N-Acetylcysteine Protects Melanocytes against Oxidative Stress/Damage and Delays Onset of Ultraviolet-Induced Melanoma in Mice

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Abstract

Purpose: UV radiation is the major environmental risk factor for melanoma and a potent inducer of oxidative stress, which is implicated in the pathogenesis of several malignancies. We evaluated whether the thiol antioxidant *N*-acetylcysteine (NAC) could protect melanocytes from UV-induced oxidative stress/damage *in vitro* and from UV-induced melanoma *in vivo*.

Experimental Design: *In vitro* experiments used the mouse melanocyte line melan-a. For *in vivo* experiments, mice transgenic for hepatocyte growth factor and survivin, shown previously to develop melanoma following a single neonatal dose of UV irradiation, were given NAC (7 mg/mL; mother's drinking water) transplacentally and through nursing until 2 weeks after birth.

Results: NAC (1-10 mmol/L) protected melan-a cells from several UV-induced oxidative sequelae, including production of intracellular peroxide, formation of the signature oxidative DNA lesion 8-oxoguanine, and depletion of free reduced thiols (primarily glutathione). Delivery of NAC reduced thiol depletion and blocked formation of 8-oxoguanine in mouse skin following neonatal UV treatment. Mean onset of UV-induced melanocytic tumors was significantly delayed in NAC-treated compared with control mice (21 versus 14 weeks; $P = 0.0003$).

Conclusions: Our data highlight the potential importance of oxidative stress in the pathogenesis of melanoma and suggest that NAC may be useful as a chemopreventive agent.

Melanoma is an increasingly prevalent cutaneous malignancy. In advanced stages, melanoma is an aggressive cancer that is generally unresponsive to conventional therapies and associated with poor prognosis (1). Individuals with personal or family history of melanoma or those with increased numbers of nevi or atypical nevi are at increased risk for developing melanoma (2). The best characterized environmental risk factor for melanoma is UV radiation from the sun (3). In the past decade, there has been an increased emphasis on melanoma prevention focusing on patient education and sun protection (4). Despite these efforts, melanoma rates have continued to increase (1).

A chemoprevention strategy would thus be useful in high-risk patients if a safe, well-tolerated, and easily administered agent could be developed targeting a critical pathway in melanomagenesis. Reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion, singlet oxygen, and hydroxyl radical, are induced in the skin by UV irradiation (5). These ROS are short-lived molecules that are capable of causing oxidative damage in DNA, as well as cellular protein and lipids, leading to permanent genetic changes and activating signal transduction pathways that promote skin carcinogenesis (6). In addition to peroxidation of cellular substrates, UV-induced ROS can damage cells by decreasing vital intracellular reductants, such as glutathione (GSH). Melanocytes are particularly sensitive to oxidative stress (7), which is chronically generated during melanogenesis (8) due to the pro-oxidant effect of melanin (9). Melanoma tissues exhibit increased products of lipid peroxidation and levels of antioxidant enzymes compared with benign nevi (10), and oxidative dysregulation is correlated with melanoma progression in a mouse model (11). These observations support the notion that UV-induced oxidative stress/damage contributes to melanoma pathogenesis (12) and could be targeted using antioxidant preventive therapy.

The thiol *N*-acetylcysteine (NAC), a cell-permeable GSH prodrug/antioxidant (13), is Food and Drug Administration approved for the treatment of acetaminophen toxicity (14) and has been used at high oral doses in humans for chronic treatment of idiopathic pulmonary fibrosis (15). In a mouse model of chronic UV-induced squamous cell carcinoma, orally administered NAC reduced tumor formation (16). Additionally, topical NAC reduced UV-mediated GSH depletion and peroxide induction in normal human skin (17). NAC also seems to have some anticancer effects independent of its role as an antioxidant. It has specifically been shown to decrease VEGF

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production in melanoma cells *in vitro* (18) and decrease matrix metalloproteinase activity in invasive tumor cells (19). Thus, in addition to its demonstrated safety record, NAC has the capacity to modulate both UV-induced oxidative stress and carcinogenesis in the skin.

In this study, we conducted a trial of orally administered NAC in a highly penetrant mouse model of melanoma (20), in which animals develop tumors following a single UV exposure in the neonatal period. Additionally, we characterized the generation of oxidative stress/damage following UV irradiation of a mouse melanocyte cell line and mouse skin. Administration of NAC to cultured cells or animals before UV irradiation resulted in a reduction in oxidative stress/damage in both melanocytes and whole skin and a significant delay in tumor formation. We conclude that NAC seems to be a promising candidate agent for melanoma chemoprevention, which may be applicable to human trials.

Materials and Methods

UV irradiation

A bank of four fan-cooled unfiltered sun lamps (FS20T12-UVB, National Biological Corp.) emitting 4 W/m^2 was used for all experiments. These bulbs emit wavelengths between 250 and 420 nm (72.6% UVB, 27.4% UVA, and 0.01% UVC), with peak emission at 313 nm, according to the manufacturer. Dosimetry was monitored using a UVB-500C meter (National Biological).

Induction and detection of ROS in melanocytes

The melan-a mouse melanocyte cell line, derived from C57BL/6 mice (21), was kindly provided by Dorothy Bennett (St. George's University of London, London, United Kingdom) and maintained in RPMI 1640 containing 10% FCS, 200 nmol/L phorbol 12-myristate 13-acetate (Sigma Chemical Co.), 200 pmol/L cholera toxin (Sigma Chemical), and antibiotics. For induction of melanocyte ROS, H_2O_2 (Sigma Chemical) was added to cultures, or cells were UV irradiated in PBS. Endogenous H_2O_2 was detected by addition of 20 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen), followed 1 h later by flow cytometry on a FACScan (BD Biosciences) using excitation and emission wavelengths of 488 and 520 nm, respectively. Data were collected and analyzed with CellQuest software (BD Biosciences). Endogenous superoxide was detected by addition of 10 μmol/L dihydroethidium (Invitrogen) followed by analysis on a CytoFluor II plate reader (Perseptive Biosystems) with excitation and emission wavelengths set at 530 and 612 nm, respectively. NAC (Sigma Chemical) was freshly prepared, the pH was adjusted to 7.4 using NaOH, and added to cultures at the appropriate concentrations 30 min before and then immediately after UV irradiation.

Oxidative damage in melanocytes and whole skin

Free thiols. Melanocytes (~1 × 10^6) were washed in PBS, and cell pellets were sonicated in 5% metaphosphoric acid containing 5 mmol/L EDTA. Lysates were centrifuged at 20,000 × *g* for 5 min at 4°C to separate the free thiol-containing supernatant from the proteinaceous cellular debris. Supernatants were combined with four volumes of buffer consisting of 400 mmol/L Tris, 5 mmol/L EDTA (pH 8.9), and 0.2 mg/mL Ellman's reagent (5,5′-dithio-bis-2-nitrobenzoic acid, Sigma Chemical; ref. 22) and incubated for 30 min at room temperature in the dark. Protein pellets were dissolved in lysis buffer containing 2% SDS, 50 mmol/L Tris, and 10% glycerol, and protein concentration was determined by standard bicinchoninic acid assay (Pierce Chemical Co.). Free low molecular weight thiols in supernatants were determined by measuring absorbance at 412 nm using a Beckman DU 530 spectrophotometer (Beckman Coulter) and using a standard curve generated by similar treatment of commercially available GSH (Sigma Chemical). Free thiol content was then normalized to the pellet protein concentration. Free thiol determinations in mouse skin were done similarly; however, lysates were generated by vigorous mechanical disruption with a mortar and pestle of skin samples (~50 mg). Data were then generated as above but with normalization to the milligram weight of the skin specimen.

8-Oxoguanine. The presence of 8-oxoguanine (8-OG) was detected by immunohistochemistry using a mouse anti–8-OG antibody (Genox Corp.). After trypsinization and washing in PBS, cells were fixed by suspension in 10% neutral buffered formalin for 1 h and then embedded in 4% low-melting agarose (GeneSieve, ISC Bioexpress) in PBS. Cell containing agarose plugs were then further fixed by suspension in 10% neutral buffered formalin on a rotator overnight and then paraffin embedded and sectioned. Slides were baked at 60°C overnight. Following deparaffinization in xylene, dehydration in ethanol, and washing in water, sections were incubated in 3% H_2O_2 for 10 min and then washed in PBS. Antigen retrieval was enhanced by incubation in 0.1% trypsin (Sigma Chemical) in PBS for 30 min at 37°C. After washing in PBS, sections were stained with a 1:20 dilution of anti–8-OG for 1 h at 37°C. Blocking and antibody dilution buffers, biotinylated antimouse antibody, and Vectastain reagents were supplied in the M.O.M. kit (Vector Laboratories) and used according to the supplier's recommendations. Positive nuclear staining was visualized by reaction with aminoethylcarbazole (AEC kit, Invitrogen), followed by counterstaining with hematoxylin (Sigma Chemical), washing in water, and application of Clearmount (Invitrogen). A total of 300 cells for each condition were analyzed by light microscopy. The same procedure was used for detection of 8-OG in whole skin, with the exception that 3,3′-diaminobenzidine (BioGenex Laboratories) was used as the peroxidase substrate.

Cyclobutane pyrimidine dimers. UV-induced cyclobutane pyrimidine dimers (CPD) were detected by Southwestern dot-blot assay (23). Briefly, genomic DNA was spotted onto nitrocellulose discs (BioTrace NT, VWR Scientific) and stained with a 1:1,000 dilution of TDM-2 antibody (kindly provided by Toshio Mori, Nara Medical University, Nara, Japan; ref. 24) in 5% nonfat milk overnight at 4°C. Reactivity was visualized by staining with peroxidase-conjugated antimouse immunoglobulin, followed by enhanced chemiluminescence and autoradiography (25).

UV-induced tumors

Male hepatocyte growth factor-Tg (BL6 MH19) mice on a C57BL/6 background (26), kindly provided by Glenn Merlino (National Cancer Institute, Bethesda, MD), were mated with female homozygous Dct-survivin mice on a C3H/HeN background (20). Visibly pregnant females (approximately 2-3 days before delivery) were placed in separate cages, and animals received either deionized water or water containing NAC (7 mg/mL; neutralized to pH 7.4 with NaOH). Assuming an average mouse weight of 25 g and daily water consumption of 6.7 mL,[6] the estimated daily maternal dose was 1.9 g/kg/d. Water containing NAC was freshly prepared on Mondays, Wednesdays, and Fridays until 2 weeks after delivery, after which time only normal drinking water was supplied. Approximately 1 to 2 days after delivery, each adult female was temporarily removed from the cage and the pups were spaced apart in the bedding in a prone position. Uncovered cages were placed in the UV box described above, irradiated (~4,000 J/m^2), and then adult mice were returned to their respective cages. Litters were weaned at 3 weeks of age, and hepatocyte growth factor–negative animals were euthanized because these do not form tumors (27); the remaining animals were monitored weekly thereafter for an additional 37 weeks. Tumor diameters were documented weekly by visual inspection with intermittent caliper confirmation. Animals developing tumors ≥1 cm in diameter before 40 weeks of age were

[6] http://depts.washington.edu/compmed/veterinary/pdf/MouseFact_sheet.pdf

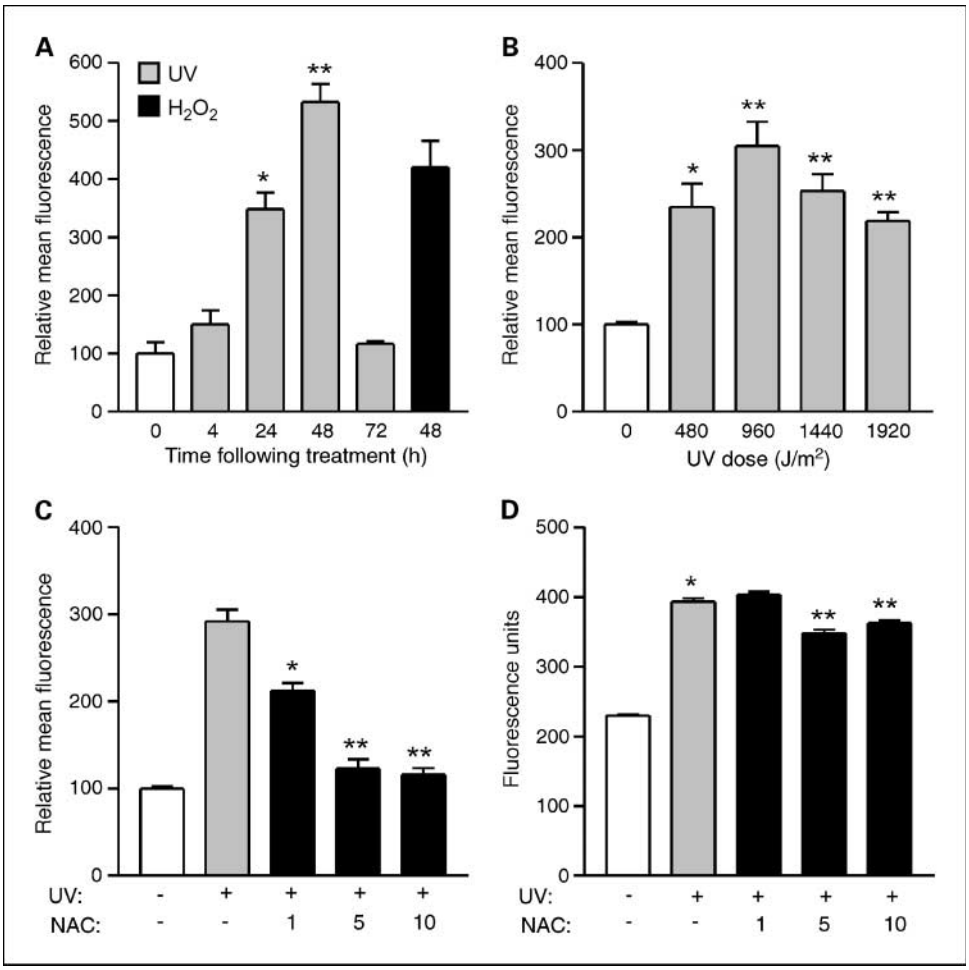

Fig. 1. Induction of ROS by UV and modulation with NAC in melan-a cells. *A,* untreated cells (*white column*) or cells treated with 960 J/m^2 UV (*gray columns*) or 100 μmol/L H_2O_2 (*black column*) were incubated for the indicated times before addition of 2′,7′-dichlorodihydrofluorescein diacetate. Columns, mean fluorescence values of untreated cells were normalized to 100 (triplicate determinations); bars, SE. *, $P = 0.003$; **, $P < 0.001$, comparisons with untreated cells. *B,* untreated cells (*white column*) or cells UV irradiated at the indicated doses (*gray columns*) were cultured for 48 h before addition of 2′,7′-dichlorodihydrofluorescein diacetate. *, $P = 0.02$; **, $P < 0.01$, comparisons with untreated cells. *C,* cells were treated with 960 J/m^2 UV in the absence (*gray column*) or presence (*black columns*) of the indicated millimole per liter concentrations of NAC. Untreated cells (*white column*) served as a control. After 48 h, 2′,7′-dichlorodihydrofluorescein diacetate was added, and cells were analyzed by flow cytometry. *, $P < 0.01$; **, $P = 0.001$, comparisons with untreated cells. *D,* cells were treated with 1,440 J/m^2 UV in the absence (*gray column*) or presence (*black columns*) of the indicated millimole per liter concentrations of NAC. Untreated cells (*white column*) served as a control. After 24 h, dihydroethidium was added, and cells were analyzed on a fluorimeter. *, $P < 0.001$, comparisons of UV-treated cells with untreated cells; **, $P < 0.01$, comparisons of UV/NAC – treated cells with UV-treated cells.

euthanized. Most skin lesions ≥3 mm in diameter were excised from animals reaching experimental end points, a subset of animals were necropsied, and tissues were processed for histologic analysis as described elsewhere (27). Tumors displaying cellular or nuclear pleomorphism, enlarged nuclei, or increased mitotic figures were considered cytologically atypical. Lymph node metastasis was interpreted as >50% effacement of node by pigmented cells or definitive presence of cytologically atypical cells. Slides were examined in a blinded fashion by a dermatopathologist (S.R.F.). Lung metastasis was identified by the presence of macroscopic dark dots on lung tissue.

Statistics

Data were subjected to various analyses (log-rank test, unpaired *t* test, or Fisher's exact test) using Prism (Graphpad Software). *P* values ≤0.05 were considered statistically significant.

Results

NAC reduces UV-induced ROS in melanocytes. To study the potential protective capacity of NAC in a mouse model of UV-induced melanoma, we began by characterizing effects on UV-induced ROS in a mouse melanocyte cell line. Treatment of melan-a cells with a single dose of UV resulted in increased levels of endogenous peroxide that were detected using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate as early as 4 h following UV exposure, peaked at 48 h, and returned to baseline levels by 72 h (Fig. 1A). The 3- to 5-fold increases in endogenous peroxide induced by UV were comparable with that induced by addition of exogenous H_2O_2 (Fig. 1A). Increases in endogenous peroxide were observed at 48 h following a range of UV doses but was highest at 960 J/m^2 (Fig. 1B). Addition of NAC (1-10 mmol/L) under these conditions resulted in dose-dependent reductions in formation of UV-induced peroxide (Fig. 1C). Concentrations of 5 to 10 mmol/L NAC restored baseline peroxide levels (Fig. 1C). We next examined the effect of NAC on endogenous superoxide using the fluorescent probe dihydroethidium under optimal conditions for UV-induced peroxide generation (960 J/m^2 UV, 48 h). The level of endogenous superoxide increased 2-fold following UV treatment, and addition of 5 to 10 mmol/L NAC resulted in very modest yet statistically significant reductions (Fig. 1D).

NAC protects melanocytes from UV-induced oxidative damage. We next investigated whether NAC-mediated reduction of UV-induced ROS translated into protection from oxidative damage in melanocytes. Melan-a cells were UV irradiated for optimal ROS generation and the low molecular weight (free) thiol fraction was quantitated using Ellman's reagent (22). Free thiols primarily represent a measure of the cellular reservoir of GSH (28) that may initially be depleted during oxidative stress. We observed that free thiols were depleted in UV-treated cells and that such depletion was not seen with the addition of NAC before and immediately after UV

irradiation (Fig. 2A). Because higher levels of free thiols were found in NAC-treated cells following UV treatment than in unirradiated cells (Fig. 2A), we investigated the possibility that the elevated free thiols simply represented residual NAC (which is not only a GSH prodrug but also a low molecular weight thiol). We added NAC to unirradiated cells and determined free thiol levels over time after washing. As shown in Fig. 2B, although free thiol recovery was increased 12 to 24 h following UV treatment, levels had declined to baseline (levels in unirradiated cells) by 48 h. These results suggest that the elevated free thiols measured in cells 48 h after UV treatment likely represent NAC-mediated GSH accumulation rather than residual NAC and that NAC can protect melanocytes against UV-induced depletion of free thiols. We next examined whether NAC also conferred protection against oxidative DNA damage by staining for 8-OG. Nuclear 8-OG staining was seen following UV exposure in an increased fraction of cells, relative to unirradiated cells, and this was significantly decreased in a dose-dependent fashion on addition of NAC (Fig. 2C). Finally, we examined UV-treated cells in the absence or presence of NAC for formation of CPD photoproducts, generally thought to be a direct modification of DNA by UV and not mediated by ROS. Consistent with this notion, we did not observe any effect of NAC on CPD formation (Fig. 2D). Thus, although NAC protected melanocytes against UV-induced oxidative sequelae, its presence did not affect oxidation-independent formation of UV-induced photoproducts.

Delivery of NAC transplacentally and during nursing protects skin from UV-induced oxidative damage. To study antioxidant function of NAC *in vivo*, we used a delivery route that would be

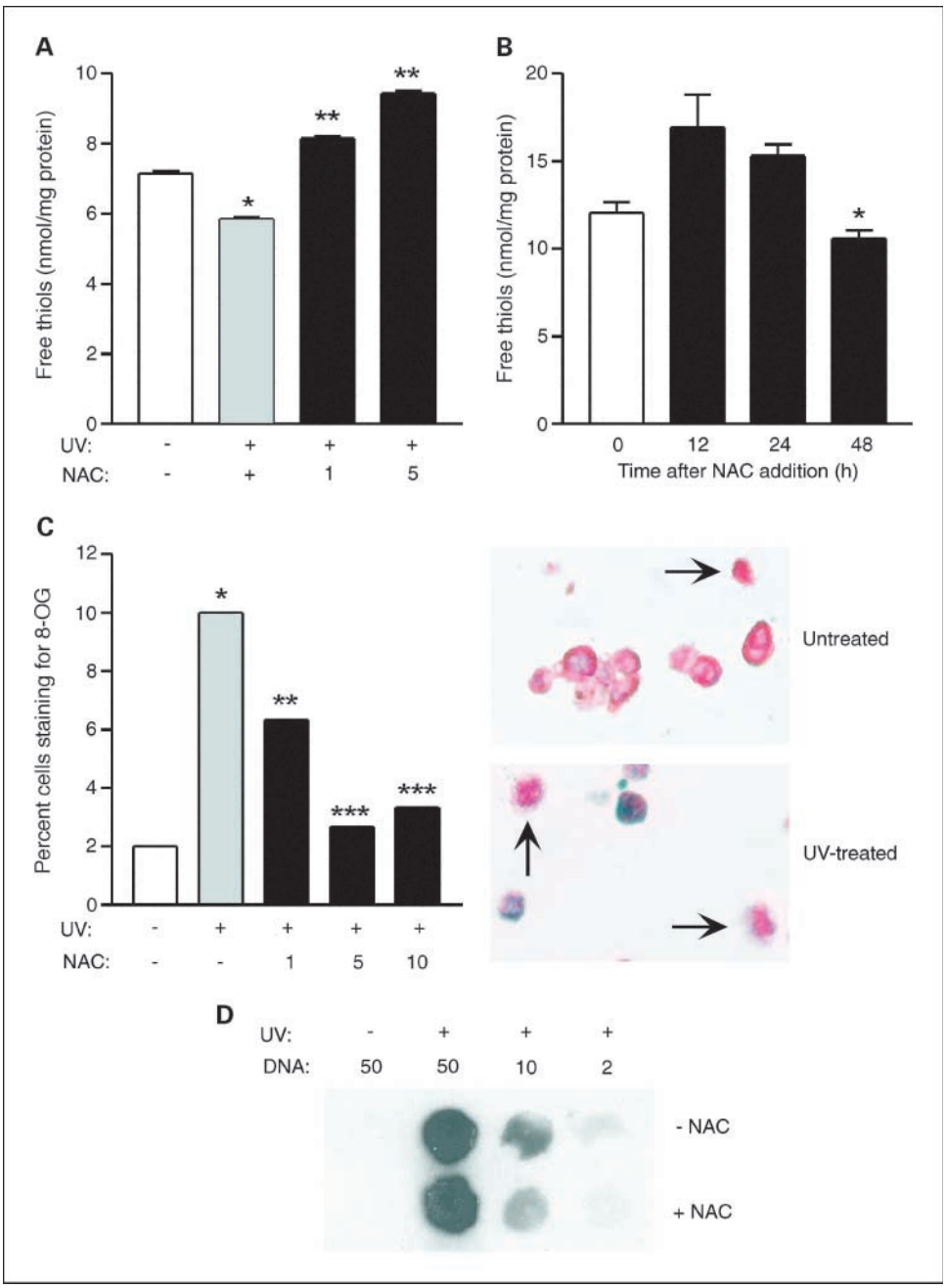

Fig. 2. NAC-mediated protection against UV-induced oxidative sequelae in melan-a cells. *A,* cells were treated with 960 J/m^2 UV for 48 h in the absence (*gray column*) or presence (*black columns*) of the indicated millimole per liter concentrations of NAC. Untreated cells (*white column*) served as a control. Free thiols were quantified and normalized to protein for each sample. Columns, free thiols (at least three determinations); bars, SE. *, $P < 0.0001$, comparisons of UV-treated cells with untreated cells; **, $P < 0.0001$, comparisons UV/NAC – treated cells with UV-treated cells. *B,* cells were treated with 5 mmol/L NAC (*black columns*) and untreated cells (*white column*) served as a control. *, $P = 0.15$, comparisons of untreated cells with cells 48 h after NAC addition. *C,* left, cells treated with 960 J/m^2 UV in the absence (*gray column*) or presence (*black columns*) of the indicated concentrations (millimole per liter) of NAC. Untreated cells (*white column*) served as a control. After 48 h, cells were embedded in agarose and stained for 8-OG. *, $P < 0.0001$, comparisons of untreated cells with UV-treated cells; **, $P = 0.05$; ***, $P < 0.0001$, comparisons of UV/NAC – treated cells with UV-treated cells, Fisher's exact test. Right, representative fields corresponding to untreated and UV-treated cells. Arrows, positive cells with red-staining nuclei. *D,* cells were treated with 960 J/m^2 UV in the absence or presence of 5 mmol/L NAC. Untreated cells served as a control. Genomic DNA was isolated and the indicated amounts (nanograms) were spotted on a membrane, which was then stained with anti-CPD antibody.

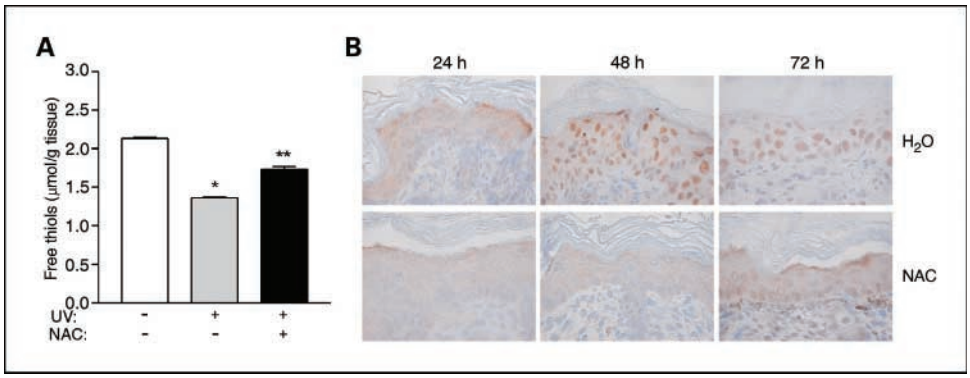

Fig. 3. NAC delivery protects skin from UV-induced oxidative sequelae. *A,* pregnant females were provided either water or water containing NAC. Resulting 2-day-old litters were treated with 3,900 J/m^2 UV. After 48 h, skin was harvested from untreated mice (*white column*), UV-treated mice (*gray column*), and UV/NAC – treated mice (*black column*). Free thiols were quantitated and normalized to tissue weight for each sample. Columns, free thiols (triplicate determinations); bars, SE. *, $P = 0.0005$, comparisons of UV-treated mice with untreated mice; **, $P = 0.005$, comparisons of UV/NAC – treated mice with UV-treated mice. *B,* pregnant females in separate cages were provided either normal water or water containing NAC. Resulting 2-day-old litters were treated with 3,900 J/m^2 UV, and skin was harvested 24 to 72 h later for 8-OG staining.

amenable to our model of UV-induced melanoma in which animals transgenic for both hepatocyte growth factor and the apoptosis inhibitor survivin form tumors following a single neonatal UV exposure (20). The pharmacologic challenge in this model is the need for drug delivery during the neonatal period. We investigated whether administration of NAC transplacentally and in the mother's milk to neonates (by addition of NAC to the mother's drinking water) could modulate UV-induced oxidative stress/damage in neonatal skin. Pregnant females were provided either deionized water or water containing NAC, and then 2-day-old pups were UV irradiated and skin was analyzed for free thiol content and formation of 8-OG. Because a previous study (29) was unable to detect NAC in serum of mice given comparable dosing, we examined metabolites and markers of oxidative damage in tissue as done in other studies (29, 30). Neonatal UV exposure was associated with significant free thiol depletion that was partially restored by NAC treatment (Fig. 3A). Under these same experimental conditions, 8-OG formation was substantially reduced by NAC in UV-irradiated skin (Fig. 3B). These data show that orally delivered NAC can modulate UV-induced oxidative stress/damage in skin.

Effect on melanoma development. Litters of mice born of mothers either treated or untreated with NAC were UV irradiated as above and then monitored for 40 weeks for tumor development, growth, and metastasis. When pups reached 2 weeks of age, NAC-containing water was replaced with deionized water. As shown in Fig. 4A, NAC exposure before and for 2 weeks after UV treatment resulted in a significant delay in melanocytic tumor onset. The mean time to first tumor onset was 13.8 and 20.6 weeks in control and NAC-treated mice, respectively (Fig. 4A). In addition, tumors were significantly smaller in treated animals compared with controls at early time points (Fig. 4B). Over time, however, average tumor size in control and NAC-treated animals became comparable (Fig. 4B). A subset of tumors from each group was subjected to histologic examination and partial necropsy was also done on animals from each group for examination of lymph nodes and lung tissues. Although tumors in NAC-treated animals showed higher rates of cytologic atypia and metastasis to lymph nodes and lungs, the differences were not statistically significant (Fig. 4C). Thus, the predominant effect of orally delivered NAC before (and briefly following) UV irradiation was a delay in tumor formation, as by the time all animals had formed tumors (32 weeks) there were no differences in tumor number, histologic pattern, or prevalence of metastasis.

Discussion

In this report, we present the novel use of NAC for the prevention of UV-induced oxidative stress and damage in melanocytes *in vitro* and the delayed onset of tumors in a highly penetrant mouse model of UV-induced melanoma *in vivo*. Specifically, we have shown that at NAC concentrations ranging from 1 to 10 mmol/L, the accumulation of intracellular H_2O_2 in melanocytes was decreased following UV irradiation. Interestingly, protection from UV-induced superoxide was only modest compared with that seen with H_2O_2. This may be because superoxide formation reflects a direct interaction of UV with melanin (31) that is not affected by cellular GSH levels. Consistent with its mechanism as a GSH prodrug (13), NAC addition was associated with a dose-dependent protection from UV-induced depletion of reduced thiols. We found that NAC also protected melanocytes from UV-induced oxidative DNA damage as measured by the oxidative DNA lesion 8-OG. Protection by NAC was specific for oxidative damage in that no decrease was observed in the formation of CPD photoproducts, which arise directly from UV irradiation of DNA. Before assessing the chemopreventive activity of NAC in a mouse model of UV-induced melanoma, we first showed that NAC delivery to nursing pups through maternal drinking water conferred similar oxidative protection to UV-irradiated skin. Administration of NAC was associated with a significant delay in tumor onset; however, the incidence of malignant tumor histology and metastasis were not statistically different from the control group by completion of the experiment. These findings indicate a role for oxidative stress/damage in the pathogenesis of UV-induced melanoma and suggest the potential utility of NAC as a chemopreventive agent.

It is interesting to note, however, that the protective effect of NAC *in vivo* became less pronounced over time and was no longer evident by the conclusion of the experiment. The diminished effect of NAC over time may be due to the fact that it was not administered chronically, but only during the period

before and (for 2 weeks) following UV treatment. Although it would be interesting to examine the effects of chronic NAC administration in this model, the animal study directly followed from our *in vitro* data in melanocytes examining protection against acute sequelae of UV irradiation and oxidative injury. The animal study was therefore designed in this way as we hypothesized that protection from the acute oxidative insult (the single neonatal UV dose) would attenuate the initiation of the tumor phenotype. Although it is unclear whether tumors developed in the NAC-treated mice due to premature cessation of NAC or failure to protect against other oncogenic mechanisms, our data at least show that acute oxidative damage is a contributing factor to UV-induced melanoma. As mentioned, it is possible that several additional factors may be involved in the development of tumors in NAC-treated animals. First, the animal model of UV-induced melanoma used was highly penetrant as 100% of the animals formed tumors. Thus, overcoming this extreme tendency toward the development of melanocytic tumors would require particularly robust protection. Second, although there is evidence that oxidative stress is pathogenic in the development of melanoma (11, 12), there are clearly additional pathways such as oxidation-independent UV-induced DNA damage (indicated by formation of CPDs) that we found were unaffected by NAC. In addition, other UV-induced pathways leading to cell cycle deregulation and modulation of apoptosis resistance may contribute to melanoma in this model and not be amenable to protection by NAC.

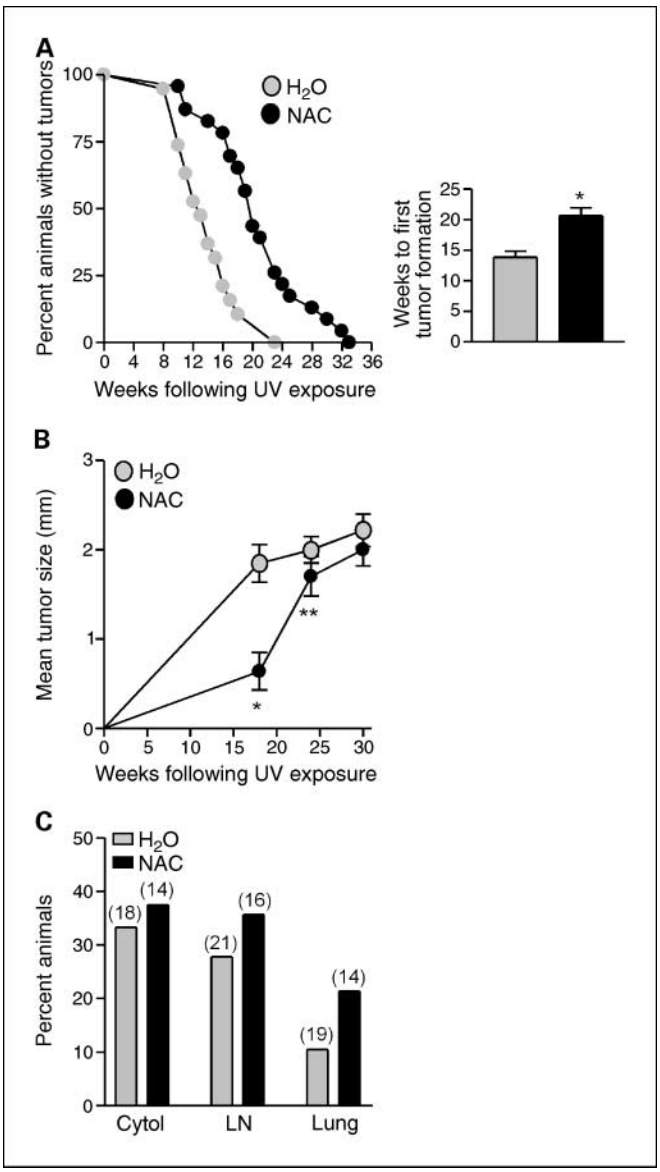

Fig. 4. NAC delivery delays onset of UV-induced melanoma. *A,* pregnant females were provided either water or water containing NAC. Resulting 2-day-old litters were treated with 3,900 J/m^2 UV, and mice (normal water, $n = 19$, *gray circles*; NAC, $n = 23$, *black circles*) were monitored for 40 wks for tumor formation. $P = 0.0003$, log-rank test. Right, mean time to onset of first tumor in control animals and animals exposed to NAC. *, $P = 0.0003$. *B,* columns, mean tumor size; bars, SE. *, $P < 0.001$; **, $P = 0.14$, comparisons of NAC-treated animals with control (water) animals. *C,* percentage tumors displaying cytologic atypia (*Cytol*), lymph node metastasis (*LN*), and lung metastasis (*Lung*). Number of tumors indicated in parentheses. $P = 1.0$, $P = 0.71$, and $P = 0.63$, comparisons of tumors from NAC-treated mice with control (water) mice for cytologic atypia, lymph node metastasis, and lung metastasis, respectively, Fisher's exact test.

Several strategies for antioxidant chemoprevention of melanoma have been promulgated in the literature. Perhaps, the best studied among these is epigallocatechin-3-gallate, a polyphenol antioxidant present in green and black teas with numerous biological activities *in vitro* that suggest its potential as an anticancer chemopreventive agent (32). Orally delivered epigallocatechin-3-gallate has also been shown to inhibit tumor metastasis in the mouse B16 melanoma model (33). Although these data for epigallocatechin-3-gallate are encouraging, there are no published studies showing an effect on *de novo* melanoma and the data in humans are mixed (32). Other proposed agents for melanoma chemoprevention include vitamin E, β-carotene, lycopene, flavonoids, grape seed extract, resveratrol, and selenium, but as with epigallocatechin-3-gallate, there is an absence of data in a *de novo* animal model system and the human data are conflicting (32). Thus, there is currently insufficient evidence to make any recommendations about implementation of antioxidants for the chemoprevention of melanoma in humans. In fact, the antioxidants vitamin E and β-carotene have been correlated with both an increase and decrease in overall cancer risk in separate randomized controlled trials and meta-analyses (34, 35). Although many of these agents are not regulated as drugs by the Food and Drug Administration, they nevertheless may have potentially deleterious health effects, and as such will require rigorous study before general recommendations can be made.

The antioxidant used here, NAC, seems to be an excellent candidate for further study in the chemoprevention of melanoma. To our knowledge, this report presents the first demonstration of antioxidant-mediated protection from the development of *de novo* melanoma in a mouse model system. Moreover, in contrast to the other agents mentioned above, NAC is commercially available as a Food and Drug Administration–approved drug (for acetaminophen toxicity) with established pharmacokinetics and toxicology. It has been safely administered over long periods to large numbers of patients with idiopathic pulmonary fibrosis (15) and short-term to protect against contrast-induced nephropathy (36). In both short-term and long-term studies, there have not been any reported severe adverse events. In the skin, NAC has been shown to have biological activity insofar as reducing parameters of photoaging after topical delivery in human volunteers (17) and decreasing the burden of UV-induced squamous cell

carcinoma in a mouse model (16). Although high oral doses of NAC have been administered safely to patients for up to 1 year (15), there may be potential hazards associated with chronic use of any antioxidant (37). Thus, NAC may have even greater utility as a chemopreventive agent in the context of acute UV (sun) exposure, taken prophylactically or immediately after exposure to reduce UV-induced oxidative stress/damage in the skin, which may ultimately contribute to melanoma development in humans.

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