

MT1-MMP induction of ROS involves β1 integrin–mediated adhesion to ECM components

Because induction of ROS requires MT1-MMP proteolytic activity localized to the cell surface, and MT1-MMP degrades and remodels the ECM (10), we asked whether MT1-MMP can induce ROS by mediating cell interactions with the ECM. To address this hypothesis, LNCaP/GFP and LNCaP/MT1-MMP-GFP cells were cultured in serum-free medium on tissue culture plates coated with selected ECM components. Cell attachment to tissue culture plastic has been shown to depend directly on the ability of the tissue culture plastic material to selectively adsorb ECM proteins such as fibronectin and vitronectin (29–31). The cells, themselves, produce and secrete ECM proteins that become adsorbed onto the tissue culture plastic and influence cell attachment. We chose to control the interactions of our experimental cells with the substratum by culturing cells in serum-free media on tissue culture plastic plates coated with collagen I, collagen IV, fibronectin, laminin, vitronectin, or BSA control.

The cells displayed morphologic variations on different ECM substrates with very little cell attachment and spreading on collagen types I and IV and, as expected, on BSA (Fig. 4A, left). Measurement of adhesion 2 hours after cell seeding confirmed our visual observation of low adhesion to collagen I, collagen IV, vitronectin, and BSA and high levels of adhesion in cells cultured on fibronectin and laminin (Fig. 4A, right). We noted, furthermore, that LNCaP/MT1-MMP-GFP cells displayed greater cell adhesion to all ECM substrates tested and were markedly greater in the cases of fibronectin and laminin (Fig. 4A, right). These results suggested that MT1-MMP plays a key role in MT1-MMP induction of ROS.

As we were studying the interaction between the cells and the ECM, we chose to quantify intracellular ROS of adherent cells in situ, by photographing the cells at constant exposure times and measuring intracellular PF-H2TMRos fluorescence intensity via image analysis software. We had observed that intracellular ROS levels quantified by this method were highly reproducible and closely matched results obtained using flow cytometry.

LNCaP/MT1-MMP-GFP displayed significantly increased ROS relative to LNCaP/GFP cells when cultured on laminin or fibronectin substrates, as shown by quantitative determination of PF-H2TMRos fluorescence (Fig. 4B, left) and in fluorescent micrographs (Fig. 4B, right). In contrast, no significant differences in ROS level between LNCaP/MT1-MMP-GFP and LNCaP/GFP cells were noted when cells were cultured on collagen I, collagen IV, vitronectin, or BSA (data not shown). These data indicated that ROS induction by MT1-MMP occurred only on substrates to which the cells adhered well, in particular, laminin and fibronectin. Because LNCaP/MT1-MMP-GFP cells displayed markedly greater adhesion to laminin and fibronectin than control LNCaP/GFP cells, this suggested that cell adhesion plays an important role in MT1-MMP–mediated induction of ROS.

Discussion

As the functional interactions of cells with the ECM are known to involve integrins (32), we asked whether disruption of cell adhesion using anti-integrin antibodies can also prevent induction of ROS in MT1-MMP–expressing cells. Seeding of LNCaP/MT1-MMP-GFP cells in the presence of anti-β1 integrin antibody (2 μg/mL) lowered their adhesion to all ECM substrates tested including laminin, fibronectin, and vitronectin (Fig. 4C, left). In contrast, culture of these cells in the presence of an α6 integrin antibody (2 μg/mL) had no significant effect on cell adhesion to any ECM substrates tested (Fig. 4C, left). Inhibition of adhesion by anti-β1 integrin antibody also resulted in loss of LNCaP/MT1-MMP-GFP ability to induce ROS when cultured on laminin, as shown using DHE staining (Fig. 4C, right). These results suggested that adhesion of LNCaP/MT1-MMP-GFP to the ECM was mediated specifically by β1 integrin, and that this adhesion plays a key role in MT1-MMP induction of ROS.

To confirm the role of MT1-MMP and cell adhesion on ROS induction, we also assessed adhesion of DU145/GFP shRNA and DU145/MT1 shRNA1 in serum-free media on collagen I, collagen IV, fibronectin, laminin, vitronectin, and BSA. We found that, unlike LNCaP cells, both control DU145/GFP shRNA and DU145/MT1 shRNA adhered well to collagen I, collagen IV, as well as to fibronectin and laminin (Fig. 4D, left). Adhesion was, as for LNCaP cells, inhibited with anti-β1 integrin antibodies and not by anti-α5 integrin antibodies (Fig. 4D, middle). Anti-β1 integrin antibodies also inhibited ROS production in DU145/GFP cells (Fig. 4D, right) and in DU145/MT1 shRNA (not shown), as evidenced by moderately decreased PF-H2TMRos intracellular fluorescence intensity (Fig. 4D, right). These results further lend support for the role of β1 integrin–mediated cell adhesion in ROS induction by MT1-MMP.

Induction of ROS does not require MT1-MMP activation of pro-MMP-2

Among the many proteolytic functions of MT1-MMP is its ability to convert the zymogen pro-MMP-2 to its active form. Because induction of ROS by MT1-MMP requires active MT1-MMP, we asked whether MT1-MMP activation of pro-MMP-2 is involved in the pathway. To address this question, we cultured LNCaP/GFP and LNCaP/MT1-MMP-GFP cells in serum-free media with or without recombinant active MMP-2 for up to 24 hours (Fig. 5A). As shown in Figure 5A and B, active MMP-2 did not increase the level of ROS in LNCaP/GFP cells. These results indicate that MMP-2 activation by MT1-MMP is not required for its pro-ROS activation function.

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MT1-MMP Induction of ROS in Prostate Cancer Invasion

exemplified by LNCaP, ectopic expression of MT1-MMP triggered induction of ROS. In DU145 cells, an androgen-independent cell line that possesses a more malignant phenotype than LNCaP cells (6), MT1-MMP knockdown resulted in approximately 50% decrease in ROS. Cellular ROS levels were quantified by staining adherent cells with DHE and CM-H2DCFDA, followed by cell lifting with trypsin-EDTA and flow cytometric analysis. This method provides a reliable measurement of ROS in adherent cells, as the ROS dyes oxidized by elevated ROS are retained in the cells (37, 38) even after the cells are placed in suspension. Because ROS determinations using redox-sensitive dyes can be argued as having nonspecific effects (39), we confirmed our findings using aconitase as an endogenous oxidative substrate. Because aconitase activity is inhibited by oxidation, our results showing more than 3-fold decreased aconitase activity in LNCaP/MT1-MMP-GFP cells than in control LNCaP/GFP cells provide evidence that MT1-MMP expression leads to oxidative damage to an endogenous enzyme. Oxidative stress can lead to progressive accumulation of oxidative DNA damage, which is thought to contribute to increased genomic instability and greater malignancy (40). In accordance with this idea, previous studies have shown that exposure of mammary epithelial cells to low levels of hydrogen peroxide over a prolonged period resulted in conversion to a more malignant phenotype (28). Radisky and colleagues (14) showed that MMP-3 was able to induce genomic instability in breast cancer cells through a ROS-mediated pathway. Of interest, results from a number of different studies have showed that expression of MT1-MMP in epithelial cells caused chromosomal instability and aneuploidy (41) and leads to malignant transformation (41, 42).

In the current study, we have shown that MT1-MMP expression in prostate cancer cells caused these cells to acquire a more aggressive phenotype, as displayed by increased migration, invasion, and anchorage-independent growth and that these qualities were dependent on enhanced ROS production. We noted that inhibition of ROS with the antioxidant NAC had an antiproliferative effect on both LNCaP/MT1-MMP and control LNCaP/GFP in soft agar. This is consistent with the well-documented observations that low levels of ROS have a growth-promoting effect on stress is provoked and the role it plays in prostate cancer development and etiology are poorly understood. The "oxidative aging" theory proposes that accumulated oxidative damage associated with aging increases an individual’s risk of developing cancer (33, 34). This is supported by findings of increasing oxidative mitochondrial damage and mutations with age (35, 36).

In this report, we show a mechanism by which expression of MT1-MMP, a molecule associated with cancer cell invasion and metastasis (8) in prostate cancer cells triggers an oxidative stress pathway leading to aggressive malignancy. In cells without appreciable levels of MT1-MMP, as

MT1-MMP induction of ROS is influenced by integrin-mediated adhesion to ECM components. A, Control LNCaP or LNCaP/GFP and LNCaP/MT1-MMP-GFP cells were cultured for ~24 hours on various ECM substrates, including collagen I (COL I), collagen IV (COL IV), laminin (Ln), fibronectin (Fn), and vitronectin (Vn), and control bovine serum albumin (BSA) in serum-free media. Differences in cell spreading between LNCaP and LNCaP/MT1-MMP-GFP (A, left) and adhesion (A, right) on different ECM substrates are shown. #, P < 0.01 between LNCaP/GFP and LNCaP/MT1-MMP-GFP on each respective ECM; *, P < 0.01; **, P < 0.001 relative to BSA control. B, ROS was increased in LNCaP/MT1-MMP-GFP only in cells cultured on laminin and fibronectin, as shown graphically (B, left), and as displayed in a representative image (B, right). Intracellular ROS levels were determined on adherent cells by quantifying the mean intracellular fluorescence intensity per cell area of images of PF-H2TMRos-stained cells treated (as described in B).
cultured mammalian cells (43–46). A modest level of ROS (≈3 μmol/L) in cells, rather than promoting oxidative stress, is thought to play an integral role in the signaling pathway regulating cell proliferation (45). Thus, the antiproliferative effect of ROS inhibition in cells that are not in a state of oxidative stress is consistent with the notion that a low level of ROS is important for regulating cell proliferation. However, treating cells with higher levels of ROS (≈120 μmol/L) have been reported to profoundly alter the cellular gene expression profile (45). Exposure of the mouse mammary epithelial cells NMuMG to similarly high concentrations of ROS caused their transition to a more invasive phenotype (28). While we do not know the intracellular ROS concentration of our experimental prostate cancer cells, our results indicate that MT1-MMP expression in these cells promote oxidative stress, as evidenced by increased 8-OHdG levels. The changes brought on by oxidative stress, including altered expression of numerous genes, as previously reported (45), can facilitate prostate cancer cells to acquire a more invasive phenotype and to enhance anchorage-independent growth (28).

Induction of ROS required MT1-MMP proteolytic activity, as evidenced by a loss of ROS induction in MT1/E240A-transfected COS-1 cells relative to full-length MT1-MMP–transfected cells. Loss of the transmembrane domain, rendering MT1-MMP soluble, rather than membrane-bound, also consistently resulted in decreased ROS induction, suggesting the importance of membrane localization. It is not directly clear from our results why neither the MT1/E240A nor the MT1Δ535 mutant constructs inhibited ROS to the level of vector control. We have found that ROS levels of LNCaP/MT1/E240A-GFP stable cell lines were also not decreased to the level of control LNCaP/GFP cells (data not shown), which supports the notion that catalytic activity by MT1-MMP, alone, cannot activate ROS to the level of full-length MT1-MMP. The observation that both the MT1/E240A mutant and the MT1Δ535 partially decreased ROS level relative to vector control leads us to hypothesize that both catalytic activity and membrane localization by MT1-MMP are required for full activation of ROS.

Activation of pro-MMP-2 does not appear to play a significant role in MT1-MMP–mediated ROS production, as evidenced by lack of ROS response in LNCaP cells to addition of active MMP-2. These results suggest that ROS induction occurs via direct MT1-MMP proteolytic activity at the cell surface, rather than indirectly via pro-MMP-2 activation. Local targets in close proximity to the cell surface, such as components of ECM or associated with the ECM, or molecules located on the cell surface would be the most likely targets of MT1-MMP proteolytic activity along the pathway leading to oxidative stress. Because MT1-MMP interacts with and can proteolytically remodel the ECM, we chose to investigate whether MT1-MMP induction can be influenced by the presence of different ECM substrates.

We found that induction of ROS by MT1-MMP requires adhesion to ECM substrates such as collagen, laminin, and fibronectin and this adhesion can be regulated by β1 integrin. LNCaP/MT1-MMP-GFP cells displayed both greater adhesion and generated significantly greater ROS than control LNCaP/GFP cells when cultured on either laminin or fibronectin. ROS levels in LNCaP/MT1-MMP and in DU145/GFP shRNA control cells were decreased by the presence of anti-β1 integrin antibodies, which also abrogated adhesion in these cells. These results suggest that one of the ways MT1-MMP promotes oxidative stress is via modulating cell adhesion to the ECM.

These results raise the question of how cell adhesion to the ECM plays a role in MT1-MMP–mediated generation of oxidative stress. Our laboratory had previously determined that MT1-MMP promigratory influence is dependent on signaling by the small GTPase Rac1 (17). Activated GTP-Rac1 is a regulator of the membrane-associated NADPH oxidase (Nox) enzymes that reduce molecular oxygen to superoxide, which then go on to generate a variety of different ROS species (47). Furthermore, Nox1-mediated generation of ROS has been shown to be regulated by Rac1 (48). We have recently found that treating MT1-MMP-GFP cells with the Nox inhibitor diphenyliodonium eliminated much of the observed ROS increase (Nguyen and colleagues, unpublished data). Rac acts reciprocally with Rho in actin assembly and focal adhesion stability. Rho promotes the formation of stress fibers and focal adhesions whereas Rac1 can suppress Rho activity, destabilizing focal adhesions and support focal complexes such as lamellipodia (49). It is interesting to note that MT1-MMP has been reported to destabilize focal adhesion complexes (50), suggesting that it may play an important role in the same or parallel pathway as that in which Rac1 resides. While data presented here do not fully elucidate the pathway by which MT1-MMP produces oxidative stress, the evidence that cell adhesion to the ECM accompanied by MT1-MMP proteolytic activity is involved, that ROS is generated by the Nox system, and that increased ROS influences cell migration, collectively suggest that the Rac1-Nox pathway is involved. Additional research along this line of thought will need to be pursued to better understand the role of MT1-MMP in this pathway.

Data presented here suggest that MT1-MMP expression modulates adhesion of cancer cells to the ECM. These results appear consistent with previous data that showed prolonged exposure of cultured cells to hydrogen peroxide induced upregulation of select integrins (28). We showed here that both, adhesion to the ECM and MT1-MMP proteolytic activity, are important for MT1-MMP–mediated ROS induction. It is uncertain from these results whether MT1-MMP proteolytic activity regulates cell adhesion or if the proteolytic activity is required subsequent to cell adhesion in ROS generation.

We also found that inhibition of ROS with NAC abrogated ability of both LNCaP/GFP and LNCaP/MT1-MMP-GFP to form colonies in soft agar. These findings are in agreement with previous reports indicating that the transformed state of cancer cells is regulated by ROS (51, 52). However, they also raise the question of...
the role of integrin-mediated adhesion in ROS induction in the setting of anchorage-independent growth, where there is presumably little or no adhesion involved. We have observed that LNCaP/MT1-MMP-GFP displayed increased ROS than LNCaP/GFP, rapidly after addition to tissue culture plates, before significant adhesion was apparent (Supplementary Fig. S1), suggesting that while adhesion to the ECM can play an important role in eliciting ROS, MT1-MMP can also induce ROS independently of adhesion to the ECM.

Our results nevertheless show that MT1-MMP expression elicits oxidative stress in prostate cancer cells and that this oxidative stress plays an important role in cell migration and invasion, promotes oxidative DNA damage, and contributes to increased malignancy. These results suggest a novel pathway by which MT1-MMP, an important component of the cancer cell migration and invasion machinery, can contribute to prostate cancer malignancy by triggering oxidative stress. Additional studies will be needed to further dissect the signaling pathway(s) involved.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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