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Increased Reactive Oxygen Species Are Associated with Low Density and Migration in a Metastatic Human Prostate Cancer Cell Line

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Abstract: Increased ROS in prostate cancer cells has been associated with cellular proliferation; however, the roles of ROS during invasion or metastasis are less well defined. While proliferation leads to higher density, invasive cells leave the cell mass and exist in a lower density state. Our aim was to determine if ROS levels vary with cell density and determine if ROS play a role in motility, adhesion or invasion. We found that ROS are increased in prostate cancer cells grown at low density and that this increase was particularly apparent in migrating cells. Inhibition of ROS reduced cell motility, adhesion to matrix substrates and invasion through Matrigel. Catalase activity was found to be down-regulated at low density but SOD activity and glutathione ratio were unaltered. Reduced catalase activity may contribute mechanistically to increased ROS at low density and this ROS appears to promote motility, adhesion and *in vitro* invasion.

Key words: Prostate cancer, reactive oxygen, invasion, motility, cell density

Introduction

Prostate cancer cell lines and tumor tissues have been shown to possess upregulated quantities of reactive oxygen species (ROS) (Lim *et al.*, 2005) and these ROS are becoming increasingly associated with several aspects of prostate cancer progression including not only carcinogenesis but also tumor cell proliferation and invasion. In prostate cancer, oxygen radicals are reported to arise from several sources within the cells including the NADPH oxidase (Lim *et al.*, 2005), mitochondrial glycerophosphate-dependent ROS (Chowdhury *et al.*, 2005), xanthine oxidase and nitric oxide synthases (Chiarugi, 2003). The cell's net redox state is a balance between oxygen radical synthesis and breakdown, and net ROS generation in prostate cancer has also been reported to develop from downregulated levels or activities of the scavenger enzyme systems catalase, superoxide dismutase I (Zn 2+/Cu 2+ SOD) and II (MN-SOD), and glutathione peroxidase (Chiarugi, 2003).

While most attention has focused on ROS mediation of carcinogenesis and proliferation, some evidence also associates ROS with tumor invasion and metastasis. In metastatic cell lines derived from the human prostate cancer line LNCaP, Lim (2005) reports elevated levels of nox1 and H_2O_2. Some potential targets of ROS activity during invasion have been identified. For instance, in the human prostate cancer line PC-3, ROS appear to regulate matrix metalloproteinase-2 (MMP-2), a type IV collagenase which degrades the basement membrane (Shariftabrizi *et al.*, 2005). Oxygen radicals in this cell line also induce VEGF which can promote angiogenesis (Gao *et al.*, 2004). ROS may also serve

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as second messengers to mediate adhesive signals from integrins or cadherins to the focal adhesion kinase (FAK) (Chiargi, 2003). Although adhesive signaling may contribute to enhanced migration, a critical role of ROS in prostate cancer cell motility and migration has not been previously reported.

In this study, we wished to first determine if ROS varied as a function of prostate cancer cell density. We found that low cell density is associated with the generation of ROS. The behavior of ROS-positive cells in culture suggested that ROS may influence the migration of cells away from tumor cell clusters and therefore we assessed the effect of ROS on migration, adhesion to specific matrix substrates and *in vitro* invasion. We also compared the activities of two major ROS catabolic pathways and the glutathione redox ratio between low and high density cells. Present results link increased ROS in relatively isolated prostate cancer cells with *in vitro* migration and invasion.

Materials and Methods

Cell Culture and Treatments

PC3M cells were a gracious gift from Dr. Isiah Fidler (MD Anderson Cancer Center, Houston TX). These cells were maintained in Minimal Essential Medium with Earle's salts (MEM), supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin, 100 ug mL^{-1} streptomycin, and 2.0 mM L-glutamine. To detect reactive oxygen species, cells were treated with 5-(and-6)-chloromethyl-2',7'- dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, purchased from Molecular Probes). This probe is a cell permeant ester which is hydrolyzed in the cytoplasm of live cells. When oxidized by molecules such as superoxide anion or hydroxyl radical, CM-H_2DCFDA fluoresces with excitation/emission spectra peaks at 495 and 525 nm, respectively. To load CM-H_2DCFDA into cells, monolayer cultures were washed using Hank's Balanced Salt solution with calcium and magnesium (HBSS-Ca, Mg), then incubated at 37°°C for one hour in a solution of HBSS-Ca, Mg containing 10 uM CM-H_2DCFDA. Cells were then washed in two consecutive changes of MEM containing 0.1% bovine serum albumin (BSA) for 30 minutes each. Following washes, cells were viewed and imaged under epifluorescence with a Zeiss Axioplan 200 microscope using an FITC-Alexafluor 488 compatible filter set (Chroma Filters No. 41017); images were captured with an Olympus Q Capture 5 camera and Q Capture Pro software. Quantitative fluorescence measurements were obtained using a Spectra Max 2 fluorescent spectrophotometer at excitation 485 and emission 530 nm. Fluorescence intensity (in units) was divided by the cell number in each well to yield an intensity per 10,000 cells.

For ROS inhibition studies, two compounds were used: the SOD mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, free radical (TEMPOL, purchased from Sigma Chemicals) and N-acetyl cysteine (NAC, Sigma Chemicals), a more water soluble form of the amino acid cysteine which is a component of the antioxidant glutathione. Tempol was diluted in HBSS-Ca, Mg and used at a final concentration of 2.0 mM; NAC was diluted in HBSS, neutralized to pH 7.0 and used at a final concentration of 2.0 mM.

Motility Assays

To quantitate the horizontal movement of cells, the scratch assay was used. For this assay, a confluent monolayer of cells is established and then a scratch is made through the monolayer in an "X" pattern using a standard plastic 1-200 uL pipet tip. This creates an *in vitro* wound approximately 700 um wide. Cancer cells fill in the scratch area by migrating as single cells from the confluent sides. The width of the scratch was measured using Q-Capture Pro software each day until the gap filled in

completely. Eight replicate wells from a 24-well plate were used for each experimental condition. The scratch gap in each well was measured in four separate areas and these measurements were averaged to give the overall measurement for that well. Data was analyed using linear regression (GraphPad Prism software, San Diego, CA).

To quantitate vertical motility, an *in vitro* motility assay was used. In this assay, FluoroBlok transwell inserts with 8 uM pores (BD Falcon) were inserted into the top of 24-well tissue culture plates to create a two-chamber culture. PC3M cells (10^5 total) were plated into the upper chamber and allowed to migrate vertically through the pores to the lower chamber. In this motility assay, both upper and lower chambers will filled with maintenance culture media and after one day, cells which migrated through the pores to the lower membrane surface were visualized and counted. To easily identify the cells on the lower membrane surface, the transwell insert was incubated in a 100 uM solution of Calcein Blue fluorescent indicator (Molecular Probes) diluted in HBSS. After one hour, the calcein Blue removed using two fifteen minutes. incubations of the insert with HBSS. This treatment produced a bright blue fluorescence in live cells on the lower membrane while the FluoroBlok coating blocked fluorescence coming from the cells in the upper chamber.

To assay *in vitro* invasion, Fluoroblok inserts were pre-coated with a 100 uL solution of Matrigel diluted in growth medium to 1 mg mL^{-1}. Matrigel was allowed to dry onto the upper surface of the membrane overnight to evenly coat the surface. One-hundred thousand PC3M cells were plated into the upper chamber as described above and quantitated after two days using Calcein Blue dye uptake. Data for motility and invasion assays were analyzed by t-test.

Cell Proliferation

Cells were plated at an initial concentration of 10,000 cells per well in 24-well plates in maintenance media and treated with tempol or NAC at 2 mM each. At two day intervals, cells from four replicate wells for control and each treatment were counted using a hemocytometer.

Adhesion Assay

Twenty-four well tissue culture plates were coated overnight with 20 ug well of human fibronectin or vitronectin (both purchased from Sigma Chemicals), Matrigel (BD Biosciences, San Jose, CA) or type IV collagen (Rockland Immunochemicals, Gilbertsville, PA). The coated wells were washed twice with PBS then blocked with 1 mL of 1% BSA in PBS for 1 h at 37°C. Following a second PBS wash, 1×10^5 prostate cancer cells were seeded onto the matrices or into BSA only-blocked wells for a background measurement for 1 h at 37°C. Non-adherant cells were removed by aspiration and all wells were then washed twice with PBS. Adherent cells were removed by trypsinization and counted. Results are the mean and standard deviation of four wells for each cell line and inhibitor-treated populations were compared to untreated populations using one-way ANOVA.

Glutathione Ratio (Reduced Glutathione/Oxidized Glutathione)

Detection and quantification of total and oxidized glutathione was performed as described (Anderson, 1985, Griffith, 1980). Briefly, to assay total glutathione, a 50 µL aliquot of protein sample (cell lysate) was combined with 700 µL 0.298 mM NADPH, 100 µL Ellman's reagent, 100 µL dH2O, and 50 µL glutathione reductase. Standards were prepared in the same manner, substituting 50 µL 5% 5-sulfosalicylic acid in place of sample. Chromogenic product formation was quantitated at 412 nm using a Multiskan Spectrum spectrophotometer (Thermo Electron Corporation, Waltham, MA). All chemicals were purchased from Sigma-Aldrich, St. Louis, MO. To assay oxidized glutathione, a

30 μL aliquot of cell lysate was mixed with 2 uL 2-viynlpyridine and incubated at room temperature for sixty minutes. A 5% solution of 5-sulfosalicylic acid (30 μL) was then added to the lysate and samples were analyzed as for total glutathione. Reduced glutathione content was calculated by subtracting oxidized glutathione from total glutathione. Final values were calculated based on equivalent amounts of total protein.

Superoxide Dismutase (SOD) Assay

SOD activity was measured using an SOD Assay Kit according to the manufacturer's protocol (Dojindo Molecular Technologies, Inc., Gaithersburg, MD).

Catalase Assay

To determine catalase activity, cells were suspended in 50mM potassium phosphate buffer pH 7.0 and sonicated. Aliquots of cell homogenate (2-5 mg total protein mL^{-1}) were activated with 30 mM H_2O_2, and then assayed at 240 nm every 30 sec for 2 min. This assay has been previously described (Aebi, 1984; Beers and Sizer, 1952).

Results

ROS Levels Become Increased in Prostate Cancer Cells at Lower Densities

PC3M cancer cells were plated at 5000 cells/well or 150,000 cells/well in 24-well plates and allowed to attach overnight. Accumulated ROS were visualized by loading cells with CM-H$_2$DCFDA indicator, which generated green fluorescence upon oxidation. At the low density level, approximately 80% of cells were visibly green (Fig. 1A, phase contrast image shows all cells present in field of view). Cells at high density, contained less than 15% of cells with ROS fluorescence (Fig. 1B). The intensity of green fluorescence varied somewhat in the population; however ROS positive cells at low density setting appeared to be flat and spreading while intense ROS positive cells in the confluent setting were rounded.

To quantify the inverse association of ROS with density, PC3M cells were plated at various densities using serial dilution beginning with 150,000 cells/well. Fluorescence was quantified and is expressed on a cell basis (Fig. 1C). This experiment demonstrated a marked increase in cellular ROS as the density decreased. The level of fluorescence appeared to plateau at densities below 10,000 cells/well (approximately 5000 cells cm^{-2}).

PC3M Migration In Vitro Can Be Modulated by Inhibiting and Enhancing Reactive Oxygen Levels

The appearance of increased ROS in cells at low density suggests that the reactive oxygen might play a role in motility, adhesion or proliferation (Mooney *et al.*, 1992). To determine whether ROS production is involved in migration, we investigated the effect of reducing net ROS utilizing tempol and NAC in a scratch assay.

In this assay, PC3M cells were plated to confluence, treated with inhibitors, and then the monolayer surface of cells was scratched. The cancer cells migrate across the gap from both sides filling in the area (Fig. 2A, upper panel). Individual cells which left the confluent population lining the gap and migrated into the space showed a notable incidence of ROS-induced fluorescence compared with cells on the side (Fig. 2A, middle and lower panel). To measure the effect of inhibiting ROS on this horizontal migration, cells were treated with tempol and NAC and the width of the gap was measured on a daily basis. Untreated PC3M filled in the scratched area in five days; however
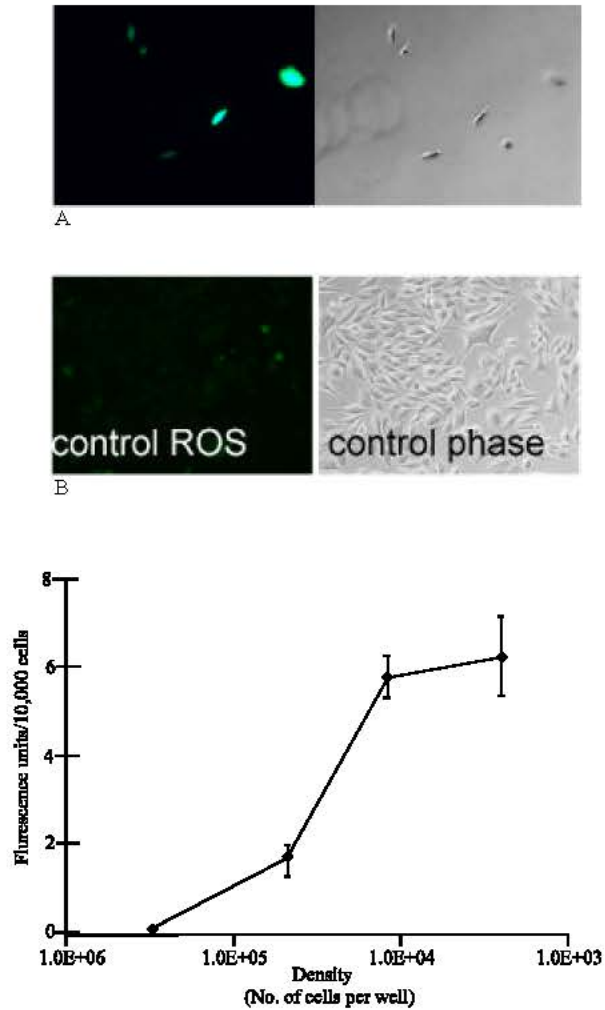
Fig. 1:   Reactive Oxygen Species (ROS) in PC3M cells at low density
   A: Green fluorescence indicative of ROS oxidation of intracellular CM-H$_2$DCFDA indicator in cells plated at 5000 cells/well (100x magnification). Fluorescent cells are shown (left frame) compared to a phase contrast image of all cells in the field of view (right frame)
   B: Green ROS fluorescence in high density cells. Fluorescent cells only (left frame), phase contrast of all cells (right frame)
   C: Fluorescent intensity from ROS increases as cell density decreases. PC3M cells were plated in 24-well plates at four dilutions. The cells were then loaded with CM-H$_2$DCFDA indicator and assayed using fluorescence spectroscopy. Results are expressed as fluorescence units per 10,000 cells vs. final cell density. Values represent mean of 4 replicates ± SEM

2A
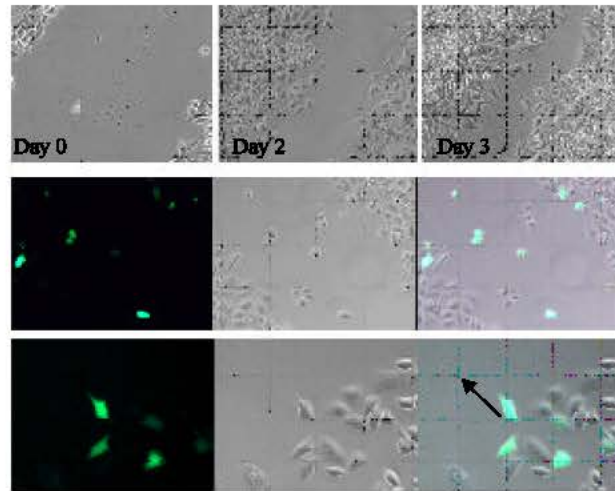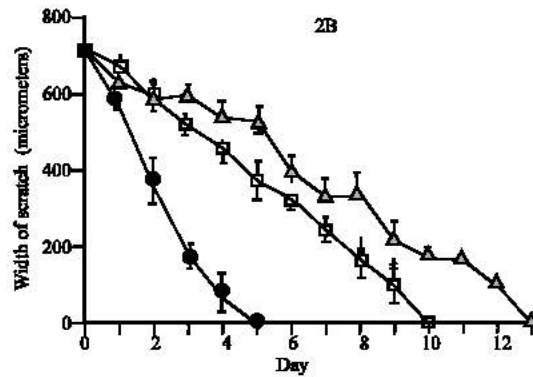
Fig. 2: Decreased rates of migration are associated with inhibition of ROS by tempol and NAC
    A: Upper panel: Phase contrast images (100x) show the time course of cellular migration resulting in filling of the gap created in the scratch assay. Middle panel: Cells which have migrated into the gap show a greater incidence of ROS-induced fluorescence than cells within the confluent sides (100 x image). Left frame shows fluorescence cells only, middle frame shows all cells present (in phase contrast) and right frame is an overlay of left and middle frames. Lower panel: 200x image showing greater detail of cells migrating away from confluent side into open gap. Arrow points to direction of the gap
    B: Time course of cell migration resulting in decreasing gap. Control (•) and treatments with tempo (□), and N-acetyl cysteine (NAC; △) are shown as the average ± S.D. of 8 replicate wells. Treatment samples had significantly different slopes than controls (linear regression analysis, 95% confidence interval)
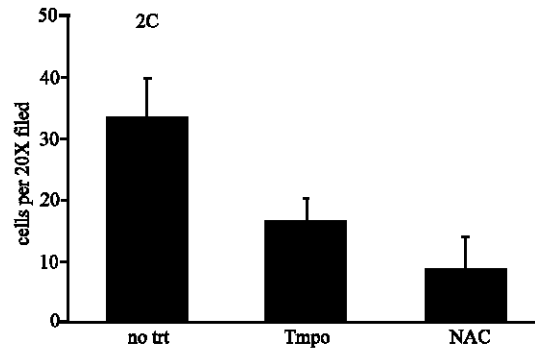
Fig. 2C: Vertical migration of cells through FluorBlok transwell chambers is inhibited by treatment
with 2 mM tempol (Tmpo) and 2 mM N-acetyl cysteine (NAC). Values represent the mean
± S.D. of cells per field of view counted through a 20x objective. Five random areas were
counted and averaged per insert and 4 inserts per treatment condition were used. Both
tempol and NAC-treated cells migrated significantly slower (p<0.05; t-test) compared with
no treatment (no trt)

tempo-treated cells required 10 days to fill in the area and NAC-treated cells required a 13-day
period (Fig. 2B). Thus, agents that enhance the breakdown of ROS inhibit motility of PC3M.

In addition to the horizontal migration measurable with the scratch test, the effect of inhibiting
ROS on migration was also tested using transwell inserts in a vertical migration assay. In this assay,
inserts with 8 uM pores were inserted into 24-well plates and cells which migrated through the pores
from the upper to lower chambers were enumerated. In this *in vitro* assay, both tempo and NAC
treatment inhibited migration by 52 and 76%, respectively (Fig. 2C). These data correlate well with
the scratch results and further support a role for ROS in directing cellular migration in this case through
a porous barrier.

ROS could also promote growth in PC3M cells; however, we performed a proliferation assay and
found that over a 6 day period, neither tempo nor NAC at the concentrations which inhibited motility
significantly altered proliferation in PC3M over a 6 day period (data not shown).

Tempol and NAC Reduced Adhesion of PC3M on Selected Matrix Substrates
PC3M cells were pretreated overnight with tempol or NAC then assayed for adhesion to vitronectin,
fibronectin, Matrigel, which is a tumor-cell derived basement membrane consisting primarily of laminin
and collagen (Kleinman *et al.*, 1982) or type IV collagen (Fig. 3A). Control PC3M cells showed greater
levels of adhesion to fibronectin and Matrigel and adhesion to these two extracellular matrix
components was significantly reduced by both tempol and NAC. In addition, NAC significantly
reduced adhesion of PC3M to vitronectin. No altered adhesion to type IV collagen was observed. The
finding that tempol and NAC inhibit adhesion as well as migration suggested the possibility that ROS
inhibition might also reduce invasion.

Tempol and NAC Inhibit In Vitro Invasion
To assay invasion, transwell inserts were employed as above but in this assay the inserts were
coated with Matrigel. Tempo and NAC treatment significantly reduced transwell invasion (Fig. 3B).
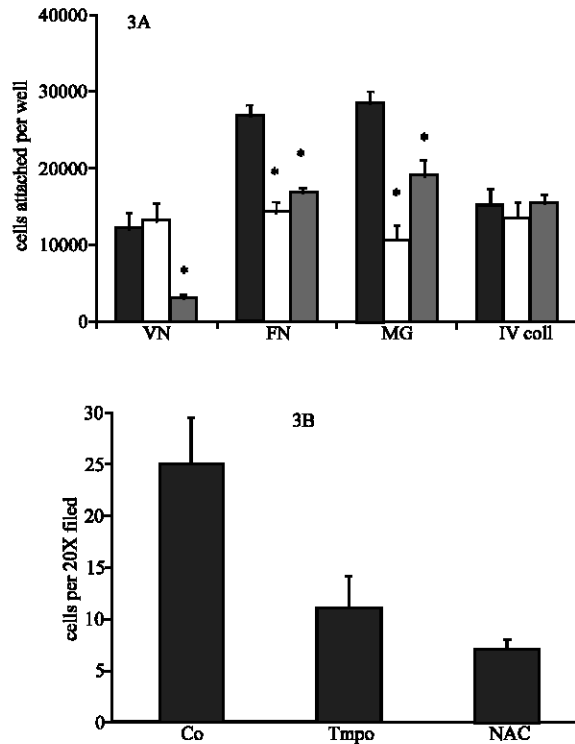
Fig. 3: Adhesion and invasion of prostate cancer cells is reduced by treatment with 2 mM tempol
and 2 mM NAC

A: Prostate cancer cells were untreated (black bars) or treated with tempol (white bars) or NAC
(grey bars) and plated into matrix-coated wells in a 96-well plate. Following a one-hour period
for attachment, the cells were detached and counted as described in Methods. Data is
expressed as the number of adherent cells; error bars show the SD from quadruplicate wells.
* indicates a significant difference compared to the untreated cells for each matrix condition.
Data were analyzed using one-way ANOVA

B: *In vitro* invasion of PC3M cells is inhibited by 2 mM tempol and 2 mM NAC treatment.
Cells plated in Matrigel-coated Fluoroblok inserts invaded through the extracellular matrix to
the lower surface of the transwell membrane. Values calculated as described in 2C. Both
tempol and NAC treatments significantly inhibited invasion (p<0.05; t-test)

E. Levels of ROS Metabolizing Enzymes May Be Altered as a Function of Cell Density

To assess the activity of major degrading enzymes for ROS, activities were measured for the
enzymes superoxide dismutase (SOD) and catalase in low vs. high density PC3M cells. In addition,
the ratio of reduced to oxidized glutathione was compared. These assays revealed that catalase activity
in low density cells was approximately one-half the activity in high density cells (Table 1). Neither
SOD activity not the glutathione ratio varied significantly with density.

Table 1: Comparison of ROS scavenging systems in PC3M cells grown to low or high densities shows that catalase activity is reduced at low density. Catalase and superoxide dismutase (SOD) activities are expressed as units/mg total protein

	Low Density	High Density
Catalase activity	43.91±1.57	95.89±15.2
Total superoxide dismutase activity	5.3 ± 0.35	6.0 ± 0.17
Glutathione ratio (GSH/GSSG)	5.5 ± 0.41	5.0 ± 0.30

Discussion

Cancer metastasis is a complex process involving tumor cell separation from the primary tumor mass, extracellular matrix proteolysis, migration, motility, altered adhesion, and angiogenesis (Kawaguchi, 2005). It has been recently suggested that ROS could play important roles in growth and angiogenesis (Lim *et al.*, 2005); however, in this study, our objective was to determine if ROS is associated with invasion-associated characteristics such as low density, motility, altered adhesion and *in vitro* invasion. Present results demonstrate that ROS are related to low cell density and are necessary for maximal directional motility, adhesion and invasion. These results suggest that prostate cancer cells may induce oxygen metabolites during the invasive process. These ROS could be by-products of increased energy production as Chowdhury *et al.* (2005) have previously shown that malignant prostate cells are more glycolytic than normal epithelia. Previous reports have indicated that ROS can activate several signaling pathways including the PI-3-kinase (PI3K) and Akt pathway in DU145 prostate cancer cells (Gao *et al.*, 2004), extracellular signal-regulated kinases (Erk)-1/2 in gastric cancer (Kim *et al.*, 2005a) and oxidant-sensitive transcription factors AP-1, CREB, and nuclear respiratory factor 1 (Felty *et al.*, 2005) in breast cancer cell lines. Activation of these pathways is implicated in cancer metastasis (Poser and Bosserhoff, 2004; Kim *et al.*, 2005a,b) and therefore the induced ROS in individual low density cells may contribute to development of a more invasive phenotype.

In our adhesion assay, we found that inhibition of ROS reduces adhesion to fibronectin and Matrigel basement membrane but this correlation did not extend not to type IV collagen. This suggests that ROS may selectively alter some components responsible for attachment. Whether these observations extend into the *in vivo* environment remains to be tested. Our observations from the scratch test indicate that only a portion of migrating cells intensely oxidized the indicator fluorochrome. This observation leads us to hypothesize that ROS upregulation during invasion and/or metastasis is a transient event and may not be associated with later stages of metastasis or with tumor establishment and high levels of homotypic adhesion.

The present findings are unique in that they suggest density is a key element in the induction of ROS in specific cells which are more motile and invasive. However, our data supports previous findings that have associated ROS with other invasion characteristics (Lim *et al.*, 2005; Shariftabrizi *et al.*, 2005; Gao *et al.*, 2004; Chiarugi, 2003).

In order to further clarify the role of ROS in prostate cancer invasion and metastasis, it is necessary to elucidate specific pathways linked to migration and adhesion which are altered by the increased ROS. These pathways could be signaling pathways such as FAK phosphorylation (Chiarugi, 2003), modification of other growth factor pathways, integrins or matrix proteases.

Acknowledgements

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