Published in final edited form as:

Free Radic Biol Med. 2009 January 1; 46(1): 42–50. doi:10.1016/j.freeradbiomed.2008.09.020.

Altered Redox Status Accompanies Progression to Metastatic Human Bladder Cancer

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Abstract

The role of reactive oxygen species (ROS) in bladder cancer progression remains an unexplored field. Expression levels of enzymes regulating ROS levels are often altered in cancer. Search of publicly available micro-array data reveals that expression of mitochondrial manganese superoxide dismutase (Sod2), responsible for the conversion of superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), is consistently increased in high grade and advanced stage bladder tumors. Here we aim to identify the role of Sod2 expression and ROS in bladder cancer. Using an *in vitro* human bladder tumor model we monitored the redox state of both non-metastatic (253J) and highly metastatic (253J B-V) bladder tumor cell lines. 253J B-V cells displayed significantly higher Sod2 protein and activity levels compared to their parental 253J cell line. The increase in Sod2 expression was accompanied by a significant decrease in catalase activity, resulting in a net increase in H_2O_2 production in the 253J B-V line. Expression of pro-metastatic and -angiogenic factors, matrix metalloproteinase 9 (MMP-9) and vascular endothelial derived growth factor (VEGF), respectively, were similarly upregulated in the metastatic line. Expression of both MMP-9 and VEGF were shown to be H_2O_2 -dependent, as removal of H_2O_2 by overexpression of catalase attenuated their expression. Similarly, expression of catalase effectively reduced the clonogenic activity of 253J B-V cells. These findings indicate that metastatic bladder cancer cells display an altered antioxidant expression profile, resulting in a net increase in ROS production, which leads to the induction of redox-sensitive pro-tumorigenic and pro-metastatic genes such as VEGF and MMP-9.

Keywords

Bladder; Cancer; Superoxide Dismutase; Sod2; Catalase; hydrogen peroxide

Introduction

An estimated 67,000 new cases of bladder cancer will be diagnosed in the United States in 2007, with approximately 13,750 deaths attributed to the disease, annually [1]. Bladder cancer occurs at a higher frequency and is the fourth most commonly diagnosed cancer in men. While

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the majority of patients presenting with localized bladder tumors can be effectively treated by resection and have a high survival rate, a subset of bladder tumors become locally invasive and malignant. The five-year survival rate for all patients with bladder cancer is 45% and for patients displaying regional and distant metastatic lesions, it drops to < 47% and < 7%, respectively [1]. The molecular mechanisms regulating the pro-metastatic phenotype of aggressive bladder cancer require further investigation for the development of potential therapeutic strategies.

The production of reactive oxygen species (ROS) is an inevitable outcome of aerobic respiration in all mammalian cells. Antioxidant enzymes are part of the physiologic defense mechanisms that detoxify the oxidants into less toxic or inert molecules. The repertoire of antioxidant enzymes include: the family of superoxide dismutases that dismutate superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen; and the prominent H_2O_2 detoxifying enzymes catalase and glutathione peroxidase. ROS have been implicated in numerous disease processes including carcinogenesis, atherosclerosis and connective tissue disorders. It has been proposed that a net pro-oxidant state, which occurs when there is a loss of homeostasis between ROS generation and detoxification i.e. oxidative stress, results in DNA damage leading to carcinogenesis [2]. H_2O_2 is a well established mitogen whose proliferative activity can be restricted by treatments with catalase [3]. Several reports have also demonstrated that increases in the oxidant/antioxidant ratio is directly correlated with tumor progression, angiogenesis and migration/invasion [4-11].

While ROS and antioxidant enzymes have been studied in many tumor systems, the role of these important molecular players has not been examined in detail in bladder cancer. Here, we define several key components that control the redox characteristics of bladder cancer cells and determine the role of oxidative stress on metastasis-related growth factors and the malignant phenotype.

Materials and methods

Cell lines and Reagents

The poorly tumorigenic, non-metastatic human bladder cancer cell line, 253J, and its highly tumorigenic and metastatic variant 253J B-V, which was selected through repeated *in vivo* bladder orthotopic implantations in nude mice, were utilized in these experiments [12]. The cells were cultured in modified Eagle's MEM supplemented with 10% fetal bovine serum. 3-Amino-1,2,4-triazole and N-acetyl cysteine were purchased from Sigma. Rabbit anti-VEGF was purchased from Santa Cruz Biotechnologies, CA. Sheep anti-Sod2 was obtained from Calbiochem, CA. Adeno-virus catalase was purchased from Gene Transfer Vector Core, University of Iowa. 100 MOI of the virus or control (beta galactosidase, Lac-Z) was used to infect cells grown in a 35mm dish.

SOD Activity Gel

Copper zinc superoxide dismutase (Sod1) and manganese superoxide dismutase (Sod2) enzymatic activity were determined on non-denaturing SDS-PAGE gels, as described previously [13]. Briefly, cells were sonicated in potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 50 μ g of protein lysate electrophoresed in a non-denatured polyacrylamide gel. To visualize Sod activity, gels were incubated for 15 min in the dark with 2.5mM nitro blue tetrazolium, 30 mM TEMED, 0.028 mM riboflavine, 50 mM phosphate buffer, pH7.8, washed twice in deionized water, and then exposed to light. Quantitative analyses of cleared zones on the gels, indicating Sod activity, were performed with ImageJ program.

Catalase Activity Assay

Catalase enzymatic activity was determined by measuring the decomposition of H_2O_2 by ultraviolet spectroscopy at 240 nm wave length, as described previously [14]. Cells were sonicated in potassium phosphate buffer, pH 7.0 and approximately 15 μg of total protein was added to 20mM H_2O_2 in 50mM potassium phosphate, following the decomposition of H_2O_2 by catalase over time at 240 nm. Specific activity of catalase was calculated from the equation: specific activity (units/mg of protein/min) = $\Delta A_{240 \text{ nm}} (1 \text{ min}) \times 1000/43.6 \times \text{mg protein}$.

Glutathione Peroxidase (GPx) Activity and Glutathione Assay

GPx activity assays were essentially carried out as described by A. L. Tappel [15]. Briefly, GPx was measured indirectly by spectrophotometrically monitoring the oxidation of NADPH at 340 nm in a coupled assay system containing glutathione and glutathione reductase and with Cumene hydroperoxide as the substrate. A unit of glutathione peroxidase is defined as the oxidation of 1 μmol NADPH per min at 37°C. Reduced glutathione (GSH) and oxidized glutathione (GSSG) assays were carried out as previously described [16].

Measurement of cellular H_2O_2

The concentration of intracellular H_2O_2 can be determined based on the rate of irreversible inactivation of catalase by 3-Amino-1,2,4-triazole, which forms a covalent complex with the catalase intermediate compound I. [17]. $[\text{H}_2\text{O}_2] = k/k_1$, where k is the first-order rate constant determined empirically for catalase inactivation in the cell, and k_1 is the rate of compound I formation ($1.7 \times 10^7 \text{ M/s}$). The method was carried out as previously described [18].

Measuring ROS with redox sensitive green fluorescent protein (RoGFP)

The novel redox-sensitive green fluorescent protein constructs (RoGFP) have been engineered to contain two surface-exposed cysteines (147 & 204) that form disulfide bonds upon oxidation, which promotes protonation of the GFP chromophore, shifting the excitation spectrum peak of GFP from around 470 to 410 nm [19,20]. Mitochondrially targeted RoGFP was kindly provided by Dr. S.J. Remington (University of Oregon, OR) [20] and modified by site directed mutagenesis to incorporate 3 additional amino acid substitutions, rendering the protein more specific for changes in oxidation (RoGFP-R12) [21]. Following transfection, fluorescence was visualized on a Zeiss Axio Observer. Z1 Microscope. The oxidized and reduced RoGFP were visualized using excitation spectra at 410 and 470 nm, respectively with exciter filter sets DH410/30 and DH470/20 (Zeiss). The same beamsplitter, 500dcxr, and emission filter, HQ535/50, were used for both filter sets. Data was analyzed using Ratio/AxioVision 4.6 software (Zeiss) and expressed as a ratio between fluorescence observed at 410nm and 470 nm exposure, with higher ratio 410/470 indicating an increased oxidized state of RoGFP within the mitochondria.

Matrix metalloproteinase zymography

MMP-2 and -9 enzymatic activity were determined on non-denatured SDS-PAGE gel, as described previously [22]. Briefly, medium was collected and incubated with gelatin-agarose beads (Sigma, MO) at 200 μl / 1 ml of medium at 4°C overnight. The amount of medium incubated with beads was adjusted to cell number. Beads were washed and gelatin-bound MMPs eluted in 4X ESB buffer (9% SDS, 36% glycerol, 180 mM Tris, pH 6.8, 0.01% bromophenol blue), followed by electrophoresis on a 10% non-denatured acrylamide gel impregnated with 1 mg/ml gelatin (Sigma). The gel was washed twice in 2.5% Triton 100, 50 mM Tris, pH7.5, 5 mM CaCl_2 , 1 μM ZnCl_2 and incubated in 1% Triton 100, 50 mM Tris, pH7.5, 5 mM CaCl_2 , 1 μM ZnCl_2 at 37°C for 24 hours. Following staining by coomassie blue the cleared bands of MMP-2 (at 72 kDa) and MMP-9 (at 92kDa) were quantitative analyzed using Image J software.

MMP-9 RT-PCR and VEGF semi-quantitative real time RT-PCR

RNA was isolated using TRizol Reagent (Invitrogen) and reverse transcribed using superscript II reverse transcriptase (Invitrogen), according to manufacturer's protocol. RT-PCR for MMP-9 was performed as described previously [22] and products visualized by agarose/ethidium bromide electrophoresis.

Real time semi-quantitative RT-PCR for VEGF was carried out on an Applied Biosystems 7500 Real Time PCR cycler, using SYBR green reagent (BioRad) and the following primer pairs: VEGF sense 5'- AGC CTT GCC TTG CTG CTC TA -3', VEGF antisense 5'- GTG CTG GCC TTG GTG AGG -3'. After amplification, specificity of the reaction was confirmed by melt curve analysis. Data was analyzed using the comparative CT method with values normalized to β -Actin levels and expressed relative to controls.

Clonogenicity Assay

Clonogenicity Assays were carried out essentially as described previously [23]. Briefly, cells were counted using a haemocytometer and 100 cells plated in each well of a 6-well culture plate or 1000 cells in a 100 mm dish. Cells were allowed to grow for 7 days, followed by crystal violet staining. Colonies containing at least 50 cells were scored and colony number in each plate was counted using ImageJ program. Survival fractions were calculated as (colonies/100 lacZ infected cells)/(colonies/100 Cat infected cells).

Results

Increased Sod2 mRNA levels correlate with stage and grade of human bladder cancer

Screening of publicly available expression data from cancer microarrays indicates that mitochondrial manganese superoxide dismutase (Sod2) expression is consistently elevated in bladder cancer specimen of increasing grade and stage (Figure 1; oncomine.org). High grade tumor specimen had significantly higher Sod2 levels than low grade tumors (Figure 1A). Invasive bladder tumors, as reflected by both increased T and N stages, indicating local invasiveness and spread to the lymph nodes, respectively, displayed enhanced expression of Sod2 (Figure 1B-D). In contrast, levels of catalase remained unchanged in most of these studies, with the exception of one study that showed a statistically significant decrease in catalase expression in invasive compared to superficial bladder cancer specimen (Figure 1E). This implicates elevated Sod2 expression and a concomitant increase in mitochondrial ROS (H_2O_2) production in the aggressiveness of bladder tumors.

Increased Sod2 activity in the human metastatic bladder cancer cell line 253J B-V

A highly metastatic line of human transitional bladder carcinoma cells, 253J B-V, that was derived from a non-metastatic parental line, 253J, following 5 successive passages after bladder implantations in athymic nude mice, was used as an *in vitro* model to further investigate the role of Sod2 in bladder cancer. The 253J B-V cells are known to exhibit enhanced baseline clonogenic activity and growth when compared to the parental cells [12]. In addition, this metastatic derivative was able to spread and form lung metastases following orthotopic implantation in nude mice and shown to exhibit enhanced anchorage-independent growth compared to the 253J line [12]. Further, characterization of this cell line showed altered expression of adhesion molecules and displayed increased movement through matrigel matrix [12]. Using this metastatic cell model, analysis of cellular Sod activity indicated a pronounced increase in the activity (Figure 2A & B) and immunoreactivity of Sod2 (Figure 2C) in the 253J B-V cells relative to their 253J parental cell lines. No changes were observed with respect to the activity of the cytosolic CuZn containing superoxide dismutase (Sod1; Figure 2A). The activity of the H_2O_2 detoxifying enzymes catalase was significantly decreased in the more

metastatic line (Figure 2D), whereas activity of another H_2O_2 detoxifying enzyme, glutathione peroxidase (GPx), remained relatively unchanged between the two cell lines (Figure 2E). Total glutathione levels were not significantly different between the two cell lines (253J 1.59 ± 0.83 $\mu\text{mol/mg}$ protein; 253J BV 0.99 ± 0.14 $\mu\text{mol/mg}$) and while also not statistically significant, the more metastatic cell line 253J-BV showed a trend towards a lower reduced/oxidized Glutathione ratio, which may be a consequence of elevated levels of H_2O_2 and the cells redox buffering capacity, as a result of enhanced Sod2 and reduced catalase levels in these cells (Figure 2F).

Elevated H_2O_2 production in the metastatic 253J B-V cells

These changes in antioxidant profile suggested that there may be an overall net increase in H_2O_2 production in the more metastatic 253J B-V cells, given their enhanced dismutating activity of Sod2 and decreased levels of catalase. Indeed, 253J B-V cells displayed an approximately 1.5 fold increase in H_2O_2 production compared to 253J cells, as determined by inhibition of complex I formation of catalase using amino 1, 2, 4-triazole (Figure 3A). Similarly, we observed increased oxidation of a novel redox sensitive GFP construct (RoGFP [19-21]) targeted to the mitochondria, when expressed in the 253J B-V line. This indicates increased mitochondrial ROS production, likely resulting from the enhanced mitochondrial Sod activity in these cells when compared to their parental line 253J (Figure 3B).

Metastatic 253J B-V cells display enhanced, H_2O_2 -dependent, MMP-9 and VEGF expression

Increased expression and activity of Sod2 have been reported to transcriptionally enhance the expression of several matrix metalloproteinase family members in distinct cellular systems [24,25]. Analysis of MMP-9 levels indicated that the expression of this gelatinase is similarly enhanced in the 253J B-V cells relative to the non-metastatic parental cell line, as observed by gelatin zymography and RT-PCR analysis (Figure 4A-C). 253J B-V cells displayed a 3-fold increase in MMP-9 activity compared to 253J cells (Figure 4B). The other human gelatinase, MMP-2, was not highly detected by zymography in either cell line, suggesting that MMP-9 is the primary gelatinase secreted by these bladder cell lines. The expression of the key angiogenic factor VEGF has been reported to be redox sensitive [10,26,27]. Analysis of VEGF protein levels in the 253J B-V cells showed a significant increase in its immunoreactivity, relative to the parental cells (Figure 4D & E). In addition we observed 8-fold higher VEGF message levels in 253J-BV cells compared to 253J cells (Figure 4F).

Expression of both MMP-9 and VEGF is known to be sensitive to alterations in the steady state production of H_2O_2 [10,24,26-30]. Enhanced Sod2 activity has been reported to increase the intracellular production of H_2O_2 at near diffusion limiting rates [31,32]. To evaluate whether an increase in H_2O_2 production in response to enhanced Sod2 expression was responsible for the increases in MMP-9 and VEGF expression in the metastatic variant 253-BV, we over-expressed the H_2O_2 -detoxifying enzyme catalase by stable adenoviral infection. Figure 5A displays the relative increases in catalase activity in the 253J and 253J B-V cells following catalase infection compared to controls (Lac-Z). Oxidation of the redox sensitive RoGFP probe was significantly reduced indicating a decrease in ROS levels following catalase expression (Figure 5B). Catalase overexpression led to a corresponding decrease in the MMP-9 activity on gelatin zymography in both the parental as well as the metastatic cells (Figure 5C & E). Similarly, catalase overexpression resulted in decreased VEGF message and protein expression as assessed by immunoblotting (Figure 5D & F). To further show the role of ROS on the levels of MMP-9 and VEGF expression in our system, cells were treated with the antioxidant N-acetylcysteine and the catalase inhibitor 3-Amino-1,2,4-triazole. As can be seen in Figures 5G & H, N-acetylcysteine had significant effects in reducing the MMP-9 and VEGF levels in 253J-BV cells. While 3-Amino-1,2,4-triazole had little effect on MMP-9 protein levels in the 253J-

BV cells, likely due to already saturating levels of MMP-9 protein in the untreated controls, VEGF message levels further increased in response to inhibition of catalase.

Removal of H₂O₂ inhibits the high clonogenicity of 253J B-V cells

As previously reported, the metastatic 253J B-V line displayed enhanced clonogenicity compared to its parental non-metastatic line 253J [Figure 6A; [12]]. Growth of both cell lines was attenuated by overexpression of catalase, suggesting the involvement of H₂O₂ in this phenotype (Figure 6B). Overall these findings suggest that oxidants, primarily H₂O₂, play a role in the regulating the activity of several key mediators of the metastatic phenotype of 253J B-V cells.

Discussion

Previous studies performed in a variety of organisms have demonstrated that decreases in oxidant burden can prolong lifespan and prevent tumor development, while a pro-oxidant state promotes carcinogenesis and tumor progression [2,4-11]. Environmental factors (*e.g.* ionizing radiation, polycyclic aromatic hydrocarbons, smoking) are capable of inducing the steady-state production of ROS which can act as both initiators and promoters of carcinogenesis [2,8,33, 34]. Redox reactions can contribute to progression to malignancy by increasing mutagenesis, inhibiting differentiation, promoting mitogenesis, converting protooncogenes into oncogenes and inactivating tumor-suppressor genes [8,33,34]. In general, malignant cells exhibit enhanced production of ROS and an altered expression of cellular antioxidants that metabolized ROS, such as Sod2 and catalase [8].

Various breast cancer cells demonstrate a variable level of expression of Sods and catalase. Adenoviral mediated overexpression of Sod1 and Sod2 resulted in decreased cell growth and survival, as well as decreased xenograft growth rate [35]. Sod2 activity has been found to be lower in the androgen independent prostate cancer cells (PC-3), relative to the nonmalignant immortalized prostate epithelial cells, as well as in prostate tumor specimen [36-38]. The plasmid mediated Sod2 overexpression in these cells was associated with inhibition of cell proliferation and suppression of tumor growth [36]. In contrast, when nude mice are injected into the flank with MIA PaCa-2 human pancreatic cancer cells, they occasionally develop intra-abdominal metastatic deposits and ascites that are associated with an increase in Sod2 activity [39]. Lewis *et al.* studied the ascetic fluid of metastatic tumors and Capan-1 (metastatic) cell lines and noted that during metastatic progression, the MnSOD, CuZnSOD, and GPx protein and activity were increased compared to the parental MIA PaCa-2 cells. They concluded that metastatic disease is associated with changes in the content and activity of antioxidant enzymes with an associated change in growth characteristics [39]. In recent years epidemiological studies have shown that increases in immuno-reactive Sod2 in a variety of tumor types are associated with poor patient outcome and more aggressive disease [40-48]. From the microarray expression data and *in vitro* data presented here it appears that Sod2 expression is similarly upregulated and associated with more metastatic forms of bladder cancer. Single nucleotide polymorphisms in Sod2 that alter its activity have been linked to an increase in the frequency of both breast cancer and prostate cancer [49,50]. The rationale for the differences in tumor cell responses to Sod2 activity have not been clearly defined, but are possibly due to differences in the stage of the cancer and other complicating genetic mutations.

While the oxidant and antioxidant profiles have been studied in several cancer cell lines, information on the oxidants and antioxidant pathways in human bladder cancer is quite limited. Pendyala *et al* reported the levels of glutathione to be significantly higher in bladder cancer cells compared to the benign cells, but the status of Sods, Glutathione Peroxidase (GPx) or catalase was not discussed [51]. One study has reported increase in the activity of GPx and catalase in cancerous bladder tissue while others report a decrease in the catalase activity in

malignant tissue, relative to the benign controls [52,53]. We have observed that the acquisition of metastatic phenotype (i.e. from parental 253J cells to metastatic 253J B-V cells), is associated with a significant increase in the expression and activity of Sod2, and decreased activity of catalase. This was associated with enhanced steady-state production H_2O_2 levels in the 253J B-V cells, as well as increased activity and expression of MMP-9 and VEGF. The observation that the VEGF protein appears to migrate slightly differently on SDS-PAGE from protein isolates of the two cell lines is intriguing (Figure 4D). VEGF has been shown to exist as different isoforms in tumor cells. For example, Cheng *et al.* have reported that the VEGF189 isoform may be less active in promoting angiogenesis than the other two isoforms (VEGF165 and 121) reported in the study [54]. In addition, VEGF has been reported to be glycosylated, thereby changing its migration on SDS-PAGE [54]. While we saw two bands in lysates from 253J cells in Figure 4D we did not consistently observe this (Figure 5D). However it would be of interest to determine which isoforms are the major proteins found in either cell line and if VEGF is differentially glycosylated. This may impact the role of VEGF signaling and angiogenesis in tumors derived following orthotopic implantation of these cells in *in vivo* tumorigenicity assays.

This is the first report demonstrating a link between oxidative stress and metastatic progression in bladder cancer cells. Overexpression of catalase resulted in a significant inhibitory effect on the metastatic cells, by decreasing MMP-9 activity and suppressing the clonogenic activity. It appears that the development of the metastatic phenotype in bladder cancer may be associated with alterations in the redox-state of the cell. It is likely that the increase in the steady state H_2O_2 levels, because of increased Sod2 activity, results in overexpression of a number of metastasis-related growth factors and may enhance their metastatic behavior. While our data suggest that changes in oxidant production alter cellular signaling events that regulate the expression of pro-metastatic genes such as VEGF and MMP-9, it remains to be determined whether these changes in ROS contribute to overall genetic instability and higher mutation rates in the more metastatic 253J-BV line resulting in enhanced expression of oncogenes and/or repression of tumor suppressor genes. However, the finding that the metastatic phenotype of the variant can be attenuated by catalase-mediated detoxification of H_2O_2 opens the window for the evolution of antioxidant based therapeutic intervention for the treatment of invasive bladder carcinoma.

Acknowledgements

Grant Support: Research described in this article was supported by Philip Morris USA Inc. and Philip Morris International. We thank Xiao Fang Ha for her excellent technical support.

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Figure 1.

An increase in Sod2 mRNA levels correlates with stage and grade of bladder cancer. Representative data from bladder cancer array studies are displayed as box and whisker plots. Boxes represent the interquartile range marking the 25th to 75th percentile; mid line indicates the median value; whiskers mark the 10th to 90th percentile range. Significant increases in Sod2 mRNA expression were observed in high (n=69) vs low grade bladder carcinoma specimen [n=12; **A** [55]]; in invasive (n=13) vs superficial transitional cell carcinoma [n=27; **B** [56]]; in high N stage (N2 n=7) vs low N stage bladder carcinoma [N1 n=5; N0 n=38; **C** [57]]; and high T stage (T4 n=13) vs low T stage bladder carcinoma (T3 n=13; T2 n=6; T1 n=9; Ta n=16; **D** [57]). * p<0.05, ** p<0.01, *** p<0.001, Student's T-test. **E.** Significant decrease in catalase mRNA expression was observed in invasive (n=81) vs superficial (n=28) bladder carcinoma [58]. *** p<0.001, Student's T-test.

Figure 2.

A. Levels of Sod2 activity are increased in 253J B-V compared to 253J cells, as assessed by in-gel Sod activity assay. **B.** Quantitative analysis of Sod2 in-gel activity assays (Image J). Data represent mean $\pm$ SEM, $n=7$. $**p<0.01$, Student's T-test. **C.** Sod2 protein levels are increased in 253J B-V compared to 253J cells, as assessed by immunoblotting. **D.** Catalase Activity Assay of 253J and 253J B-V cells was carried out as indicated in Materials and Methods Section. Data represent mean $\pm$ SEM, $n=6$, $*p<0.05$, Student's T-test. **E.** Glutathione peroxidase activity assay of 253J and 253J B-V cells. Assay was carried out as indicated in the Materials and Methods Section. Data represent mean $\pm$ SEM, $n=4$. **F.** Reduced glutathione (GSH) / Oxidized Glutathione (GSSG) ratio of 253J and 253J B-V cells. Glutathione levels were assessed as described in Materials and Methods. Data represents mean $\pm$ SEM, $n=3$.

Figure 3.

A. Levels of H₂O₂ in 253J and 253J B-V cells as assessed using aminotriazole-mediated inactivation of catalase. Data points are expressed as inverse log of catalase activity following aminotriazole treatment at different time points over catalase activity without treatment. H₂O₂ concentrations were derived as described in methods (n=2). **B.** Measuring mitochondrial ROS production with RoGFP. Cells were transfected with mitochondrially targeted RoGFP-R12. 24 hrs post-transfection fluorescent images were taken at excitation 410 and 470 (n=5). Oxidation of RoGFP (ie ROS production) in 253J compared to 253J B-V cells was plotted as a ratio of GFP fluorescence observed at excitation 410 over 470nm (mean +/- SEM, p=0.055).

Figure 4.

A. An increase in MMP-9 activity is observed in 253J B-V compared to 253J cells, as assessed by in-gel zymography assays. **B.** Quantitative analysis of MMP-9 activity in 253J and 253J B-V cells ($n=7$, mean $\pm$ SEM, ** $p<0.01$, student's T-test). **C.** Representative RT-PCR of MMP-9 **D.** Levels of VEGF protein are increased in 253J B-V cells compared to 253J cells, as assessed by immunoblotting. **E.** Quantitative analysis of VEGF immunoblotting in 253J and 253J B-V cells ($n=5$, mean $\pm$ SEM, ** $p<0.01$). **F.** VEGF Real time semi-quantitative RT-PCR. RNA was isolated, reverse transcribed and real time semi-quantitative RT-PCR carried out with VEGF specific primers. Values were normalized against β -actin levels and expressed relative to levels in 253J cells ($n=5$, mean $\pm$ SEM, ** $p<0.01$, student's T-test).

Figure 5.

A. Catalase activity following adenoviral infection of catalase (CAT) or control (Lac-Z) in 253J and 253J B-V cells. **B.** Decreased oxidation of RoGFP following catalase expression in 253J-BV cells. RoGFP oxidation was assessed as described in Fig. 3B. Oxidation of RoGFP in catalase infected 253J B-V cells as compared to control infected cells (LacZ) was plotted as a ratio of GFP fluorescence observed at excitation 410 over 470nm (n=20, mean $\pm$ SEM, * p<0.05).

C. MMP-9 activity decreases in both 253J and 253J B-V cells following catalase expression, as assessed by gelatin zymography. **D.** Decreased VEGF protein expression following catalase adenoviral infection, as demonstrated by immunoblotting. **E.** Quantitative analysis of MMP-9

activity in 253J and 253J B-V cells following catalase overexpression. (n=3, mean +/- SEM, * p<0.05 catalase infected compared to lacZ infected cells, student's T-test). **F.** VEGF Real time semi-quantitative RT-PCR following catalase over-expression. RNA was isolated, reverse transcribed and real time semi-quantitative RT-PCR carried out with VEGF specific primers. Values were normalized against β -actin levels and expressed relative to lacZ infected controls (n=3, mean +/- SEM, ** p<0.01 catalase infected compared to lacZ infected cells, student's T-test). **G.** MMP-9 activity levels following catalase inhibition by aminotriazole or treatment with the antioxidant N-acetylcysteine, as assessed by gelatin zymography (n=4, mean +/- SEM, *** p<0.001 statistical significance relative to un-treated controls of each cell line, Student's T-test) **H.** VEGF Real time semi-quantitative RT-PCR following catalase inhibition by aminotriazole or treatment with the antioxidant N-acetylcysteine Values are expressed relative to levels in non-treated 253J cells (n=3, mean +/- SEM, * p<0.05, ** p<0.01 statistical significance relative to un-treated controls of each cell line, Student's T-test).

Figure 6.

A. Clonogenicity of 253J and 253J B-V cells. 1000 cells were seeded in 100 mm dishes and allowed to form colonies for 7 days. Colonies formed were stained by crystal violet and counted (n=4, mean $\pm$ SEM, *** p<0.001). **B.** Catalase expression decreases clonogenicity of 253J and 253J B-V cells. Cells were infected with catalase (CAT) or control (Lac-Z) adenovirus. 100 cells of each cell line were grown in 6-well plate for 7 days and the colonies formed stained with crystal violet and counted (n=4, mean $\pm$ SEM, * p<0.05; ** p<0.01).