OXIDATIVE STRESS AND PROSTATE CANCER PROGRESSION ARE ELICITED BY MEMBRANE-TYPE 1 MATRIX METALLOPROTEINASE (MT1-MMP)

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ABSTRACT

Oxidative stress caused by high levels of reactive oxygen species (ROS) has been correlated with prostate cancer (PCa) aggressiveness. Expression of membrane-type 1-matrix metalloproteinase (MT1-MMP), which has been implicated in cancer invasion and metastasis, is associated with advanced PCa. We demonstrate here that MT1-MMP plays a key role in eliciting oxidative stress in PCa 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 ROS. Expression of MT1-MMP increased oxidative DNA damage in LNCaP and in DU145 cells, indicating 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 (NAC), 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. Employing cells expressing MT1-MMP mutant cDNAs, we demonstrated that ROS activation entails cell surface MT1-MMP proteolytic activity. Induction of ROS in PCa cells expressing MT1-MMP required adhesion to extracellular matrix (ECM) proteins and was impeded by anti-β1 integrin antibodies. These results highlight a novel mechanism of malignant progression in PCa 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.
INTRODUCTION

Prostate cancer (PCa), 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, PCa progresses from an indolent to a more aggressive state that is refractory to conventional therapy. The factors underlying PCa transition to the more advanced state remain poorly understood and present a significant challenge for improving treatment and survival of PCa patients.

Oxidative stress, a state in which cellular reactive oxygen species (ROS) level exceeds the ability to detoxify the ROS or to repair the ROS-mediated damage, has been demonstrated to significantly increase the risk of developing PCa (2-5). Oxidative stress in PCa cells has been shown to be necessary for their invasive phenotype, with more aggressive PCa cells displaying greater oxidative stress than less aggressive cells (6). While there is mounting evidence that oxidative stress plays an important role in development and progression of PCa, there is incomplete understanding of the source and role of oxidative stress in PCa.

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 PCa (8, 9). MT1-MMP’s ability 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 PCa cell lines, increased MT1-MMP has been associated with the transition from androgen-dependent to androgen-independent growth (11, 12). Higher MT1-MMP mRNA levels were reported in human pre-cancerous prostatic intraepithelial neoplasia and in PCa tissue than in benign epithelial tissue (13). In spite of a body of evidence
implicating MT1-MMP in PCa invasion and in promoting PCa 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 if there is also an association between MT1-MMP and enhanced ROS production. We report herein that MT1-MMP expression triggers oxidative stress in PCa cells. The presence of MT1-MMP produced a more aggressive phenotype in PCa 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. Since MT1-MMP’s degradation and remodeling of the ECM plays an integral role in promoting cell migration and invasion (10), we asked if 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 PCa progression that entail integrin-mediated cell adhesion to the ECM, together with MT1-MMP proteolytic activity, which collectively generates oxidative stress and enhanced PCa aggressiveness.
MATERIALS AND METHODS

Cell culture

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

Detection and quantitative determination of ROS

Cells were stained with 25μM dihydrorhodamine 6G (DHR), 1μM H₂-PFTMRos, 5μM dihydroethidium (DHE), or 25μM 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluoresceindiacetate (CM-H₂DCFDA) and viewed with 510-560 nm excitation and 575 -590 nm emission for DHR and H₂-PFTMRos and 450-490nm excitation and 505-520 nm emission for CM-H₂DCFDA. All dyes were purchased from Invitrogen Corp. (Carlsbad, CA).

To quantify ROS by in fluorescence images of adherent cells, cell images were captured with a Nikon Digital Sight camera attached to a Nikon TE2000S microscope (Nikon Instruments, Inc., Melville, NY). Mean intracellular fluorescence intensity of 10 field images, containing over 200 cells, were automatically calculated for each group using NIS Elements BR 3.0 imaging software.

To quantify ROS by flow cytometry, adherent cells were stained with either DHE or CM-H₂DCFDA for 45 minutes in PBS at 37°C in the dark. DHE is oxidized by superoxide in live cells to ethidium (16). Cells were lifted with Trypsin-EDTA, washed, and fluorescence data was acquired within 60 minutes using the BD FACSCalibur (Franklin Lakes, NJ), with 488nm laser and 585nm (for CM-H₂DCFDA) and 585nm (for DHE) bandpass filters.

Western blotting
Cells were lysed using SDS sample buffer and protein concentrations were determined using the BCA method (Thermo Fisher Scientific, Rockford, IL). Equal amounts of protein were electrophoresed through a NuPAGE 4-12% Bis-Tris acrylamide and transferred according to manufacturer's instructions (Invitrogen Corp, Carlsbad, CA). Western blotting was performed as previously described (15).

8-OHdG

Genomic DNA was extracted using the DNEasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to manufacturer’s instructions. 10 μg of each DNA sample was denatured with heat, digested with S1 nuclease, and dephosphorylated with shrimp alkaline phosphatase. 8-OHdG measurement was performed by ELISA (Cell Biolabs, Inc. San Diego, CA) according to manufacturer’s instructions.

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

Cell migration and cell scattering

Cell migration was performed using the BD Falcon™ FluoroBlok™ 96 system (BD Biosciences, San Jose, CA). Cells labeled with 5 nM of DilC18 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., Sunnyvale, CA) at Ex/Em wavelengths 549/565 nm.

To assay scattering, cells were seeded at 20,000 cells/mL in 0.2% rat tail Type I collagen (BD Biosciences, San Jose, CA) 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 FITC filter sets and color coded depth was rendered on Zeiss LSM Image Browser version 4.2.0.121 software.

**Xenograft prostate tumor growth**

1.5 x $10^6$ LNCaP/MT1-MMP and LNCaP/GFP cells were suspended in 50 μl of PBS, mixed with 50 μl Matrigel (BD Biosciences, New Bedford, MA), and injected subcutaneously in the right flank of 6 week old athymic (nu/nu) male mice (Taconic farms, Germantown, NY). Animals were weighed, tumor dimension was measured with calipers, and volume was determined by the formula: Length x 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% of 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 50μL of 0.35% agar over a base of 50μL of 0.5 % agar in complete medium. Live colonies in the soft agar were counted in 3 replicate wells 14 days after seeding. Relative number of viable cells in each well was monitored by adding an Alamar Blue (Invitrogen Corp, Carlsbad, CA) 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 MTΔPEX construct was prepared as previously described (18). In brief, an N-terminal MT1-MMP fragment using the MT1-MMP forward primer (5' to 3', CACGAATTCCGGACCATGTCTCCGCACCCCAGA) and reverse primer (5' to 3', AGCCGCCTACCCGCCCCACAGATTTGCGGCCCATA) and a C-terminal fragment using the forward primer (5' to 3', GGGGCGGTGAGCGCGGCTGCCGTG) and reverse MT1-MMP primer (5' to 3', ACCCTGGATGGCGTAGAAGCTGCTTGG) were generated from the MT1-MMP cDNA template. The products were used as template to generate MT1-MMP with a deleted PEX domain using MT1-MMP forward and reverse primers. 18 hours after transfection, medium was replaced with fresh serum-free DMEM containing recombinant proMMP-2 (19). Conditioned media from the transfected cells were analyzed by gelatin zymography as previously described (15).

Fluorescence-labeled gelatin degradation assay

Acid-washed coverglasses 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 coverglasses were washed once with 70% ethanol and air-dried, and equilibrated with PBS before use. Transfected COS-1 cells were seeded at 50,000/cm², cultured for 18 hours overnight, washed twice with PBS, fixed in 4% paraformaldehyde for 10 minutes, and mounted onto Fluoromount G mounting medium (Southern Biotech, Birmingham, AL). Coverglasses were viewed and photographed at Ex/Em wavelengths 549/565 nm.

Cell adhesion

Cells were pre-labeled with 5 μM Calcein AM (Invitrogen Corp. Carlsbad, CA), counted on the Cellometer™ Auto T4 (Nexcelom Bioscience, Lawrence, MA) cell counter and seeded at 1.5 x 10⁵ cells/cm² in triplicate wells of 96-well Millipore Millicote™ ECM-coated plates (collagen I, collagen IV, laminin, fibronectin, vitronectin, and BSA) with or without anti-integrin antibodies in
serum-free media for 1-2 hours. ECM-coated plates were purchased from Millipore (Billerica, MA). Pre-labeled cells seeded with 2 μg/mL monoclonal anti-β1 integrin (clone 4B4, Beckman-Coulter, Fullerton, CA) or monoclonal anti-α6 integrin (Mab 13444-20, Millipore, Billerica, MA) antibodies were pre-incubated with the antibodies for 30 minutes at room temperature before seeding. Fluorescence was determined at Ex/Em 487/520 nm before and after washing twice with serum-free media. Adhesion was expressed for each respective well, as the percent fluorescence after washing relative to before washing.

**Aconitase assay**

All reagents and chemicals were purchased from Cayman Chemical Company (Ann Arbor, MI) and the assay was performed 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 800g for 10 minutes at 4°C. The cell pellet was resuspended in 50 mM Tris, pH 7.4, containing sodium citrate, sonicated to homogenize, and spun at 20,000g for 15 minutes at 4°C. Total protein level was determined using the bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL) on the supernatant. 50μg 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 aconitase activity for each cell sample was determined by the following formula:

\[
\frac{[\text{Slope (Sample)}] - [\text{Slope (Sample – Inhibitor)}]}{0.0313 \mu M^{-1}} \times \frac{\text{Total Volume}}{\text{Volume Sample}}
\]

Where 0.313 μM⁻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 PCa cells

Both oxidative stress (2, 3, 6) and MT1-MMP expression (12, 13, 20, 21) have been reported to be important for PCa pathogenesis and aggressiveness. To test if there is a link between MT1-MMP expression and ROS activity in PCa, we assayed ROS activity in MT1-MMP-expressing PCa cells, using a number of different dyes designed to detect ROS. These include dihydroethidium (DHE), dihydrorhodamine 6G (DHR), 5-(and 6-)chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA), and PF-H$_2$TMRos (Figure 1).

Utilizing 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 compared to LNCaP/GFP or untransfected LNCaP cells (Figure 1A, left panel). We were unable to use CM-H$_2$DCFDA for flow cytometry in these cells because the GFP excitation/emission properties in these cells overlapped with those of CM-H$_2$DCFDA and interfered with flow cytometry measurements. To confirm results obtained from DHE staining, we used DHR, which, like CM-H$_2$DCFDA, is sensitive to oxidation by hydrogen peroxide and PF-H$_2$TMRos, 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-H$_2$TMRos by fluorescence microscopy, in MT1-MMP-GFP-expressing LNCaP cells relative to LNCaP/GFP (Figure 1A, right panel). These observations were consistent with a link between overexpression of MT1-MMP and elevated ROS.

To further confirm the role of MT1-MMP in ROS induction in PCa, we utilized DU145 cells, an invasive, androgen-independent PCa cell line, which produces high levels of endogenous MT1-MMP. Two independent DU145 PCa cell lines each infected with different
retrovirus constructs encoding MT1-MMP shRNA (MT1 shRNA1 and MT1 shRNA2) resulted in downregulated MT1-MMP expression by real time RT PCR (Figure 1B, left) and by Western blotting (data not shown), compared to DU145 cells expressing an irrelevant GFP shRNA (DU145/GFP shRNA). We assessed ROS production in these cell lines using PF-H$_2$TMRos and CM-H$_2$DCFDA. Intracellular CM-H$_2$DCFDA fluorescence intensity, as determined by flow cytometry, showed significantly decreased mean fluorescence intensity in both DU145/MT1 shRNA1 and DU145/MT1 shRNA2 cells compared to uninfected DU145 cells or DU145/GFP shRNA (Figure 1B, middle panel). Decreased redox potential, as indicated by PF-H$_2$TMRos intensity, was also observed in DU145/MT1 shRNA, compared to DU145/GFP shRNA cells (Figure 1B, right panel). 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 1μM of the broad spectrum MMP inhibitor BB3103 (Figure 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-hydroxydeoxyguanosine (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 PCa 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 compared to LNCaP/GFP and to untransfected LNCaP cells (Figure 1D, left). Conversely, DU145/MT1 shRNA1 cells had significantly decreased level of 8-OHdG relative to control DU145/GFP shRNA (Figure 1D, left). To determine if MT1-MMP expression can cause PCa 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 were significantly greater within 25 days in mice injected with MT1-MMP/GFP transfected
LNCaP cells as compared to 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 as compared to LNCaP/GFP cells (Figure 1D, right), consistent with findings by 8-OHdG ELISA in the cell lines (Figure 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 over 3-fold lower aconitase activity compared to control LNCaP/GFP cells (Figure 1E). Inhibition with the aconitase competitive inhibitor oxalomalate (OMA) inhibited all aconitase activity down to non-detectable levels (N.D.) in both LNCaP/GFP and LNCaP/MT1-MMP-GFP, confirming the specificity of the assay for aconitase activity.

Taken together, we have demonstrated for the first time that MT1-MMP, expressed in transfected cells or produced endogenously, induces ROS activity and causes oxidative stress in PCa.

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

To determine if 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 1mM of the ROS scavenger N-acetylcysteine (NAC). We had verified independently that 1mM NAC is not cytotoxic, by Alamar Blue 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 variations 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 (DIC), and confocal laser scanning microscopy with color-coded depth, demonstrate 3-dimensional scattering of LNCaP/MT1-MMP-GFP cells, with cells in the same field displayed 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.

Since 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 1mM NAC. In agreement with previous reports (10, 24, 27), MT1-MMP promoted LNCaP cell migration as compared to controls (Figure 2B). Interestingly, addition of NAC reduced MT1-MMP-enhanced cell migration to the level of GFP expressing LNCaP cells (Figure 2B), suggesting that MT1-MMP-mediated PCa 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 sub-lethal doses of hydrogen peroxide, over as short a period as two days, caused an increase in invasive behavior.

Previous studies had suggested that oxidative stress was correlated with an aggressive phenotype in PCa cells (6). Accordingly, we asked whether increased oxidative stress mediated by MT1-MMP can lead to a more aggressive cancer phenotype. To address this question, we employed an in vitro soft agar assay. We found that LNCaP/MT1-MMP-GFP had significantly
enhanced ability to proliferate in soft agar, as assayed by Alamar Blue fluorescence in the first 7 days (Figure 2C, left). Alamar Blue dye was added to a set of cells immediately after seeding to monitor the initial number of viable cells. Viable cells were first detectable by Alamar Blue on the second day after cells were seeded in soft agar and approximately 24 hours after the dye was added. Although all cells were counted and diluted to the same seeding density, actual cell count on the second day after seeding revealed that there were more viable LNCaP/GFP cells than LNCaP/MT1-MMP-GFP. Nevertheless, by the 7th day after cell seeding, proliferation rate of LNCaP/MT1-MMP-GFP cells was significantly greater than of control, LNCaP/GFP cells (Figure 2C, left). All cells cultured in the presence of a sublethal dose of 1mM NAC displayed profound inhibition of growth in soft agar, even by the second day after cell seeding (Figure 2C, left). We were able to count cell colonies by 14 days after cell seeding and found that LNCaP/MT1-MMP-GFP had enhanced ability to form colonies in soft agar compared to LNCaP/GFP cells (Figure 2C, middle & right). Consistent with results from determining Alamar Blue fluorescence, addition of a non-toxic concentration of NAC inhibited colony formation in soft agar for both LNCaP/GFP as well as for LNCaP/MT1-MMP-GFP (Figures 2C middle & right).

These results collectively support the notion that increased ROS production, triggered by PCa cell expression of MT1-MMP, can lead to a more invasive phenotype and to enhanced malignancy.

*Induction of ROS requires MT1-MMP proteolytic activity and membrane anchorage*

To shed light on the mechanism by which MT1-MMP elicits oxidative stress in PCa cells, we began by asking which functional domains of MT1-MMP are important in inducing ROS. We had found that full-length MT1-MMP can induce ROS in COS-1 African green monkey kidney epithelial cells, and that COS-1 cells can be transfected more efficiently than LNCaP cells. We thus chose to compare ROS levels of COS-1 cells transfected with full-length MT1-MMP to
those of COS-1 cells transfected with mutant MT1-MMP constructs, in order to assess the roles of different domains of MT1-MMP in ROS induction. Accordingly, we transiently transfected COS-1 cells with a control empty vector, a vector expressing full-length MT1-MMP, or deletion mutant constructs that included a deleted PEX domain (MTΔPEX), a non-functional catalytic domain mutant with glutamine to alanine substitution at position 240 (MT1E_{240}→A) (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 40-60%, 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 (Figure 3A, right).

Full-length MT1-MMP was able to proteolytically activate proMMP-2 to its fully active form, as demonstrated by gelatin zymography, whereas MT1-MMP deletion mutants were unable to convert proMMP-2 to active MMP-2 (Figure 3B). COS-1 cells transfected with full-length MT-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 (Figure 3C). Significant Texas Red-labeled gelatin degradation was also observed by COS-1 cells transfected with MTΔPEX (Figure 3C), suggesting that the PEX domain of MT1-MMP is not required for direct degradation of ECM substrates. However, cells transfected with either MT1/E_{240}→A or MT1Δ535 were unable to effect degradation of Texas Red-labeled gelatin to a visually appreciable extent (Figure 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 three independent experiments were normalized relative to full-length MT1-MMP-transfected cells, and the mean ROS levels are displayed...
graphically (Figure 3D). Of the mutants tested, the MT1/E<sub>240→A</sub> catalytic domain mutant displayed ~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 ~27% reduction in ROS, relative to full-length MT1-MMP-transfected cells (Figure 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 transfectants did not display decreased ROS, indicating that the PEX 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**

Since induction of ROS requires MT1-MMP proteolytic activity localized to the cell surface, and MT1-MMP degrades and remodel the ECM (10), we asked if 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 demonstrated 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 bovine serum albumin (BSA) control.

The cells displayed morphological variations on different ECM substrates with very little cell attachment and spreading on collagen types I and IV and, as expected, on BSA (Figure 4A, left panel). 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 (Figure 4A, right panel). 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 (Figure 4A, right panel). These results suggest a role for MT1-MMP in altering adhesion of cells on ECM substrates.

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 (Figure 4B, left) and in fluorescent micrographs (Figure 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. Since 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.

As the cells’ functional interactions with the ECM are known to involve integrins (32), we asked if 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 (2μg/mL) lowered their adhesion to all ECM substrates tested, including laminin, fibronectin, and vitronectin (Figure 4C, left). In contrast, culture of these cells in the presence of an α6 integrin antibody (2μg/mL) had no significant effect on cell adhesion to
any ECM substrates tested (Figure 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 (Figure 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 (Figure 4D, left). Adhesion was, as for LNCaP cells, inhibited with anti-β1 integrin antibodies and not by anti-α6 integrin antibodies (Figure 4D, middle). Anti-β1 integrin antibodies also inhibited ROS production in DU145/GFP cells (Figure 4D right) and in DU145/MT1 shRNA (not shown), as evidenced by moderately decreased PF-H2TmRos intracellular fluorescence intensity (Figure 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 proMMP-2*

Among the many proteolytic functions of MT1-MMP is its ability to convert the zymogen proMMP-2 to its active form. Since induction of ROS by MT1-MMP requires active MT1-MMP, we asked whether MT1-MMP activation of proMMP2 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 (Figure 5A). As shown in Figure 5A and 5B, 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.
DISCUSSION

An association between oxidative stress and PCa is well-established, implicating oxidative stress in both the pathogenesis and progression of PCa (4-6, 33). However, the mechanism by which oxidative stress is provoked and the role it plays in PCa 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 demonstrate a mechanism by which expression of MT1-MMP, a molecule associated with cancer cell invasion and metastasis (8) in PCa cells triggers an oxidative stress pathway leading to aggressive malignancy. In cells without appreciable levels of MT1-MMP, as exemplified by LNCaP, ectopic expression of MT1-MMP triggered induction of ROS. In DU145, an androgen-independent cell line that possesses a more malignant phenotype than LNCaP cells (6), MT1-MMP knockdown resulted in ~50% decrease in ROS. Cellular ROS levels were quantified by staining adherent cells with DHE or CM-H₂DCFDA, followed by cell lifting with trypsin-EDTA and flow cytometry analysis. This method provides a reliable measurement of ROS in adherent cells, since the ROS dyes oxidized by elevated ROS are retained in the cells (37, 38) even after the cells are placed in suspension. Since ROS determinations using redox-sensitive dyes can be argued as having non-specific effects (39), we confirmed our findings using aconitase as an endogenous oxidative substrate. Since aconitase activity is inhibited by oxidation, our results showing over 3-fold decreased aconitase activity in LNCaP/MT1-MMP-GFP cells compared to control LNCaP/GFP cells provides 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).
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 et al. (14) demonstrated 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 demonstrated 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 PCa 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 anti-oxidant NAC had an anti-proliferative 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 cultured mammalian cells (43-46). A modest level of ROS (~3μM) in cells, rather than promoting oxidative stress, is thought to play an integral role in the signaling pathway regulating cell proliferation (45). Thus, the anti-proliferative 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 (~120μM) have been reported to profoundly alter the cellular gene expression profile (45). Exposure of mouse NMuMG mammary epithelial cells 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 PCa 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 PCa cells to acquire a more invasive phenotype and to enhanced anchorage-independent growth in soft agar.
Induction of ROS required MT1-MMP proteolytic activity, as evidenced by a loss of ROS induction in MT1/E240-A 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/E240→A nor the MT1Δ535 mutant constructs inhibited ROS to the level of vector control. We have found that ROS levels of LNCaP/MT1/E240→A -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/E240→A 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 proMMP-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 proMMP-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. Since 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 compared to 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 pro-migratory 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 et al., unpublished communications). Rac acts reciprocally with Rho in actin assembly and focal adhesion stability. Rho promotes the formation of stress fibers and focal adhesions, while 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 it may play an important role in the same or parallel pathway as that in which Rac1 resides. While data presented here does 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 are 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 in order to better understand MT1-MMP’s role 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 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 compared to LNCaP/GFP, rapidly after addition to tissue-culture plates, before significant adhesion was apparent (Figure 1 supplement), 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 demonstrate that MT1-MMP expression elicits oxidative stress in PCa 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 PCa malignancy by triggering oxidative stress. Additional studies will be needed to further dissect the signaling pathway(s) involved.
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FIGURE LEGENDS

Figure 1. Expression of MT1-MMP in prostate cancer cells elicits oxidative stress.

A. Left panel. 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 490/585 nm. Data represents the geometric mean of 3 experiments, each result of which was normalized to LNCaP/GFP ± SEM. Statistical significance was determined with a 2-tailed Student’s t-test. * P<0.05. Right panel. LNCaP/GFP and LNCaP/MT1-MMP-GFP were stained with PF-H₂TMRos (upper panels) to assess intracellular redox potential and dihydrorhodamine 6G (DHR; lower panels) to stain for ROS. Bar represents 50μm.

B. Right panel. Real-time RT-PCR analysis of MT1-MMP levels from total RNA extracted from DU145/GFP shRNA, DU145/MT1 shRNA1, and DU145/ MT1 shRNA2. Results shown are the mean of 2 replicates ± SEM, normalized to the human hypoxanthine phosphoribosyltransferase 1 (HPRT1) gene. Middle panel. Wild-type DU145, DU145/GFP shRNA, DU145/MT1 shRNA1, and DU145/MT1 shRNA2 cells were stained with 10 μM CM-H₂DCFDA and fluorescence intensity of 10,000 cells per group was analyzed by flow cytometry at excitation/emission 490/520 nm. Data represents the mean of 3 experiments, each result of which was normalized to DU145/GFP shRNA ± SEM (black bar). **P<0.01, ***P<0.001. Right panel. Representative results of PF-H₂TMRos staining in DU145, DU145/GFP shRNA, and DU145/MT1. Bar represents 50μm

C. DU145/GFP shRNA and DU145/MT1 shRNA1 cells were treated for 1 hour with 1 μM BB3103 before staining with 10 μM CM-H₂DCFDA and fluorescence intensity determined as described in (B). Data shown represent the mean of three experiments normalized to DU145/GFP shRNA ± SEM (black bar). *P<0.05.
D. *Left panel.* 8-OHdG content from prostate cancer cell genomic DNA were assayed by ELISA. 4 μg of DNA from each sample were loaded into each of duplicate wells; results shown are the mean ± SEM. 8-OHdG level in DU145/MT1 shRNA was below the detection limit of the 8-OHdG standard curve used and was considered not detectable (N.D.). Statistically significant differences between each group were determined using a 2-tailed student’s t-test. * P<0.05 for the respective groups. *Right panel.* Nude mice were implanted with tumors derived from LNCaP/GFP or from LNCaP/MT1-MMP-GFP. Tumors were dissected, fixed in formalin and embedded in paraffin. 5μm paraffin sections from these tumors were stained with a goat anti-8-OHdG antibody, HRP-conjugated anti-goat IgG, DAB substrate (shown in brown), and counterstained with hematoxylin (purple). Images in the upper panels were viewed and photographed at 200X, and those in the lower panels at 400X magnification. Bars in upper and lower right panels represent 100μm and 50μm, respectively.

E. Aconitase activity was measured from 50μg of protein lysates prepared from LNCaP/GFP and LNCaP/MT1-MMP-GFP cells. Aconitase activity shown was measured in untreated lysates (-) or in lysates treated with the aconitase competitive inhibitor, oxalomalate (OMA). Mean aconitase activity was determined from triplicate samples for each cell line and shown ± SEM. Levels of aconitase that were below the detection limits of the fluorescence reader was labeled not detectable (N.D.) ***P<0.001.

**Figure 2.** MT1-MMP expression promotes a more invasive, aggressive phenotype in PCa cells.

A. LNCaP, LNCaP-GFP, and LNCaP/MT-MMP-GFP were cultured in 0.2% collagen with or without 1mM NAC. On the left, cells shown were photographed under DIC microscopy 4 days after initial seeding. On the right, different views of the same cell samples were photographed under confocal microscopy and shown represented with color coded depth.
Blue to red spectrum represents cells ranging from closest to farthest away from viewer. Bar represents 50μm.

B. Relative migration of LNCaP-GFP and LNCaP/MT-MMP-GFP cells treated with 1mM NAC (light grey) or vehicle (dark grey), through BD Falcon™ HTS FluoroBlok™ 96 inserts, 7 hours after cell seeding into the inserts. Results are expressed as mean of 4 replicate wells ± SEM. Statistically significant differences determined using a 2-tailed Students t-test, *P<0.05.

C. LNCaP/GFP and LNCaP/MT1-MMP-GFP were cultured in 3 replicate wells of 96-well plates, with 1mM NAC (light grey) or vehicle (dark grey) in soft agar for 14 days. Left. Relative number of viable cells as determined by Alamar Blue staining at days 2 and 7 are shown. Alamar Blue data points below the detection limit of the fluorimeter were designated N.D. Middle. The number of viable colonies formed at 14 days, as shown graphically. Right. Representative micrograph of colonies formed at 14 days. Bars for all graphs represent the mean of 3 replicate wells ± SEM. *P<0.05 by a 2-tailed Student’s t-test. Bar in micrograph represents 500 μm.

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 (A, 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 (A, right).
B. Ability of each MT1-MMP construct to activate proMMP-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 coverglasses coated with Texas Red-labeled gelatin in serum-free medium and incubated overnight. Coverglasses were fixed with 4% PFA 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. Since transfection efficiency of COS-1 cells is ~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 5μM 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.

Figure 4. 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.
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 H₂-PFTMRos stained cells. Bar represents 100 μm. *P<0.05, **P<0.001.

C. Left panel. Cells were seeded, in the presence of 2 μg/mL of antibody to β1 integrin or to α6 integrin, onto 96-well plates coated with fibronectin, laminin, or vitronectin. Adhesion was assayed for each group relative to untreated cells, as shown graphically; only anti- β1 integrin was able to disrupt cell adhesion. Right panel. Incubation of LNCaP/MT1-MMP-GFP cells in the presence of β1 integrin also decreased ROS. A representative ROS experimental result is shown using DHE staining. Bar represents 100 μm.

D. Left panel. Adhesion of DU145/GFP shRNA and DU145/MT1 shRNA1 to collagen I, collagen IV, fibronectin, laminin, vitronectin, and BSA. Middle panel. Adhesion of DU145/GFP shRNA and DU145/MT1 shRNA1 to collagen I, collagen IV, fibronectin, and laminin in the presence and absence of 2 μM anti-β1 or anti-α6 integrins. Right panel. Representative images of DU145/GFP shRNA cells cultured on collagen IV and on fibronectin cultured with or without anti-β1 integrin antibodies. Cells stained with PF-H₂TmRos, shown on the right side, are also shown photographed under DIC, on the left. *P<0.05, **P<0.01, ***P<0.001.

**Figure 5. Induction of ROS does not require MT1-MMP activation of pro-MMP-2**

LNCaP/GFP and LNCaP/MT1-MMP-GFP cells were incubated with media containing recombinant proMMP-2.

A. Gel zymography of conditioned media from LNCaP/GFP (left lane) and LNCaP/MT1-MMP-GFP (right) incubated overnight with recombinant proMMP-2. Conditioned media from LNCaP/MT1-MMP-GFP displayed fully activated MMP-2.
B. LNCaP/GFP and LNCaP/MT1-MMP-GFP cells were cultured with conditioned media from cells treated as described in (A) containing activated MMP-2 (+MMP-2). Images were taken 24 hours after seeding, and ROS levels, in RFU of PF-H₂TmRos are shown graphically (B) and in representative images.

C. Representative images of PF-H₂TmRos-stained cells treated as described in (B).
Figure 1

A

![Bar chart showing DHE fluorescence comparison between control and MT1-MMP-GFP](image)

GFP  MT1-MMP-GFP

![Immunofluorescence images of GFP and MT1-MMP-GFP](image)

Rhodamine 6G

PP-H210Rc

Scale bar: 100 μm

B

![Bar chart showing fold expression of PTEN over HPRT1](image)

GFP-shRNA1  MT1-shRNA2

C

![Bar chart showing DCFDA fluorescence comparison](image)

GFP-shRNA1  MT1-shRNA2

D

![Western blot analysis comparing MT1-MMP-GFP and control](image)

GFP  MT1-MMP-GFP

![Immunohistochemistry images of MT1-MMP-GFP and control](image)

LNCaP  DU145

E

![Graph showing acetate activity](image)

OMA  N.D.  N.D.

GFP  MT1-MMP-GFP
Figure 3

A

<table>
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<th>Signal</th>
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B

C

D

Mean DHE fluorescence

MT1-MMP  MT1/E240-A  MTΔ355  MTΔPEX

* * *
Figure 4

A

B

C

D

Legend:

- GFP
- MT1-MMP-GFP
- GFP + anti-β1
- GFP + anti-v6
- MT1-MMP-GFP + anti-β1
- MT1-MMP-GFP + anti-v6

Statistical significance:

- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- **** p < 0.0001
Molecular Cancer Research

OXIDATIVE STRESS AND PROSTATE CANCER PROGRESSION ARE ELICITED BY MEMBRANE-TYPE 1 MATRIX METALLOPROTEINASE (MT1-MMP)

Hoang-Lan Nguyen, Stanley Zucker, Kevin Zarrabi, et al.

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