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Effects of N-acetyl-L-cysteine on adhesive strength between breast cancer cell and extracellular matrix proteins after ionizing radiation

Huiwen Cheng^{a,b}, Shin Hee Lee^a, and Shiyong Wu^{a,b,*}

^aEdison Biotechnology Institute, Ohio University, Athens, Ohio 45701, USA

^bDepartment of Chemistry and Biochemistry, Ohio University, Athens, Ohio 45701, USA

Abstract

The benefits of administering antioxidants during radiation therapy have been the subject of much controversy. It is known that both ionizing radiation (IR) and reactive oxygen species (ROS) alter the adhesive affinity between tumor cells and extracellular matrix (ECM) proteins and that IR induces production of ROS. We therefore evaluated the effect of N-acetyl-L-cysteine (LNAC), a common ROS scavenger, on the adhesive affinity between MDA-MB-231 breast cancer cells and extracellular matrix (ECM) proteins after IR. We focused mainly on fibronectin, a representative ECM protein. Our results using static cell adhesion assays indicated that continuously treating the breast cancer cells with LNAC (10 mM) for 24 h, starting immediately after IR (20 Gy), could inhibit IR-induced cell adhesion to ECM proteins at 24 h post-IR. Analyses of intracellular levels of ROS by the fluorescence dye carboxy-2,7-dichlorodihydrofluorescein diacetate and activated integrin $\beta 1$ by flow cytometry revealed that the reduction of cell adhesive affinity was correlated with a down-regulation of IR-induced ROS production and surface expression of activated integrin $\beta 1$. In addition to cell adhesion, treatment with LNAC inhibited IR-induced expression of vimentin, an epithelial mesenchymal transition marker (EMT). Interestingly, when the cells were pretreated for 1 h, the inhibitory effects of LNAC were found to be either reduced or completely abrogated followed by 24 h or 2 h treatments, respectively. Our results demonstrated that the time and duration of LNAC treatment is critical for regulating IR-induced adhesive affinity, and thus metastatic potential, as well as EMT process of breast cancer cells.

Keywords

LNAC; ionizing radiation; breast cancer; cell adhesion; integrin

Introduction

During tumor cell progression and metastasis, the extracellular matrix (ECM) plays a critical role in regulating tumor cell integrity and stability, as well as cell motility and migration (Aplin et al. 1999; Bendes 2012 and Borsig 2012). Cells are in dynamic contact with the

*To whom correspondence should be addressed. Shiyong Wu, 118 Konneker Research Center, Ohio University, Athens, OH 45701, USA, Phone: 740-597-1318, wus1@ohio.edu.

Conflicts of Interest

No conflict of interest.

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ECM via integrin receptors, which not only serve as mechanical linkages for cellular attachment, but also transmit outside-in and inside-out signals essential for cell growth and motility. Integrins are heterodimeric transmembrane molecules composed of non-covalently linked α and β subunits (Campbell and Humphries 2011). In mammals, 18 α and 8 β integrins have been characterized, which can be combined to form 24 different integrins, selectively binding with different ECM components (Fu et al. 2012). Integrins also form the bridge between the ECM and the intracellular cytoskeleton. Through this bridge, the cytoskeleton influences the structure or function of matrix adhesions. In endothelial cells, vimentin cytoskeleton associated with integrin $\alpha v \beta 3$, regulates focal adhesion size and helps to stabilize cell-matrix adhesions (Tsuruta and Jones 2003). It has been shown that cell-matrix interactions are critical for each step of the metastatic process, suggesting that integrin-ECM interactions could prove to be effective targets for therapeutics (Felding-Habermann 2003).

Reactive oxygen species (ROS), such as hydroxyl radicals, superoxide, and hydrogen peroxide can promote cancer progression through regulation of cell-matrix interactions (Chiarugi 2003). Several studies have demonstrated the positive correlation between generation of ROS and increased progression and metastatic ability of different carcinomas including prostate cancer (Lim et al. 2005), melanoma (Hyoudou et al. 2006) and lung cancer (Yan et al. 1996). Possible mechanisms involve ROS-mediated changes in integrin expression (Mori et al. 2004) and signaling (Svineng et al. 2008). In fact, reports have shown that pretreatment of highly metastatic tumor cells with ROS scavengers such as N-acetyl-L-cysteine (LNAC) suppress their metastatic potential in mice (Ishikawa et al. 2008). It is well known that radiation induces the production of ROS. Since radiation therapy is necessary for nearly all breast cancer patients (Yang and Ho 2013), the question arises as to whether radiation-induced oxidative stress may affect cell-matrix interactions in cancer cells leading to altered metastatic behavior. Therefore, the goal of the present investigation was to determine whether the ROS scavenger, LNAC, affects tumor cell-matrix interactions, especially cell-fibronectin interactions, by decreasing ROS levels induced by ionizing radiation. We assessed the extent to which LNAC affects cell adhesion, ROS levels and integrin activation under different treatment regimens after ionizing radiation.

Materials and methods

Cell culture

The human breast cancer cell line MDA-MB-231, was cultured in Minimum Essential Medium Eagle (MEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1mM nonessential amino acids, and 1% penicillin/streptomycin at 37 °C and with 5% carbon dioxide (CO₂). All cells were seeded in 100 mm cell culture dishes 24 h before each experiment.

Reagents and Antibodies

LNAC and 5-(and-6)-chloromethyl- 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) were purchased from Sigma (St. Louis, MO). Catalase from human erythrocytes, ECM cell adhesion array kits, human fibronectin-coated 96-well strips, and anti-human $\beta 1$ (activated) mAb (HUTS-4) were obtained from Millipore (Billerica, MA). FITC-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Drug Treatment

LNAC was dissolved in Tris-HCL (PH 8.8) and added to the cell culture medium at a final concentration of 10 mM. The cells were treated with LNAC according to three different time

frames indicated as: (1) “prolonged treatment”, which consisted of adding LNAC at the time of radiation, then harvesting the cells after 24 h post-irradiation; (2) “pre-short-term treatment”, which consisted of treating the cells with LNAC –1 h before irradiation, removing the LNAC by replacing the medium at 2 h post-irradiation (3 h total), and then harvesting the cells after incubating for another 22 h (24 h post-irradiation); and (3) “pre-prolonged treatment”, which consisted of treating the cells with LNAC at 1 h before irradiation, and then harvesting the cells 24 h post-irradiation (25 h total).

For the irradiation, cells were exposed to γ -rays generated from a ^{137}Cs source (J. L. Shepherd Associates) for a single dose of 20 Gy, at a dose rate of approximate 1 Gray (Gy) per minute.

Static Cell Adhesion Assay

ECM protein pre-coated 96-well strips were rehydrated with phosphate buffered saline (PBS) before the adhesion assay. At 24 h post-irradiation, cells were harvested and 10^5 cells per well were seeded onto plates and incubated at 37 °C for 2 h. After washing three times with PBS to remove unattached cells, the cells were fixed with 4% formaldehyde for 10 min, stained with crystal violet (0.5% in 2% Ethanol) for 5 min, washed three times with PBS and allowed to air dry for 30 min. Following solubilization with 2% SDS, absorbance at 550 nm was measured using a Spectra-max fluorescence plate reader (Molecular Devices Corp., Sunnyvale, CA).

Detection of intracellular reactive oxygen species

After treatment, cells were washed once with DPBS, and incubated with 5 μM CM-H₂DCFDA at 37 °C for 30 min, followed by three washes with warm DPBS. Cells were trypsinized, washed, and re-suspended in DPBS. The suspended cells were transferred to 96-well plates to be analyzed with a fluorescence plate reader at an emission wavelength of 470 nm and excitation wavelength of 529 nm. Meanwhile, the amount of living cells was determined by Trypan Blue staining. The mean fluorescence intensity of each sample was normalized by live cell numbers.

Fluorocytometric Analysis

Surface protein expression on MDA-MB-231 cells was detected using flow cytometry. The cells were detached from plates by trypsinization, and pelleted by centrifugation, washed twice with PBS, fixed with 4% formaldehyde at 37° C for 10 min and cooled on ice for 1 min. Cells were then blocked with 0.5% BSA in PBS for 10 min. Specific monoclonal antibodies against molecules of interest were first titrated to saturating concentrations. For each sample, cells were suspended in 100 μL of a saturated concentration of primary mAbs for 30 min at room temperature, washed twice, and incubated with 100 μL of FITC-conjugated secondary antibodies and kept on ice for 30 min. Cells were washed twice, then re-suspended in PBS for analysis on a FACSort analyzer (BD Biosciences, Franklin Lakes, NJ).

Western Blot Analysis

The cells were lysed with Nonidet P-40 lysis buffer (2% Nonidet P-40, 80 mM NaCl, 100mM Tris-HCl, 0.1% SDS) with a protease inhibitor mixture (Millipore, Darmstadt, Germany). After 15 min incubation on ice, the lysates were centrifuged and the supernatants were collected. The protein concentration was measured by using a Bio-Rad Protein DC Assay kit (Bio-Rad Laboratories). Equal amounts of protein were run on SDS-PAGE gels and transferred to a nitrocellulose membrane and were blocked in 5% milk in Tris-buffered saline with 0.02% Tween 20 (TBS-T) (USB, Cleveland, OH) for 30 min. The membranes

were incubated with primary antibodies at 4 °C overnight. Membranes were washed with TBS-T three times and were probed with corresponding secondary antibodies for 30 min at room temperature. Blots were then washed with TBS-T three times and with tris-buffered saline (TBS) twice. The signals were detected using West Pico Supersignal chemiluminescent substrate (Pierce, Rockford, IL).

Statistical Analysis

Data are expressed as the mean $\pm$ standard error. Statistical significance of differences between means was determined by two-tailed paired *t*-test. Statistical significance was defined as $p < 0.05$.

Results

LNAC, but not catalase, alters adhesive affinity between breast cancer cells and ECM proteins

To analyze the role of ROS in cell-matrix adhesion after IR, we first determined the extent to which the ROS scavenger, LNAC (10 mM) affects adhesion of MDA-MB-231 cells after exposure to IR. We found that adhesion between the irradiated cells and each ECM protein was increased at 24 h post-IR (Fig. 1A). Treating the cells with LNAC (10 mM) immediately after IR for 24 h resulted in decreased adhesion of the cells to fibronectin, laminin, collagen-I, and collagen-IV by 23.3%, 17.8% (not statistically significant), 29.9% and 24.0%, respectively (Fig. 1A). The effect of LNAC on the adhesion between the cells and fibronectin after IR was also visualizable under microscope (Fig. 1B).

The extent by which ROS effects cell adhesion is reported to be non-linear with respect to concentration (Yuan et al. 2013). Therefore, we evaluated ROS levels in MDA-MB-231 cells after IR. We found that intracellular ROS production was increased at 24 h post-IR (Fig. 1C). This increase was inhibited by 28.97% after treating the irradiated cells with LNAC at the time of irradiation (Fig. 1C). These results suggest that LNAC reduces cell adhesion by decreasing IR-induced generation of ROS, further suggesting that ROS plays a role in IR-induced cell adhesion. To determine whether IR-induced production of hydrogen peroxide (H_2O_2), a relatively stable ROS (Riley 1994), is involved in the regulation of cell adhesion, we examined the effect of catalase (700 U/ml), which decomposes H_2O_2 into water and oxygen, on the affinity of the cancer cells for ECM proteins. Our data indicated that treatment of irradiated cells with catalase did not alter the adhesive affinity between the cells and ECM proteins with or without IR treatment (Fig. 1D), suggesting that H_2O_2 is not a major player in ROS-mediated elevation of cell adhesion after IR.

Effects of LNAC on IR-induced cell adhesion and ROS production are dependent on time and duration of the treatment

Since ROS production after IR is dynamic, we further determined the influence of the time of administration and duration of LNAC treatment on IR-induced adhesive affinity between the cells and fibronectin as well as ROS production. We found that pre-prolonged treatment with LNAC reduced IR-induced adhesion by 13.60% (Fig. 2A) and ROS production by 25.23% (Fig. 2B). Pre-short-term treatment with LNAC did not suppress IR-induced cell adhesion onto fibronectin (Fig. 2C) and ROS production (Fig. 2D). These findings demonstrate that treating the cells with LNAC for 1 h prior to IR or shortening the length of LNAC exposure reduces the capacity of LNAC to lower cell adhesion and cellular ROS at 24 h post-IR.

Effects of LNAC treatment on IR-induced increases in cell surface expression level of activated integrin $\beta 1$ and vimentin expression

Our recent study indicated that $\beta 1$ integrin is activated and plays a role in regulating cell adhesion after IR (submitted for publication). To determine whether LNAC can inhibit IR-induced activation of $\beta 1$ integrin, we measured the expression of an activation-dependent integrin $\beta 1$ epitope (integrin $\beta 1$ active form) by flow cytometry. We found that prolonged treatment of LNAC reduced the IR-induced surface expression of activated $\beta 1$ integrin by 10.9%, but did not alter the background level of the protein in the absence of IR (Fig. 3A). Pre-prolonged or pre-short-term treatment of LNAC did not change the surface expression of the activated $\beta 1$ integrin in the presence or absence of IR treatment (Figs. 3B and 3C). The surface expression of total $\beta 1$ integrin was not altered under any of the LNAC treatments used in this study (prolonged, pre-prolonged and pre-short-term. Data not shown). These results suggest that changes in cell adhesion levels onto fibronectin after LNAC treatment could be due, at least in part, to a conformational change in integrin $\beta 1$ without alterations in total expression level on the cell surface.

Since cell adhesion plays a role in regulating epithelial mesenchymal transition (EMT) (Acloque et al. 2009; Yilmaz and Christofori 2009), we determined whether reduction of adhesive affinity between the cells and fibronectin alters the EMT process in MDA-MB-231 cells (mesenchymal-like breast cancer cells). In this study we used vimentin, which plays a predominant role in regulation of the EMT process (Mendez et al. 2010), as a marker. We found that IR increased vimentin expression, which was attenuated by prolonged LNAC treatment (Fig. 4A). However, pre-short-term treatment with LNAC could not attenuate the increase in vimentin expression by IR (Fig. 4B). This suggests that vimentin is a potential candidate in regulation of LNAC-inhibited cell adhesion and EMT process in the presence of IR.

Discussion

The benefits of administering antioxidants during radiation therapy have been the subject of much controversy as the results of such supplemental treatment vary widely based on the timing of administration, among other parameters (Prasad et al. 2002). In this study, we determined the effects of different times and durations of LNAC treatments on adhesive affinity between breast cancer cells and ECM proteins, especially fibronectin with or without IR treatment. Our data indicated that treating the cells with LNAC immediately after irradiation could reduce their adhesive affinity between the breast cancer cells and ECM proteins (Fig. 1A). The reduced adhesion was correlated to a reduced production of ROS (Fig. 1C) and inhibition of integrin $\beta 1$ activation (Fig. 3A), which plays an important role in tumor metastasis by modulating tumor cell adhesion and extravasation at the target organ (Cordes and Park 2007). Our results suggest that ROS plays a role in the control of cell adhesiveness, consistent with previous reports showing that ROS influences cell adhesion and spreading onto ECM proteins in fibroblasts (Chiarugi et al. 2003; Nimnual et al. 2003). Although treatment with LNAC could also slightly decrease cell adhesion of non-irradiated cells (Fig. 1A), its effect on irradiated cells was more pronounced, suggesting that ROS induced by IR might be responsible for increased cell adhesion onto ECM proteins. Our results showing that LNAC reduces integrin $\beta 1$ activity conflicts with a previous report in which treatment with thiol antioxidants (such as LNAC) enhanced, rather than reduced, integrin $\beta 1$ activity in human peripheral blood mononuclear cells through surface protein disulfide bond reduction (Laragione et al. 2003). This discrepancy could be due to the fact that the study was conducted on cells that had not undergone IR treatment. Additionally, the study was conducted with a different cell line, which may respond to LNAC treatment differently. In fact, we found that LNAC treatment did not counteract IR-induced cell

adhesion of a lung cancer cell line H1299 (data not shown). Our results and previous reports indicate that the effect of LNAC on cell adhesion is cell line dependent.

In addition to cell adhesion, our results indicated that ROS might be involved in the EMT process in irradiated cells since treatment with LNAC also reduces expression of vimentin (Fig. 4A), which is a well-known marker for EMT (Lee et al. 2006). Interestingly, addition of a 1 h pretreatment to the 24 h or a short 2 h treatment of LNAC reduced or abolished the antioxidant's ability to counteract the elevation of cell adhesion, ROS production and integrin $\beta 1$ activation induced by irradiation (Figs. 2, 3B and 3C). Our results with LNAC suggest that if antioxidant treatment is to be combined with IR therapy, time of administration and treatment duration are important variables to consider.

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Fig. 1.

Effect of LNAC (10 mM) or catalase (700 U/mL) on MDA-MB-231 breast cancer cells in the presence or absence of IR. The cells were treated with IR (20 Gy) and then immediately incubated in the medium supplied with LNAC or catalase (CAT). Static cell adhesion assay and fluorescent analysis were performed at 24 h post-IR. The cells without the treatment of IR or LNAC were used as control. (A) Relative amount of breast cancer cells bound to ECM proteins fibronectin (FN), laminin (LAM), collagen-I (COL I), collagen-IV (COL IV). The numbers were normalized by the control of each ECM components and expressed as the mean $\pm$ SE from four independent experiments. * $p < 0.05$ vs. irradiated cells without LNAC treatment. (B) Cell morphology and cell density was observed for cell adhesion assay onto fibronectin. The cells were stained with crystal violet before the imaging was taken. (C) Relative level of intracellular ROS determined by CM-H₂DCFDA fluorescence method. The fluorescence intensity was corrected with respect to cell numbers. (D) Relative amount of breast cancer cells bound to ECM proteins fibronectin (FN), laminin (LAM), collagen-I (COL I), collagen-IV (COL IV). The numbers were normalized by the control of each ECM components and expressed as the mean $\pm$ SE from four independent experiments. # $p > 0.05$ vs. irradiated cells without CAT treatment.

A. Cells bound to fibronectin **B. ROS production** **C. Cells bound to fibronectin** **D. ROS production**

Fig. 2.

Effect of time and duration of LNAC (10 mM) treatment on MDA-MB-231 breast cancer cells in the presence or absence of IR (20 Gy). Static adhesion assay and fluorescent analysis were performed at 24 h post-IR. The cells without the treatment of IR or LNAC were used as control. (A) Relative amount of breast cancer cells bound to fibronectin (FN). The cells were treated with LNAC 1 h before IR and then continue to be treated with LNAC for 24 h. The numbers were normalized by the control without receiving IR and LNAC treatment. (B) Relative level of intracellular ROS after being treated under same condition as described in A. (C) Relative amount of breast cancer cells bound to fibronectin (FN). The cells were treated with LNAC 1 h before IR and then continue to be treated with LNAC for 2 h before replacing the medium with fresh medium without LNAC. The numbers were normalized by the control without receiving IR and LNAC treatment. (D) Relative level of intracellular ROS after being treated under same condition as described in C. The cell adhesion data were normalized by control and expressed as the mean $\pm$ SE from three independent experiments. The fluorescence intensity data were corrected with respect to cell numbers. Data is expressed as the mean $\pm$ SE from three independent experiments. * $p < 0.05$ vs. irradiated cells without LNAC treatment; # $p > 0.05$ vs. irradiated cells without LNAC treatment.

Fig. 3.

Effect of time and length of LNAC (10 mM) treatment on surface expression of activated integrin $\beta 1$ on MDA-MB-231 breast cancer cells in the presence or absence of IR (20 Gy). The surface expression of activated integrin $\beta 1$ was determined by immunofluorescent staining followed by flow cytometry analysis. (A) The cells were treated with LNAC immediately after IR treatment for 24 h. (B) The cells were treated with LNAC 1 h before IR and then continue to be treated with LNAC for 24 h. (C) The cells were treated with LNAC 1 h before IR and then continue to be treated with LNAC for 2 h before replacing the medium with fresh medium without LNAC. Data is expressed as the mean $\pm$ SE from three independent experiments. * $p < 0.05$ vs. irradiated cells without LNAC treatment; # $p > 0.05$ vs. irradiated cells without LNAC treatment.

Fig. 4.

Effect of time and length of LNAC (10 mM) treatment on vimentin expression in MDA-MB-231 breast cancer cells in the presence or absence of IR (20 Gy). Western blot analysis was used to detect the amount of vimentin in total cell lysate prepared at 24 h post-IR. β -actin was used as loading control. (A) The cells were treated with LNAC immediately after IR treatment for 24 h. (B) The cells were treated with LNAC 1 h before IR and then continue to be treated with LNAC for 2 h before replacing the medium with fresh medium without LNAC. Data represent three independent experiments.