Oxidative Stress Induces ADAM9 Protein Expression in Human Prostate Cancer Cells

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Abstract

The ADAM (a disintegrin and metalloprotease) family is a group of transmembrane proteins containing cell adhesive and proteolytic functional domains. Microarray analysis detected elevated ADAM9 during the transition of human LNCaP prostate cancer cells from an androgen-dependent to an androgen-independent and metastatic state. Using a prostate tissue array (N = 200), the levels of ADAM9 protein expression were also elevated in malignant as compared with benign prostate tissues. ADAM9 protein expression was found in 43% of benign glands with light staining and 87% of malignant glands with increasing intensity of staining. We found that ADAM9 mRNA and protein expressions were elevated on exposure of human prostate cancer cells to stress conditions such as cell crowding, hypoxia, and hydrogen peroxide. We uncovered an ADAM9-like protein, which is predominantly induced together with the ADAM9 protein by a brief exposure of prostate cancer cells to hydrogen peroxide. Induction of ADAM9 protein in LNCaP or C4-2 cells can be completely abrogated by the antioxidant, ebselen, or genetic transfer of a hydrogen peroxide degradative enzyme, catalase, suggesting that reactive oxygen species (ROS) are a common mediator. The induction of ADAM9 by stress can be inhibited by both actinomycin D and cycloheximide through increased gene transcription and protein synthesis. In conclusion, intracellular ROS and/or hydrogen peroxide, generated by cell stress, regulate ADAM9 expression. ADAM9 could be responsible for supporting prostate cancer cell survival and progression. By decreasing ADAM9 expression, we observed apoptotic cell death in prostate cancer cells.

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Introduction

Prostate cancer progression, invasion, and metastasis are complex processes involving the interplay between cancer cells and microenvironment with tight regulations to ensure the growth and survival of cancer cells (1). Recent data suggest that chronic inflammation of the prostate and production of reactive oxygen species (ROS) could contribute to DNA damage and genomic instability, which may facilitate subsequent progression of cancer cells (2). It also has become apparent that oxidative stress such as toxins, dietary fat consumption, or high level of androgen may be important etiologic factors in the development and progression of prostate cancer (3). Fisher et al (4) showed that oxidative and osmotic stresses on tumor cells increase the shedding of pro-heparin-binding epidermal growth factor (EGF), which is processed by proteins of the adenyl cyclase, specifically ADAM9, ADAM10, and ADAM17. There are concomitant inductions of ADAM9 (5) and ROS (6) in prostate carcinogenesis. It seems that ROS can induce expression of the ADAMs via p38 mitogen-activated protein kinase activation (4). The induced ADAM proteins are responsible for release of processed heparin-binding EGF, which further promotes cancer cell growth and survival through an EGFR-dependent mechanism.

Recent studies showed a relationship between the elevation of ADAMs and the progression and metastasis of cancer cells (7–11). Among them, ADAM9 has been shown to possess potent biological activities. The metalloprotease domain of ADAM9 could cleave insulin β-chain, tumor necrosis factor α, gelatin, β-casein, and fibronectin (12, 13), and induce shedding of EGF, fibroblast growth factor receptor 2IIIB (14), or heparin-binding EGF-like growth factor (4, 15). The disintegrin domain of ADAM9 contains an ECD (Glu-Cys-Asp) motif, which may mediate cellular adhesion through α5β1 (16) and α6β4 integrins (17). The cytoplasmic tail of ADAM9 has potential SH3 binding motifs, which have been reported to interact with potentially important regulatory proteins, endophilin 1 (SH3GL2), SH3PX1 (18), and mitotic arrest deficient 2β (19). The abilities of ADAM9 to degrade specific extracellular matrix substrates, release active growth factors, interact with key regulatory factors, and appear in the invasion front of several tumor metastases suggest that ADAM9 provokes cancer cell growth, invasion, and metastasis. This conclusion is further supported by a recent report where ADAM9 knockdown mice, when crossed with transgenic mice bearing prostate carcinoma, exhibited reduced tumor growth and local invasion (14).

In this communication, we show increased ADAM9 expression in prostate cancer cells on androgen-independent progression and by exposing prostate cancer cells to stress conditions. Our findings on the roles of ROS in the induction of ADAM9 protein expression provide a framework for the development of future antioxidant therapies to arrest the growth of clinical human prostate cancer and its distant metastasis.
Materials and Methods

Cell lines, cell culture, and chemical reagents. The androgen-dependent and androgen receptor–positive parental LNCaP human prostate cancer cell line and its lineage-derived androgen-independent, androgen receptor–positive, metastatic C4-2 and C4-2B sublines were used for this study. Unless otherwise stated, these cell lines were cultured in T-medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Sigma, St. Louis, MO), 100 IU/mL penicillin G, and 100 μg/mL streptomycin at 37°C under 5% CO2. Ebselen (2-phenyl-1,2-benzisothiole-3-thione) (Sigma), actinomycin D, or cycloheximide (Sigma) was freshly reconstituted with DMSO before use.

Generation of human prostate cancer cell lines that stably express catalase. Construction and overexpression of catalase was done as previously described (20). In brief, human catalase cDNA was recovered from the pCI-neo vector and ligated into the HindIII site of the pZipNeo vector. A clone containing the catalase in the sense orientation (pZipNeo/catalase) was verified by DNA sequencing. Transfection of the cDNA to prostate cancer cells was carried out with Fugene 6 (Roche Applied Science, Indianapolis, IN) according to the instructions of the manufacturer. After 2 days, the cells were subcultured into 10-cm plates and treated with 0.4 mg/mL zeocin (Invitrogen) until individual colonies formed.

Quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR (RT-PCR) was done with the iCycler thermal cycler system (Bio-Rad, Hercules, CA). Total RNA (5 μg) from cells at 80% to 95% confluence was used as the starting material for reverse transcription using the Moloney murine leukemia virus reverse transcriptase system (Invitrogen). In brief, quantitative RT-PCR was done with a CYBR green RT-PCR kit (Applied Biosystems, Foster City, CA). A 25-μl reaction contained 1× CYBR green reaction mix with 50 ng of primers and 0.4 μL of cDNA from the reverse transcription. Forty cycles of PCR reactions were done with 30 seconds of denaturing at 95°C, 30 seconds of annealing at 60°C, and 1 minute of PCR reaction at 72°C. Fold of induction of expression was determined according to a published method (21). Average Ct value (n = 3) was used to measure the fold change. The formula used to calculate fold changes was ΔCt = CtSample − CtInternal. The ΔΔCt was calculated using the formula 2−ΔΔCt, and fold change was 2−ΔΔCt (21). Primers used were 18S-F1, 5′-GTCGACCTAATACGACTCACTATAGGG-3′; 18S-R1, 5′-GTGTTGTCGCTTCTCGTCAAT-3′; ADAM9-1274F, 5′-GTCGCCCTGGGCAACCCAGAC-3′; and ADAM9-1274R, 5′-CCACGGTCCCAACCATATT-3′.

Western blot. Prostate cancer cell lines cultured at 80% to 95% confluence were lysed in a lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.02% NaN3, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate) containing 100 μM phenylmethylsulphonyl fluoride and 1× Protease Inhibitor Cocktail Complete (Roche Diagnostics, Indianapolis, IN). Protein concentrations of the lysates were determined with the BCA Protein Assay Kit (Pierce, Rockford, IL). Unless otherwise stated, 20 μg of total protein were fractionated on an SDS-PAGE. Protein was transferred onto a nitrocellulose membrane, which was incubated with anti-human ADAM9 monoclonal antibody (1 μg/mL; R&D Systems, Inc., Minneapolis, MN), mixed with antihuman EFl-α or actin antibody as internal control, and visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Intensity of the detected signals by Western blot was quantified with ImageJ after scanning and inverting the images into TIFF files and converting into grayscale mode. Relative ADAM9 expression was semiquantified after normalization with either endogenous β-actin or EFl-α as indicated.

Effects of cell crowding on ADAM9 expression. Effects of the crowding on ADAM9 expression were evaluated after growing cells to 70% or 100% confluence. For low-density culture, 8 × 104 cells were plated onto 10-cm dishes to avoid overlapping and clumping of the cells. For crowding conditions, 6 × 106 cells were seeded. Cells were harvested 48 hours after seeding and Western blot analysis of ADAM9 expression was done.

Effects of hydrogen peroxide on ADAM9 protein expression. Hydrogen peroxide (H2O2) and ebselen, an antioxidant, were used to evaluate the contribution of ROS to ADAM9 expression. Cells at 90% confluence were treated with different concentrations of H2O2 for 0, 0.5, 1, 2, 4, and 8 hours. In some experiments, ebselen was added to the cells for 30 minutes before exposure to the H2O2. Cells were cultured and harvested 4 hours later. To determine whether ebselen could affect ADAM9 expression induced by cell crowding, cells were first cultured under the crowding condition for 48 hours and then treated with ebselen (0.02-20 μmol/L) for 2 hours.

ADAM9 expression in clinical prostate cancer specimens. Clinical prostate cancer tissue specimens were obtained following an institution-approved protocol. Paraformaldehyde-fixed, paraffin-embedded tissues were deparaffinized and rehydrated. Antigen retrieval was done in citrate buffer (pH 6) using an electric pressure cooker at 120°C for 5 minutes. The section was then exposed to 3% H2O2 for 5 minutes to quench endogenous tissue peroxidase I and then incubated for 30 minutes with the monoclonal anti-ADAM9 antibody (1:50 dilution). Immunohistochemical staining was visualized with the Envision system (DAKO Corporation, Carpinteria, CA) and the results were independently evaluated by two pathologists (S.I. and M.A.). The anti-ADAM9 antibody from R&D Systems has been validated by several other groups to be specific for the detection of ADAM9 protein expression in prostate cancer (8, 22), small-cell lung carcinoma (23), and hepatic cancer (24).

Short hairpin RNA mediated gene silencing of ADAM9. To determine the potential functions of ADAM9 protein in C4-2 cells, we transfected these cells with a predesigned short hairpin RNA (shRNA; Invitrogen), which was shown to knock down specifically ADAM9 mRNA and ADAM9 and ADAM9-like protein. C4-2 cells were seeded in six-well plates and cultured until 90% confluence. The cells were then transfected with 50 nM/μL ADAM9 shRNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to the recommendations of the manufacturer and cultured for an additional 6 hours in serum-free RPMI. Cells were washed and cultured in T-medium for an additional 18 hours. The cells were evaluated by Western blot analysis for expression of ADAM9 proteins with β-actin as an internal control.

Flow cytometry analysis of cell apoptosis. C4-2 cells were seeded at 2 × 104 per well in six-well dishes overnight before ADAM9 gene knockdown. shRNA knockdown of ADAM9 was done as described above with scramble shRNA or liposome as controls. Cells were cultured for an additional 48 hours before harvesting for Annexin V flow cytometry analysis according to the recommendations of the manufacturer (Invitrogen). In brief, cells were collected and washed with cold PBS and diluted in AnnexinV-binding buffer [10 mMol/L HEPES, 140 mMol/L NaCl, and 2.5 mMol/L CaCl2 (pH 7.4)] with 1 × 105 cells/mL. Cells were incubated with Annexin V at room temperature for 15 minutes before analysis by flow cytometry (BD FACScan) to determine the population of cells with or without Annexin V by the use of anti-Annexin V antibody (Invitrogen) with a green filter and counting 1 × 105 C4-2 cells.

Hypoxia culture conditions. C4-2 cells were seeded at 75% confluence overnight and then exposed to low oxygen tensions in humidified airtight chambers with inflow and outflow valves. The chambers were flushed with preanalyzed gas (1% O2, 5% CO2, balance N2; SGS, Atlanta, GA) mixtures and continuously maintained at 37°C for 24 hours. Normal cell culture conditions were defined by placing cells in an incubator with 5% CO2 and 95% air for 24 hours. Cells were harvested and total cellular protein was extracted and subjected to Western blot analysis for ADAM9 protein expression.

Assessment of endogenous ROS concentration. Intracellular ROS was determined according to a published protocol (25, 26). Confuent cells (85-90%) were rinsed with HBSS and detached by trypsin treatment. Cells were pelleted, resuspended in HBSS, and incubated in 2.5% FBS and 2 μmol/L dichlorofluorescein diacetate in the dark at room temperature for 45 minutes. Fluorescence was determined with FACSCalibur from Becton Dickinson (San Jose, CA) (excitation wavelength, 488 nm; emission wavelength, 545 nm).

Effects of actinomycin D and cycloheximide on ADAM9 expression. To examine if ADAM9 expression was regulated at the level of transcription or translation, we employed an RNA synthesis inhibitor, actinomycin D, and a protein synthesis inhibitor, cycloheximide, to inhibit the H2O2-induced ADAM9 expression. Cells at 90% confluence were exposed to H2O2 in

the presence of actinomycin D (1 μg/mL) or cycloheximide (10 μg/mL) for 2 hours. The cells were harvested and ADAM9 protein expression was assessed by Western blots.

Results

Increased ADAM9 protein expression in the lineage-related LNCaP human prostate cancer progression model. We used the lineage related prostate cancer cell lines, androgen-dependent LNCaP and androgen-independent C4-2 (27), as models to identify genes of which the expression is differentially activated in prostate cancer progression. Microarray analysis was used to compare gene expression profiles between lineage-related androgen-dependent LNCaP and androgen-independent C4-2 and C4-2B cells (27, 28). These analyses suggested a differentially elevated expression of ADAM9 in the androgen-independent derivative sublines of the LNCaP. We confirmed differential ADAM9 mRNA and protein expression in LNCalP lineage cells with quantitative RT-PCR and Western blot, respectively. As shown in Fig. 1A, a 2.8- and 3.3-fold increase in ADAM9 mRNA expression was noted in C4-2 and C4-2B cells, respectively, over that of the parental LNCaP cells. Western blot analyses corroborated that ADAM9 mRNA and protein expression in C4-2 and C4-2B cells were also 1.5- to 1.4-fold higher than parental LNCaP cells (Fig. 1B). Under stress conditions, such as cell crowding from 70% to 100% confluence, an additional increase of ADAM9 protein expression by 1.6- and 2.0-fold over those of the basal levels was observed in C4-2 and C4-2B cells, respectively (Fig. 1C). This stress induction of ADAM9 also can be found in androgen-independent, androgen receptor–negative PC3 and DU-145 cells in which cell crowding increased ADAM9 protein expression by 2.0- and 2.6-fold, respectively. Likewise, when cells were cultured under hypoxic conditions, a 1.3-fold increase in ADAM9 protein expression was noted in C4-2 cells (Fig. 1D). While evaluating ADAM9 protein overexpression under stress conditions, such as crowding and hypoxia, we noted that, in addition to the increase of ADAM9 protein expression (~ 80 kDa protein), a smaller size of immunoreactive protein, tentatively defined as an ADAM9-like protein in the molecular weight range of 60 to 65 kDa, reacted positively with ADAM9 antibody and was induced under stress conditions and by hydrogen peroxide (Figs. 1C and 3B).

Increased ADAM9 protein expression in clinical specimens. To confirm that ADAM9 and/or ADAM9-like expression in response to stress conditions in cultured human prostate cancer cells is relevant to clinical conditions, tissue microarray of ADAM9/ADAM9-like expression was done in clinical prostate cancer specimens. Tissue microarray was conducted in 200 prostate specimens (80 benign hyperplastic prostate and 120 prostate cancer; Table 1). Results of these studies revealed that 57.5% (46 of 80) of benign foci and 12.5% (15 of 120) of cancer were negative for ADAM9/ADAM9-like protein expression. In the cancerous foci, the distribution of intensity of the positively stained samples was as follows: 19.2% (23 of 120) showed 1+, 20.8% (25 of 120) showed 2+, and 47.5% (57 of 120) showed 3+. Location of the positive ADAM9/ADAM9-like stain was found to be associated with the cell membrane or apical surface of cancer

Figure 1. ADAM9 protein expression in human prostate cancer cells increases on progression from androgen-dependent to androgen-independent state. A, real-time quantitative RT-PCR analysis of ADAM9 transcripts showed significant (*, P < 0.05) elevation of ADAM9 mRNA on progression of human prostate cancer cells from androgen-dependent (LNCaP) to androgen-independent state (C4-2 and C4-2B). B, Western blot analysis of ADAM9 expression in prostate cancer cell lines. ADAM9 protein was elevated on progression of LNCaP (1.0×) to C4-2 (1.5×) and C4-2B (1.4×). C, ADAM9 protein was elevated by 1.1-, 1.6-, and 2.0-fold, respectively, in LNCaP, C4-2, and C4-2B cells, and 2.0- and 2.6-fold, respectively, in PC-3 and DU-145 cells on cell culture in crowding conditions. Note that ADAM9-like protein also increases in crowding conditions. D, ADAM9 protein was also shown to be elevated by 1.3-fold under hypoxic cell culture conditions.
In contrast, in benign hyperplastic prostate tissues, only 1+ stain (42.5%; 34 of 80) was detected. None of the samples scored 2+ or 3+. In this study, protein expression was found to be absent from prostate stromal cells (0 of 200). Because of a relatively small sample size, we cannot draw a direct correlation between increased ADAM9/ADAM9-like protein expression and the Gleason score of prostate cancer tissues (113 of 120 in Gleason 6 as opposed to 7 of 120 in Gleason ≥7). The specificity of this

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Figure 2. Immunohistochemical study of ADAM9 expression in clinical prostate cancer specimens. Human prostate cancer tissues were stained with ADAM9 antibody. A, stronger ADAM9 protein staining was observed at a magnification of ×20 in malignant (closed arrows) than in benign (open arrows, marked N) prostate tissues. B, ADAM9 knockdown by shRNA resulted in decreased expression of both 80-kDa and 65-kDa protein in C4-2 cells under crowding culture condition (95% confluence at the time of cell harvest). We observed a down-regulation of ADAM9 protein (80 kDa) in noncrowding conditions (75% confluence) at the time of cell harvest. C, knockdown of ADAM9 expression using shRNA strategy also resulted in an apoptosis of C4-2 cells as revealed by Annexin V flow cytometry analyses.
monoclonal antibody has been extensively validated by several other groups of investigators in which they showed that ADAM9 immunoreactivity was blocked by a sequence-specific ADAM9 adsorbing peptide in pancreatic carcinoma and small-cell lung carcinoma (8, 22, 23). We have also confirmed the specificity of this antibody by conducting the ADAM9 knockdown study where we showed that on ADAM9 shRNA transfection, both ADAM9 and ADAM9-like proteins were decreased as analyzed by Western blot analysis (see Fig. 2F). In addition, we routinely did isotype-specific immunoglobulin G control staining of comparable tissue specimens to ensure that there is no nonspecific staining of antigenic epitope in clinical prostate cancer specimens (29–31). In our study, we observed ADAM9 shRNA treatment of C4-2 cells induced apoptotic cell death of prostate cancer cells in culture as assessed by Annexin V FACS analysis (Fig. 2C).

**Induction of ADAM9 expression under stress is mediated through ROS.** When cells are subjected to crowding and hypoxia stress conditions, they produce high levels of ROS, primarily H2O2 (32). Figure 3A (left) shows that when C4-2 cells were exposed to exogenously added H2O2 at 0.01 to 10 mmol/L, a concentration-dependent elevation of ADAM9 protein was observed. Addition of 10 mmol/L H2O2 resulted in rapid induction of ADAM9 protein accumulation in C4-2 cells at 4 hours (Fig. 3A, right). The H2O2 induction of ADAM9 protein expression by H2O2 was more marked when cells were exposed to relatively higher concentrations of H2O2 (>5 mmol/L). The doublet bands, detected by exposing the X-ray films for shorter time, observed on H2O2 addition were ADAM9 and ADAM9-like protein, and both proteins were shown to be elevated (Fig. 3B). The role of H2O2 in the induction of ADAM9 protein expression is further supported by experiments where exogenously added H2O2 failed to induce ADAM9 protein expression in C4-2 cells that were transfected with catalase (an enzyme that converts H2O2 to water; ref. 33; Fig. 3C, C4-2/catalase). In addition, recombinant catalase added to C4-2 cells exposed to H2O2 also abolished ADAM9 induction (Fig. 3C).

To evaluate the potential pathophysiologic role of H2O2 in the expression or accumulation of ADAM9 protein in prostate cancer cells and tissues, we measured ROS and ADAM9 protein in several human prostate cancer cell lines. Figure 3D shows a linear correlation between ROS and the respective steady-state levels of ADAM9 protein in cells. These results are in agreement with a recently published report by Lim et al. (6) who showed that a key ROS-producing enzyme, Nox1, is predominately expressed in malignant rather than benign human prostate tissues.

**Increased ADAM9 in prostate cancer cells under crowding conditions is associated with ROS in a cell context-dependent manner.** Because cell crowding induced ADAM9 protein accumulation in prostate cancer cells (Fig. 1C), we assessed the potential of H2O2 as a mediator. C4-2, DU-145, and PC3 cells were grown to 100% confluence, then added with ebselen, an antioxidant, and ADAM9 protein accumulation in cells was determined. ADAM9 protein induction in C4-2 cells by H2O2 can be effectively antagonized by the antioxidant ebselen in a concentration-dependent manner with effective blockade shown at 0.2 μmol/L ebselen (Fig. 4A). As shown in Fig. 4B, the addition of ebselen decreased ADAM9 protein expression in 100% confluent cells of PC-3 from a 2.0-fold to a 1.0-fold induction and DU-145 from a 2.6-fold to a 1.95-fold induction as compared with a 75% confluent culture (see Fig. 1C). Similar effects were not detected in C4-2 cells despite the evidence suggesting that H2O2 is a potent inducer for ADAM9 protein expression in these cells.

**ADAM9 protein expression up-regulated by hydrogen peroxide requires active gene transcription and translation: effects of actinomycin D and cycloheximide.** To determine if the up-regulation of ADAM9 protein by H2O2 may require active mRNA and protein synthesis, we monitored the steady state of ADAM9 protein in C4-2 cells after exposing them to actinomycin D, an inhibitor of RNA synthesis, or to cycloheximide, an inhibitor of protein synthesis. Figure 5 shows that induction of ADAM9 protein expression in C4-2 cells by H2O2 has a rapid onset and this induction can be blocked within 1 to 2 hours by the addition of either actinomycin D or cycloheximide to the cell culture medium (Fig. 5). We showed that this short-term (1-2 hours) exposure to actinomycin D and cycloheximide did not alter the basal ADAM9 protein accumulation in prostate cancer cells.

**Discussion**

Using human prostate cancer androgen-dependent LNCaP cells and their lineage-derived androgen-independent C4-2 and C4-2B sublines as models for cDNA microarray analysis, we detected an elevation of ADAM9 mRNA expression on androgen-independent progression. These results were confirmed by quantitative RT-PCR and Western blot for ADAM9 mRNA and protein, respectively (Fig. 1). On further evaluation of ADAM9 protein expression in cultured human prostate cancer cell lines, we observed that the steady-state level of this protein was determined by certain pathophysiologic stress conditions, such as cell crowding, hypoxia, and the addition of H2O2 to cell culture medium (34). We also confirmed the earlier study by McLulloch et al. (9) that androgen stimulated ADAM9 expression and such stimulation can be blocked by the simultaneous administration of an antiandrogen (data not shown). Both the native ADAM9 and its smaller ADAM9-like protein were elevated by placing prostate cancer cells under stress conditions or exposing them to H2O2 (Figs. 1C and 3B), and the induction of both proteins was blocked by catalase (Fig. 3C). Recently, Fischer et al. (4) showed that stress can induce ROS production, which can cause the release of heparin-binding EGF through enzymatic shedding by ADAM9, ADAM10, and ADAM17. H2O2 increased ADAM9 but failed in ADAM10, ADAM15, and ADAM17 (data not shown) in LNCaP suggesting a special role of ADAM9 in prostate cancer progression through increased growth factor signaling in response to stress conditions. The roles of H2O2 in ADAM9 induction may be cell context dependent because ebselen abolished crowding-induced ADAM9 protein accumulation in PC-3 and DU-145, but not C4-2, cells (Fig. 4). It was noted, however, that destruction of endogenous H2O2 by catalase transfection also eliminated ADAM9 protein accumulation in C4-2 cells (Fig. 3C).

The increased basal and cell crowding–induced ADAM9 protein expression in metastatic androgen-independent human prostate cancer cell lines was confirmed in tissue array using human prostate cancer specimens. For example, higher and more intense staining of ADAM9/ADAM9-like proteins was associated with malignant compared with benign prostate tissues (Fig. 2A). Recent studies showed that the secreted form of ADAM9-S promotes cancer cell invasion (24). Further, increased ROS may be associated with increased stress responses that result in increased ADAM9 protein expression in prostate cancer cells. In our study, it seems that ADAM9 protein may be responsible for cell survival on prostate cancer progression in which we noted that decreased ADAM9 protein promoted apoptotic death of prostate cancer cells (Fig. 2C). This suggestion is also supported by the observations that (a)
increased ROS in prostate cancer cells (Fig. 3D) and tissues (6) correlated with increased malignant potential of the prostate cancer cells and tissues; and (b) the antioxidant ebselen (Fig. 4A) and H$_2$O$_2$-degrading enzyme catalase (Fig. 3C) effectively blocked the ability of prostate cancer cells to respond to stress- or H$_2$O$_2$-induced increases in ADAM9 protein expression by prostate cancer cells. Although we have not established a direct correlation between stress response–induced ADAM9 expression and prostate cancer metastasis, there is evidence supporting this hypothesis. For example, C4-2 and C4-2B cells expressing higher levels of H$_2$O$_2$ and ADAM9 also seemed to be more tumorigenic and metastatic than the lineage-related parental LNCaP cells (28). Shintani et al. (11) showed a close correlation between ADAM9 mRNA expression and brain metastasis from lung cancer, and recently, Petudo et al. (14) showed that ADAM9$^{-/-}$ W10 mice developed well-differentiated prostates whereas littermate controls (ADAM9$^{+/+}$ W10) had predominantly

Figure 3. The induction of ADAM9 expression by H$_2$O$_2$. A. Western blot. Left, ADAM9 expression in C4-2 cells induced by H$_2$O$_2$ in a dose-dependent manner. Right, induction of ADAM9 is rapid and effective, occurring within 4 hours of H$_2$O$_2$ exposure at 10 mmol/L. B. increased expression of ADAM9-like protein (arrow, with shorter time exposure) was observed on induction by either stress or addition of H$_2$O$_2$. Note that this protein was not expressed in LNCaP, C4-2, and C4-2B cells at 0 and 0.2 mmol/L H$_2$O$_2$ treatments; marked induction of ADAM9-like protein was observed when elevated H$_2$O$_2$ was added (5 or 10 mmol/L H$_2$O$_2$). C, the role of H$_2$O$_2$ in ADAM9 protein induction was further confirmed by aborting H$_2$O$_2$ in C4-2 cells stably transfected with catalase (C4-2/catalase) and by the addition of catalase in culture medium (catalase+). D, a positive correlation between increased endogenous ROS levels, as measured by dichlorofluorescein diacetate assay, and increased ADAM9 protein expression, as assessed by Western blot, was observed.

Figure 4. Differential blockade of ADAM9 expression by ebselen in human prostate cancer cells subjected to cell crowding conditions. A, antioxidant ebselen blocked H$_2$O$_2$-induced ADAM9 and ADAM9-like protein expression in a concentration-dependent manner in C4-2 cells. C4-2 cells were cultured in the presence of 10 mmol/L H$_2$O$_2$ for 2 hours and in the presence of different concentrations of ebselen (0.02-20 μmol/L) for an additional 2 hours. B, to assess the possible relation between induction of ADAM9 and ADAM9-like protein expression in crowding conditions, C4-2, DU-145, or PC3 cells were grown to 100% confluence. Ebselen (20 μmol/L) effectively blocked cell crowding–induced ADAM9 and ADAM9-like protein expression in PC3 and DU-145, but not C4-2, cells.
poorly differentiated and significantly larger tumors. They also showed that overexpression of ADAM9 in mouse prostate epithelium resulted in significant abnormalities, including epithelial hyperplasia at 4 to 6 months of age, and prostatic intraepithelial neoplasia after 1 year. A report by Mozzocca et al. (24) indicated that the soluble form of ADAM9 could help tumor migration by cleavage of the laminin in basement membrane. Taken together, the results suggest that ADAM9 is a stress-inducible protein involved in prostate cancer progression. ADAM9 may play a pivotal role supporting the growth, survival, and metastasis of human prostate cancer cells under pathophysiologic stress conditions (11, 14, 24). By inactivating ADAM9 in human prostate cancer cell lines, we obtained preliminary evidence that these cells become highly sensitive to radiation and chemotherapy (35).

Whereas our study suggests that \( \text{H}_2\text{O}_2 \) could play a key role in directly regulating ADAM9 protein expression in prostate cancer cells, its pathophysiologic role warrants further investigation. The reasons for this conclusion are as follows: (a) In addition to \( \text{H}_2\text{O}_2 \), several other ROS, including nitric oxide, reactive nitrogen species, and lipid peroxides, have been shown to exert regulatory roles controlling cell proliferation, survival, and differentiation (36). (b) A more pronounced induction of ADAM9 protein expression was observed at higher concentrations of \( \text{H}_2\text{O}_2 \), which could cause apoptosis albeit ADAM9 protein induction could be effectively prevented by exposing the treated cells to ebselen. The possible local ROS gradients and the heterogeneity of ADAM9 protein induction, some with live cells and others with apoptotic cells, warrant further investigation. (c) ADAM9 protein expression could be the consequence of stress conditions induced by cell cycle arrest. We have addressed this concern by using low concentrations of \( \text{H}_2\text{O}_2 \) that do not cause cell cycle arrest and yet still stimulate ADAM9 protein accumulation in prostate cancer cells. Additionally, the use of metabolic poisons, such as actinomycin D and cycloheximide, inhibited cell cycle progression but did not induce ADAM9 protein expression (Fig. 5).

Our results raise an interesting possibility about the development of androgen-independent progression and the possible role of ROS as mediators for stress and androgen-independent prostate cancer progression in prostate cancer cells. Evidence indicates the critical role of ROS in the regulation of cell growth and differentiation. Cells engineered to overexpress ROS were found to enhance cell signaling through specific growth factors, extracellular matrices, and matrix metalloproteinases that facilitate tumor growth and invasion (20, 37, 38). Likewise, cells subjected to oxidative stresses such as cell crowding and hypoxic conditions were also found to have an altered repertoire of growth stimulatory and survival factors (39, 40). It is likely that ADAM9 up-regulation is not only associated with androgen-independent progression but is also required for cells to survive and to overcome harsh oxidative stress conditions (41–46). ROS, represented by a host of oxygen radicals such as superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxy nitrate, can cause macromolecular damage and may play important roles in tumor development and aging (47–49).

The possibility exists, though unproven, that ADAM9 protein expression may be intimately linked with the critical balance between the formation and destruction of ROS. Several human cell lines derived from cancers have significantly elevated hydrogen peroxide (33). Lim et al. (6) showed that the increased capability of invasive androgen-independent human prostate cancer cell lines for ROS production and the higher level of ROS-producing enzyme, Nox1, were associated with malignant but not benign clinical prostate tumors. Higher levels of ROS could be generated from cells that have mitochondrial DNA mutations during tumor progression, tumor development, and aging (50, 51).

In summary, intracellular ROS, generated by cell stress, controls ADAM9 expression during androgen-independent and malignant progression of human prostate cancer cells and tissues. Up-regulation of ADAM9 could facilitate cancer development (14) and promote cancer cell invasion (24), migration (11), and resistance to stress-induced injuries (4). Ebselen, a lipid-soluble selenoorganic antioxidant, was shown to block ADAM9 through inhibition of ROS production. A further understanding of the downstream pathways linking ROS production and the oxidative-induced stress response could potentially be the basis for a new clinical therapeutic development for advanced and androgen-refractory prostate cancer.

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