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Differential Susceptibility of Nonmalignant Human Breast Epithelial Cells and Breast Cancer Cells to Thiol Antioxidant-Induced G_1-Delay

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ABSTRACT

Reactive oxygen species (ROS) and ROS signaling have been implicated in a variety of human pathophysiological conditions that involve aberrant cellular proliferation, particularly cancer. We hypothesize that intracellular redox state differentially affects cell-cycle progression in nonmalignant versus malignant cells. The thiol antioxidant, *N*-acetyl-L-cysteine (NAC), was used to alter intracellular redox state in nonmalignant human breast epithelial (MCF-10A) and breast cancer cells (MCF-7 and MDA-MB-231). Treatment of cells with NAC resulted in significant augmentation of intracellular small-molecular-weight thiols, glutathione and cysteine. In addition, NAC treatment decreased oxidation of a prooxidant-sensitive dye in MCF-10A cells, but not in MDA-MB-231 and MCF-7 cells. NAC-induced shifts in intracellular redox state toward a more reducing environment caused G_1 delays in MCF-10A cells without causing any significant changes in MCF-7 and MDA-MB-231 cell-cycle progression. NAC treatment of MCF-10A (but not MCF-7 and MDA-MB-231) was accompanied by a decrease in cyclin D1 and an increase in p27 protein levels, which correlated with increased retinoblastoma protein hypophosphorylation. These results show differential redox control of progression from G_1 to S in nonmalignant versus malignant cells and support the hypothesis that loss of a redox control of the cell cycle could contribute to aberrant proliferation seen in cancer cells. *Antioxid. Redox Signal.* 7, 711–718.

INTRODUCTION

Reactive oxygen species (ROS) are oxygen-containing molecules that have higher chemical reactivity than ground state molecular oxygen. ROS, including superoxide, hydrogen peroxide, hydroxyl radical, singlet molecular oxygen, and organic hydroperoxides, are constantly generated intracellularly as by-products of aerobic metabolism and have traditionally been thought of as unwanted and toxic products of living in an aerobic environment (4). Increased levels of ROS can cause oxidative stress leading to damage to nucleic acids, proteins, and cell membranes and subsequently cell death. However, recent evidence suggests that the physiological levels of ROS production are tightly regulated and serve a signaling function (4, 20). ROS and ROS signaling have been implicated in a variety of human pathophysiological conditions, including cancer, diabetes, atherosclerosis, inflammation, fibrosis, neurodegenerative diseases, and aging. As aberrant cellular proliferation is central to many of these pathophysiological conditions, studies aimed at investigating the possible regulatory roles of ROS during cellular proliferation have received much attention.

Thiol-containing compounds [cysteamine, amifostine, *N*-acetyl-L-cysteine (NAC), penicillamine, etc.] are widely used in biology and medicine because of their abilities to act as antioxidants neutralizing ROS. NAC is an aminothiol capable of scavenging ROS and acting to increase cysteine levels, which can enhance glutathione (GSH) synthesis. As GSH is the major intracellular reducing buffer, NAC-induced increase in GSH levels has been shown to shift the intracellular redox environment

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toward a more reducing state (8, 13). Redox state-dependent intra- and intermolecular thiol–disulfide exchange reactions have been suggested to activate many transcription factors and signaling pathways (9, 19), which in turn influences a number of biological processes, including cellular proliferation.

Cellular proliferation is a tightly regulated sequence of transitions from G_0/G_1 to S to G_2 to M phases, which requires assembly and activation of phase-specific cyclin and cyclin-dependant kinase (CDK) complexes (6, 16). Cyclins D (D1, D2, and D3) and E in association with CDKs (CDK2, CDK4, and CDK6) regulate progression from G_1 to S. Furthermore, CDK inhibitors [CIP/KIP family (p21, p27, and p57) and INK4 family (p15, p16, p18, and p19)] negatively regulate cyclin/CDK kinase activity. Active cyclin/CDK kinase complex partially phosphorylates the retinoblastoma (Rb) protein, which causes the release of the E2F family of proteins initiating transcription of E2F-mediated gene expression and entry into S phase (14). Although redox regulation of cell-cycle regulatory proteins is not completely understood, we and others have shown that manipulations of intracellular redox state with thiol antioxidants affect cyclin D1, p21, p27, and Rb phosphorylation (11, 13). These results support the hypothesis that fluctuations in intracellular redox environment could regulate periodic activation of cell-cycle regulatory proteins during progression from one cell-cycle phase to the next, and loss in such a redox control of the cell cycle could contribute to aberrant cell proliferation.

We have previously shown that nontoxic doses of NAC cause primarily G_1 delays in mouse embryonic fibroblasts without any significant alterations in progression through S, G_2, and M. NAC-induced G_1 delay was preceded by decreased cyclin D1, increased p27 protein levels, and hypophosphorylation of Rb protein. The present study was designed to investigate whether NAC-induced G_1 delays in nonmalignant cells differ relative to those in malignant cells.

MATERIALS AND METHODS

Cell culture and treatment

Human breast tumor cell lines MCF-7 (wild-type p53) and MDA-MB-231 (mutant p53) were cultured with RPMI-1640 medium containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin). The nonmalignant breast epithelial cell MCF-10A was cultured in Dulbecco's modified Eagle's medium and F12 (50/50) supplemented with serum and antibiotics. Stock solution of NAC (Sigma Chemicals) was adjusted to pH 7.0 with sodium bicarbonate, and appropriate aliquots were added to exponential cultures.

Immunoblots

Total cellular proteins were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotted with antibodies to cyclin D1 (Pharmingen), p27 (Santa Cruz Biotechnology), and Rb (Pharmingen). Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies and ECL chemiluminescence detection reagents (Amersham Pharmacia). Actin protein levels were used for comparison. The bands were visualized and quantitated with a computerized digital imaging system using AlphaImager 2000 software.

Intracellular prooxidant measurements

Intracellular prooxidant levels were measured using a prooxidant-sensitive fluorescent dye dihydrofluorescein diacetate (DFH-DA; Molecular Probes) and flow cytometry following previously published procedures (13). In brief, NAC-treated and control cells were incubated with 10 μ*M* DFH-DA for 15 min at 37°C and fluorescence measured by flow cytometry (excitation at 488 nm and emission at 535 nm). The mean geometric fluorescence intensity was calculated by subtracting the mean fluorescence of unstained cells from the stained population. The variations in geometric mean fluorescence intensity (MFI) were calculated relative to control cells at the time of experimental manipulations.

Measurement of intracellular thiol levels

Intracellular reduced and oxidized GSH levels as well as NAC levels, were assayed following previously published spectrophotometric and HPLC assays (1, 3, 13). Cell pellets were homogenized in potassium phosphate buffer containing 1.34 m*M* diethylenetriaminepentaacetic acid. Total GSH content was determined in sulfosalicylic acid extracts. For HPLC assay, standard NAC, GSH, and cysteine (100–100,000 fmol/20-μl injection) were derivatized with 0.5 m*M* ThioGlo® 3 (Covalent Associates Inc.) and resolved using a C18 Reliasil column (Column Engineering). Retention times of individual thiols were determined to be 7.6 min (NAC), 20.1 min (GSH), and 30.9 min (cysteine). Total cellular homogenates prepared from control and NAC-treated cells were treated with ThioGlo 3 and analyzed by HPLC. All biochemical determinations were normalized to the protein content of whole-cell homogenates using the method of Lowry *et al*. (12).

Flow cytometry assay for measurements of cell-cycle phase distributions

Asynchronously growing cells were labeled with 10 μ*M* bromodeoxyuridine (BrdUrd) (Sigma Chemicals) for 30 min at 37°C. Cells were either harvested immediately after the labeling or continued in culture in BrdUrd-free growth medium. Ethanol-fixed cells were processed for immunostaining and flow cytometry analysis following previously published procedures (13). Data from a minimum of 20,000 nuclei were acquired in list mode and analyzed using Cell Quest software. Four compartments were identified: BrdUrd-positive S-phase cells, BrdUrd-negative G_1 and G_2 phase cells, and BrdUrd-positive S-phase cells that have completed cell division (G_1^+). The fraction of G_1^+ cells (½ number of cells in G_1^+ box)/(number of BrdUrd-positive undivided cells + ½ number of cells in G_1^+ box) was used as a measurement of cell transits through S + G_2 + M. Transit through S was also measured by calculating relative movement (RM) as originally described by Begg *et al* (2). The fraction of G_1 was used to measure G_1 transit.

Experiments were repeated two or three times, and data were calculated as means, standard deviations, and standard error of means.

Table 1. Intracellular Small Molecular-Weight Thiol Levels

Cell lines	*Treatments*	*Total GSH*	*Cysteine*	*NAC*
MDA-MB-231	Control	6.1 ± 1.1	1.5 ± 0.4	ND
	NAC (20 m*M*)	16.1 ± 3.1	5.1 ± 1.2	8.2 ± 1.4
MCF-7	Control	9.6 ± 0.8	1.5 ± 0.5	ND
	NAC (20 m*M*)	36.0 ± 8.3	12.5 ± 4.2	4.4 ± 1.3
MCF-10A	Control	0.6 ± 0.1	0.6 ± 0.1	ND
	NAC (20 m*M*)	2.9 ± 1.6	9.9 ± 5.3	20.02 ± 3.8

Results are shown as the means ±SD ($n = 3$) and are in units of nmol/mg of protein. ND, not detected.

RESULTS

Thiol antioxidant-induced changes in intracellular redox environment in nonmalignant cells are absent in malignant cells

To determine if NAC-induced changes in intracellular redox environment are different in nonmalignant versus malignant cells, exponential cultures of human epithelial cells (MCF-10A) and breast cancer cells (MCF-7 and MDA-MB-231) were cultured in the presence of 20 m*M* NAC (pH 7.0) for 24 h. Cells were harvested for measurements of intracellular small-molecular-weight thiols (cysteine and NAC) by HPLC, and total GSH was determined using a spectrophotometric recycling assay (Table 1). The amounts of intracellular total GSH and cysteine in exponential cultures of MCF-10A were 0.6 ± 0.1 nmol/mg of protein and 0.6 ± 0.1 nmol/mg of protein (averages ± SD), respectively. Following exposure to NAC, intracellular total GSH levels increased approximately four-fold, whereas cysteine levels increased ~16-fold. The basal levels of total GSH and cysteine in untreated malignant MCF-7 (total GSH, 9.6 ± 0.8 nmol/mg of protein; cysteine, 1.5 ± 0.5 nmol/mg of protein) and MDA-MB-231 (total GSH, 6.1 ± 1.1 nmol/mg of protein; cysteine, 1.5 ± 0.4 nmol/mg of protein) cells were six- to nine-fold higher compared with those in nonmalignant MCF-10A cells. The intracellular small-molecular-weight thiol in both MCF-7 and MDA-MB-231 cells increased four- to five-fold following a 24-h exposure to NAC. These results show that both nonmalignant and malignant cells internalized NAC and increased intracellular total GSH and cysteine pools. Although the basal levels of total GSH and cysteine were different among the cell lines, the relative increases in total thiol pools following NAC exposure were not significantly different.

NAC-induced changes in intracellular redox environment were also estimated by measuring changes in the fluorescence of a prooxidant-sensitive dye. Cells from replicate dishes were stained with DFH-DA for 15 min and fluorescence measured by flow cytometry (Fig. 1). The relative fold change in MFI calculated from the geometric mean fluorescence of MCF-10A cells grown in the presence of NAC showed ~50% decrease following 24 h of NAC treatment compared with cells grown in the absence of NAC [Fig. 1A (middle panel) and B; $p < 0.05$]. In contrast, NAC treatment of malignant MCF-7 and MDA-MB-231 cells did not exhibit any significant changes in DFH fluorescence compared with untreated control. These results show that the apparent NAC-induced suppression in dye oxidation in nonmalignant (MCF-10A) cells is absent in malignant (MCF-7 and MDA-MB-231) cells.

Differential susceptibility of nonmalignant and malignant cells to thiol antioxidant induced G_1 delay

To determine if NAC-induced changes in intracellular redox environment differentially affect proliferation of nonmalignant versus malignant cells, exponential cultures of MCF-10A, MCF-7, and MDA-MB-231 were incubated with 0–20 m*M* NAC for 24 h. Cells were pulse-labeled with BrdUrd prior to harvest and cell-cycle phase distributions analyzed by flow cytometry. Results presented in Fig. 2 show that exponential cultures of MCF-10A nonmalignant cells had ~66% G_1, 24% S, and 10% G_2 + M. However, following exposure to NAC, cells in G_1 increased to ~87% with a concomitant reduction in S to 4% and no alterations in G_2 + M (10% in control versus 9% in NAC-treated cells). In contrast, NAC treatment of MCF-7 and MDA-MB-231 malignant cells did not result in any significant redistribution in cell-cycle phase. Exponentially growing MCF-7 cells had 55% G_1, 35% S, and 10% G_2 + M, and following 24-h exposure to NAC, the cell-cycle phase distributions were 62% G_1, 29% S, and 9% G_2 + M. Similarly, NAC treatment in MDA-MB-231 cells also did not show any significant changes in cell-cycle phase redistributions (48% G_1, 43% S, and 9% G_2 + M in control versus 44% G_1, 48% S, and 8% G_2 + M in NAC-treated cells). These results clearly show that NAC-induced alterations in intracellular redox environment result in the redistribution of the MCF-10A nonmalignant cells to G_1 of the cell cycle, whereas a similar treatment did not show any significant redistribution in cell-cycle phases of MCF-7 and MDA-MB-231 malignant cells.

To determine whether NAC-induced changes in cell proliferation are a global affect or progression through specific cell-cycle phases was affected, transits through each cell-cycle phase were measured using BrdUrd pulse-chase assay. Exponential cultures of MCF-10A and MCF-7 cells were

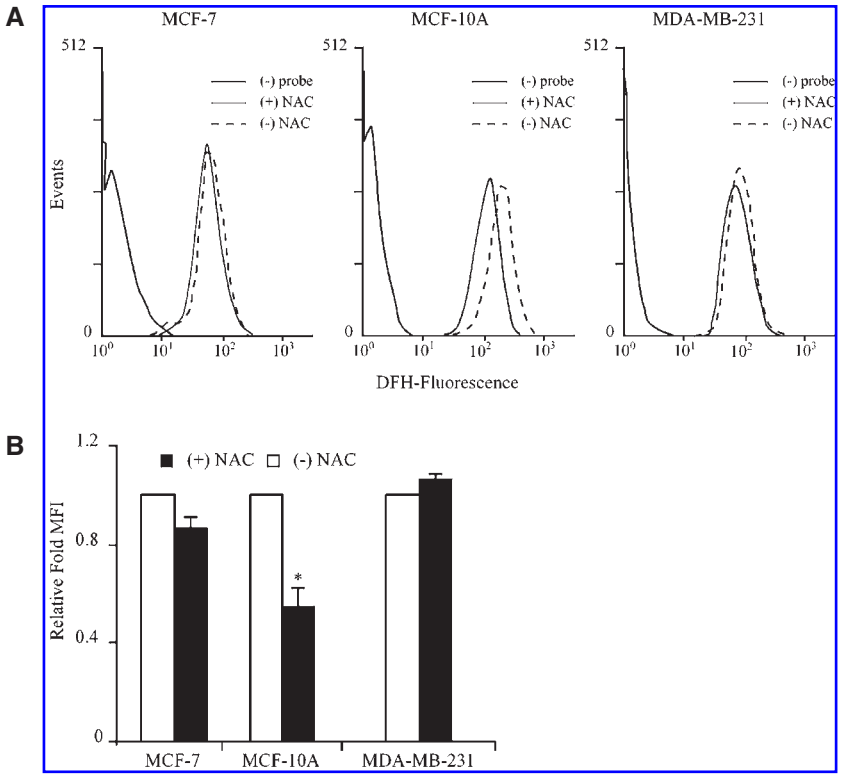

FIG. 1. Thiol antioxidant-induced decrease in prooxidant-sensitive DFH-DA fluorescence in human breast epithelial cells (MCF-10A) was absent in breast cancer cells (MCF-7 and MDA-MB-231). Exponentially growing asynchronous cultures of MCF-10A, MCF-7, and MDA-MB-231 were treated with 20 m*M* NAC for 24 h. **(A)** Cells were stained with DFH-DA for 15 min and fluorescence measured by flow cytometry. **(B)** Relative fold change in geometric MFI in each experiment was calculated relative to untreated control (*$p < 0.05$). (−) probe in A indicates autofluorescence.

pulse-labeled with BrdUrd and continued in culture in BrdUrd-free growth medium. Cells were harvested at regular intervals and cell-cycle phase distributions analyzed by flow cytometry. Fractions of BrdUrd-negative G_1, BrdUrd-positive cells that have completed cell division (G_1^+) and RM were used to determine transits through G_1, S, and S + G_2 + M phases. Results presented in Fig. 3A show that percent G_1 (number of cells in the box labeled G_1 relative to total cell number, Fig. 3D) in untreated MCF-10A cells gradually decreased, reaching 40% of 0-h control at 12 h post BrdUrd labeling followed by an increase at 24 h. In contrast, MCF-10A cells cultured in the presence of NAC showed relatively no change in percent G_1 cells. In fact, there was an increase in percent G_1 cells in NAC-treated cells compared with 0-h control, indicating that while cells are transiting from G_2 to G_1 phase, the cells' exit from G_1 was inhibited. The initial increase in percent G_1 at 2 h post BrdUrd labeling for both control and NAC-treated cells is due to a combination of daughter cells entering G_1 and a slower exit from G_1 to S. The G_1 transit time calculated at 50% level was ~10 h for control and >24 h in NAC-treated cells. Transit through S in both control and NAC-treated MCF-10A cells remained essentially unchanged (Fig. 3B). The initial RM values for both control and NAC-treated cells were 0.5. The RM value increased linearly and reached 1.0 at 6 h for both control and NAC-treated cells, indicating that the NAC treatment did not affect progression through S. Similarly, transition of the cells through S and G_2 + M, determined from fraction of G_1^+ cells, was unaffected by the NAC treatment (Fig. 3C). Thus, NAC-induced changes in intracellular redox environment in MCF-10A cells delayed transit through G_1 without any significant changes in S, G_2, and M transits.

Interestingly, NAC treatment of MCF-7 malignant cells did not show any significant change in progression through G_1, S, G_2, and M (Fig. 3E–H). G_1 transit time for both control and NAC-treated cells was calculated to be ~16 h. Transit through S was calculated to be ~14 h. Similarly, NAC treatment did not cause any change in transitions of MDA-MB-231 cells through the cell cycle (data not shown). These results clearly demonstrate a differential susceptibility of nonmalignant human breast epithelial and breast cancer cells to thiol antioxidant-induced G_1 delay.

Differential response of malignant and nonmalignant cells to NAC-induced changes in G_1 cell-cycle regulatory proteins

To determine whether NAC-induced changes in intracellular redox environment have a differential affect on G_1 cell-cycle regulatory protein levels in nonmalignant versus malignant cells, exponential cultures of MCF-10A, MDA-MB-231, and MCF-7 were exposed to 20 m*M* NAC and total protein extracts prepared

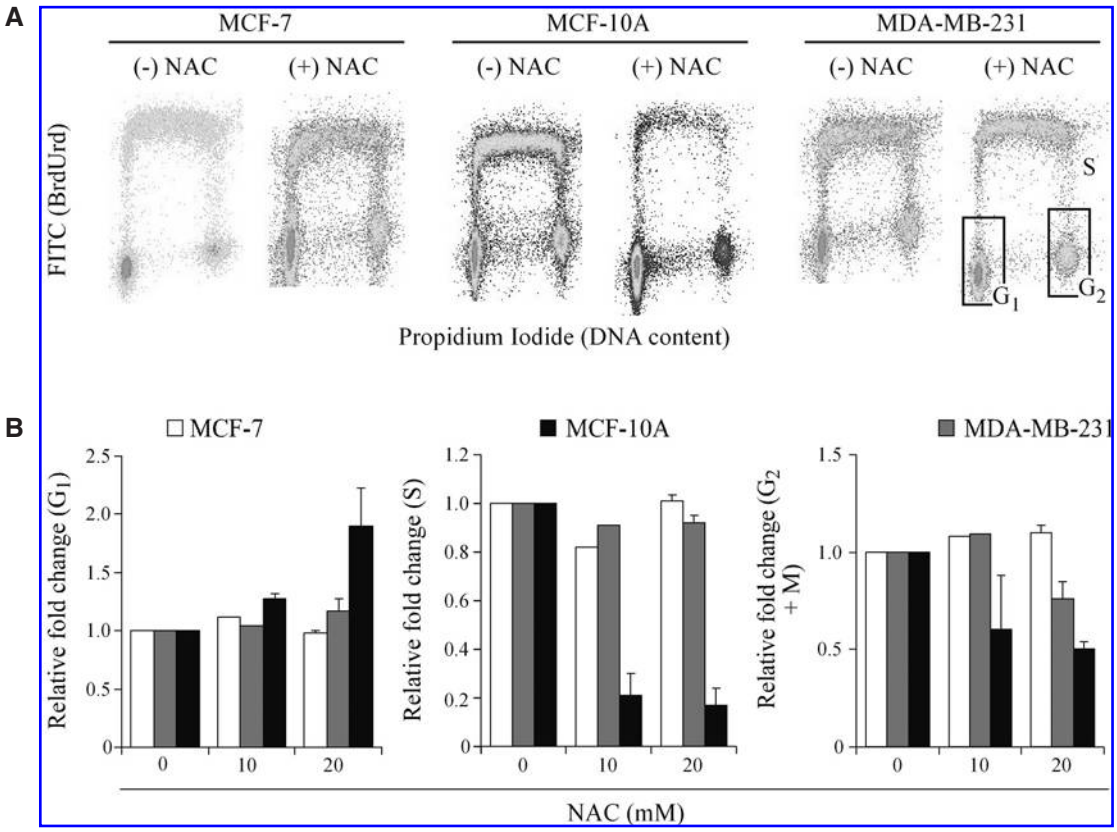

FIG. 2. Thiol antioxidant-induced decrease in S phase of nonmalignant MCF-10A cells is absent in MCF-7 and MDA-MB-231 malignant cells. Exponential cultures of MCF-10A, MCF-7, and MDA-MB-231 were cultured in the presence (10 and 20 m*M*) and absence of NAC for 24 h. Cells were pulse-labeled with BrdUrd prior to harvest and cell-cycle phase distribution analyzed by flow cytometry. **(A)** Bivariate histograms of DNA content versus log BrdUrd-fluorescein isothiocyanate (FITC) fluorescence. Positions of G_1, S, and G_2 cells are marked in the right histogram. **(B)** Quantitation of G_1, S, and G_2 phase distribution in untreated control and cells treated with 10 and 20 m*M* NAC.

at the indicated times (Fig. 4). Results presented in Fig. 4 show that NAC treatment in MCF-10A cells caused >90% decrease in cyclin D1 protein levels at 12 h and remained low at 24 h. NAC-induced decrease in cyclin D1 protein levels correlated with a three- to four-fold increase in p27 protein levels and increased Rb hypophosphorylation. In contrast, NAC treatment in MCF-7 and MDA-MB-231 malignant cells did not cause any significant change in cyclin D1 and p27 protein levels, as well as Rb phosphorylation. These results suggest that the thiol antioxidant-induced G_1-delay in nonmalignant MCF-10A cells could be regulated by redox-sensitive modulation of G_1cell-cycle proteins. Interestingly, NAC-sensitive control of G_1 cell-cycle regulatory proteins was absent in malignant cells.

DISCUSSION

Intracellular redox environment is a balance between prooxidant species produced by cellular metabolism and intracellular antioxidants. A redox imbalance could therefore result from an increased ROS formation or a compromised antioxidant defense system, which could potentially alter the structure or function of important cell components, including DNA, proteins, and lipids. Whereas many of the earlier reports suggest that ROS are toxic to many cellular processes, recently ROS have been implicated in the regulation of several physiologic processes, including cell proliferation (4). In fact, the concept of the relationship between the intracellular oxidation/reduction (redox) state and cell proliferation dates back to 1931 when Louis Rapkine first reported the periodic cycling of "soluble" SH groups during the sea urchin mitotic cycle following fertilization (18). Additional evidence for the "Rapkine cycle" was provided later by Kawamura and Dan in 1958, who demonstrated localized increase in protein-thiol staining during prophase and metaphase, followed by a dramatic decrease in telophase of the sea urchin mitotic cycle (7). Intracellular GSH levels have also been reported to vary during the mouse fibroblast cell cycle, being maximal in mitotic cell (10). Although these previous studies provide some evidence for a mechanistic link between intracellular redox reactions and cell proliferation, it is unknown if transit through specific cell-cycle phase is redox-sensitive and what specific cell-cycle regulatory processes could be responsive to changes in intracellular redox state.

We have previously shown that changes in intracellular redox state toward a more reducing environment resulted in G_1 delay without any significant change in transits through S, G_2,

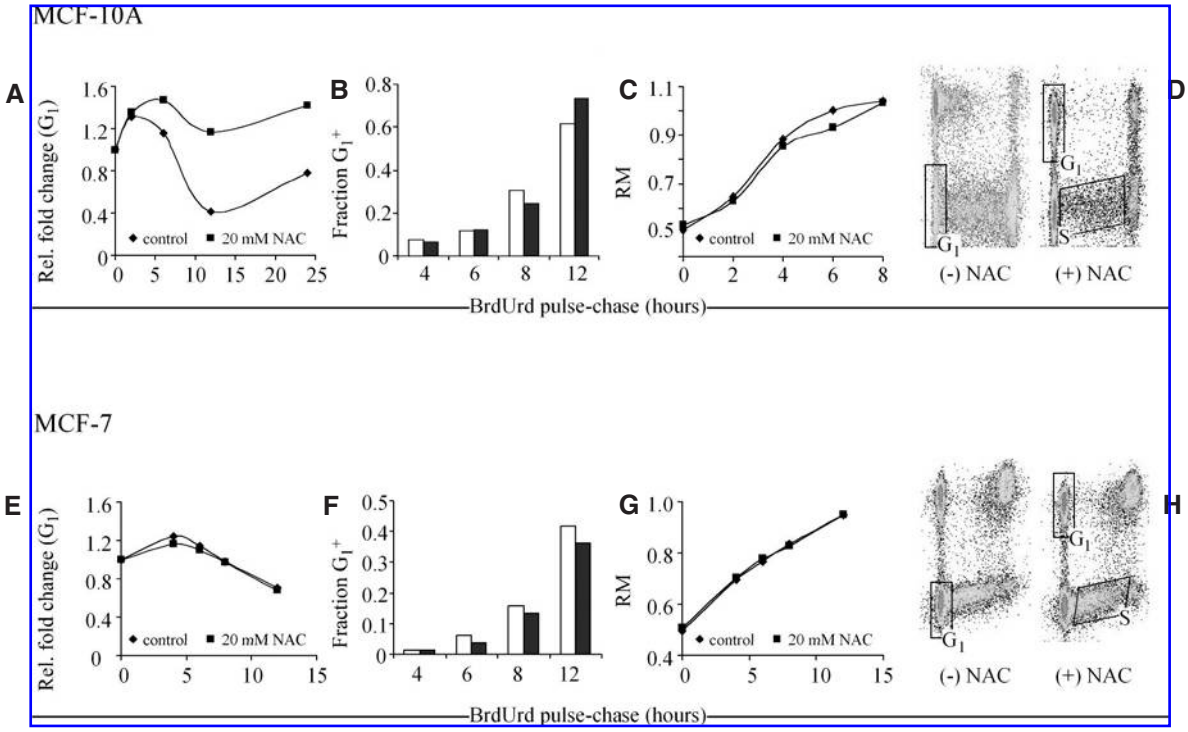

FIG. 3. Differential susceptibility of nonmalignant human breast epithelial cells and breast cancer cells to thiol antioxidant-induced G_1-delay. MCF-10A and MCF-7 exponential cultures were pulse-labeled with BrdUrd and continued in culture in BrdUrd-free growth medium and medium containing 20 m*M* NAC. Cells were harvested at the indicated times and cell-cycle phase transits analyzed by flow cytometry. Transits through G_1 (**A** and **E**), S (**B** and **F**), and S and G_2 + M (**C** and **G**) were calculated using CellQuest software and equations described in Materials and Methods. (**D** and **H**) Dual parameter propidium iodide versus FITC histograms of BrdUrd pulse-chase cells at 12 h post labeling. Populations of G_1, S, and G_1^+ are marked in D and H. G_1^+ represents BrdUrd-positive cells that have completed cell division and are used for measurements of transits through S, G_2, and M phases. Open and dark bars represent control and NAC-treated cells, respectively.

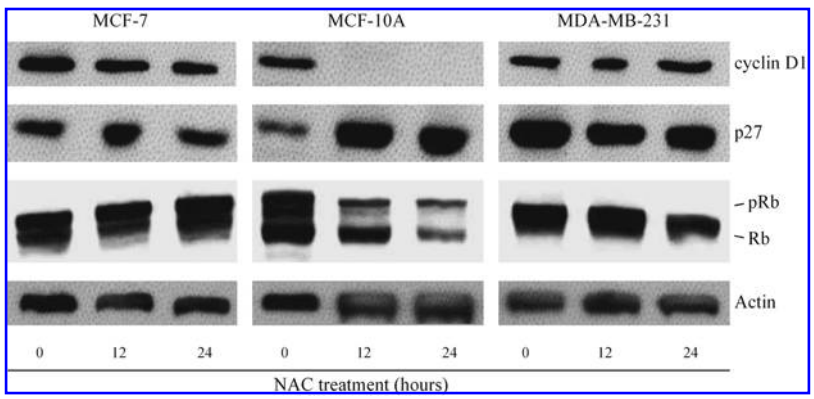

FIG. 4. Redox-sensitive regulation of G_1 cell-cycle proteins in nonmalignant cells was absent in malignant cells. Immunoblot analysis of G_1cell-cycle regulatory protein levels in control (0 h) and NAC-treated nonmalignant (MCF-10A) and malignant cells (MCF-7 and MDA-MB-231) is shown; pRb represents phosphorylated Rb protein. Actin protein levels were used for comparison.

and M of the mouse embryo fibroblast cell cycle. Furthermore, our results also showed the requirement of a transient increase in intracellular prooxidant levels prior to S entry and the cyclic nature of such a redox-sensitive event early in G_1 of the subsequent cell cycles of the daughter generations. As aberrant cellular proliferation is central to a variety of pathophysiological conditions involving growth disturbances (including cancer), the present study was initiated to determine if changes in intracellular redox environment differentially affect progression through the cell cycle in nonmalignant versus malignant cells.

Intracellular redox environment in matched pairs of human breast nonmalignant epithelial (MCF-10A) and breast cancer (MCF-7 and MDA-MB-231) cells was modulated using the thiol antioxidant NAC. NAC treatment of both nonmalignant and malignant cells increased intracellular small-molecular-weight thiols, total GSH, and cysteine levels (Table 1). HPLC measurements also showed that NAC was internalized in all three cell lines, demonstrating that the differences in cell-cycle progression and G_1-regulatory proteins (discussed below) between nonmalignant versus malignant cells were not due to the inability of cells to take up NAC and metabolize it to other thiols. Interestingly, whereas the NAC treatment in MCF-10A decreased prooxidant levels, there were no significant changes in DFH-DA fluorescence in both MCF-7 and MDA-MB-231 malignant cells (Fig. 1). These results clearly show that NAC-induced redox sensitivity of the cell-cycle in the nonmalignant cells was absent in the malignant cells. Because NAC treatment enhanced GSH and cysteine levels in all three cell types, the differential effects on cell-cycle progression and G_1-regulatory protein levels are most likely not mediated by the absolute amount of GSH.

NAC treatment caused a differential effect in cell-cycle phase distributions in nonmalignant cells versus malignant cells (Figs. 2 and 3). The S-phase percentage in nonmalignant MCF-10A cells decreased from 24% in control to 4% in NAC-treated cells. Interestingly, identical treatments in malignant MCF-7 and MDA-MB-231 did not show any significant change in cell-cycle phase distributions. Results from the BrdUrd pulse-chase assay showed that NAC-induced changes in intracellular redox environment toward a more reducing environment specifically delayed progression from G_1 to S without any significant change in progression through S, G_2, and M. These results confirm earlier reports from our laboratory, as well as others, and provide strong support for the hypothesis that intracellular redox environment in nonmalignant cells could regulate progression from G_1 to S. Because such a redox sensitivity in progression from G_1 to S was absent in both MCF-7 and MDA-MB-231 malignant cells, our results also suggest that loss of a redox control of G_1 to S progression could lead to aberrant proliferation, a hallmark of cancer cell proliferation.

Although the identity of the redox-sensitive event in G_1 needs further studies, it is possible that ROS (*i.e.*, superoxide and hydrogen peroxide) generated during mitochondrial metabolism or from NADPH oxidase enzymes in the cytosol could act as a signaling mechanism during progression from G_1 to S to coordinate metabolic processes with the cell-cycle regulatory processes. In nonmalignant cells, cellular antioxidant defense mechanisms, including enzymatic scavenging systems (superoxide dismutases, catalase, glutathione peroxidases, thioredoxin peroxidases) and thiol-reducing buffers (small-protein and nonprotein thiols), could neutralize the transient increase in ROS levels, interrupting the normal signaling process leading to entry into S. In contrast, malignant cells with enhanced metabolic activity combined with a compromised antioxidant system (*e.g.*, aberrant expression of antioxidant enzymes and/or thiol-reducing systems, such as glutaredoxin and/or thioredoxin) would lead to increased ROS production, which could subsequently lead to the loss of redox sensitivity in G_1 to S progression. In fact, loss of a specific intracellular antioxidant enzyme function [*i.e.*, manganese superoxide dismutase (MnSOD) in tumors (15)] is a common finding in cells that have undergone malignant transformation. In many ways, such a redox-sensitive regulation in G_1 to S progression resembles the "restriction point" of the cell cycle (17). Pardee showed withdrawal of growth factors after the restriction point did not affect transit through the remainder of G_1, S, G_2, and M. However, withdrawal of growth factors before the restriction point halts progression from G_1 to S. Similarly, manipulating the intracellular redox state toward a more reducing environment in cells in which the prooxidant event had already occurred would not be expected to affect cell transit through the remainder of G_1, S, G_2, and M of the cell cycle. In contrast, such a manipulation prior to the prooxidant event would inhibit progression into S. It has been postulated that cells aberrant proliferation of tumor cells is associated with a loss in regulation of the restriction point. It is possible that the redox-sensitive event during progression from G_1 to S might overlap with the restriction point.

Although the molecular mechanisms regulating redox-sensitive progression from G_1 to S are unknown at present, thiol antioxidants are known to affect cyclin D1, p21, p27, and Rb phosphorylation. In support of these observations, NAC treatment in MCF-10A cells showed a decrease in cyclin D1 and increase in p27 protein levels, and Rb hypophosphorylation. Because the decrease in cyclin D1 protein levels was rapid (>70% decrease as early as 4 h; data not shown), these results suggest that the redox properties of NAC as opposed to cell-cycle rearrangement *per se* are responsible for the decrease in cyclin D1, increase in p27 protein levels, and Rb hypophosphorylation. As NAC treatment of MCF-7 and MDA-MB-231 malignant cells did not cause any significant change in G_1 cell-cycle regulatory proteins, our results also suggest that metabolic redox reactions linking biochemical processes in G_1 to the cell-cycle machinery could be defective in malignant cells.

Indeed, intracellular redox state-dependent alterations in protein function in many ways could be analogous or even linked to phosphorylation/dephosphorylation events except that protein modification occurs on cysteines, arginine, histidine, methionine, and/or metal cofactors rather than on serine, threonine, or tyrosine residues. Although hypothetical at present, it is possible that both redox and phosphorylation/dephosphorylation modifications of proteins (a putative "redox/phos switch") could act in concert during activation (or inactivation) of key biological processes. Such a hypothetical binary switch concept, methyl/phos (acetyl/phos and ubiquitin/phos), has been proposed recently by Fischle *et al.* for the original "histone code hypothesis" (5). It is interesting to note that putative "redox/phos" sites are present in cyclin D1 (amino acid sequence **C**ap**S**, **C**iy**T**, **CT**p**T**), antioxidant enzyme MnSOD (Rav**C**gt**S)**, and perhaps a host of other antioxidant enzymes and cell-cycle regulatory proteins. Experimental verifications

of such an interesting hypothesis could have much broader implications in the near future for our understanding of various signaling cascades that act in concert in coordinating the progression of key biological regulatory processes.

In summary, we have shown a differential susceptibility in human breast nonmalignant and breast cancer cells to thiol antioxidant-induced G_1 delay. Thiol antioxidant-induced G_1 delay in nonmalignant MCF-10A cells was accompanied by decreased cyclin D1, increased p27 protein levels, and Rb hypophosphorylation. These results support the hypothesis that redox-sensitive signaling events may act as a mechanistic bridge to coordinate metabolic and gene expression pathways in preparation for cell entry into S phase. Disruption of such a controlling mechanism during transformation could contribute to the growth abnormalities seen in cancer progression.

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ABBREVIATIONS

BrdUrd, bromodeoxyuridine; CDK, cyclin-dependent kinase; DFH-DA, dihydrofluorescein diacetate; FITC, fluorescein isothiocyanate; GSH, glutathione; MFI, mean fluorescence intensity; MnSOD, manganese superoxide dismutase; NAC, *N*-acetyl-L-cysteine; Rb, retinoblastoma protein; redox, oxidation and reduction reactions; RM, relative movement; ROS, reactive oxygen species.

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