Effect of N-Acetyl Cysteine on Oxidative DNA Damage and the Frequency of DNA Deletions in Atm-Deficient Mice

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ABSTRACT
Ataxia telangiectasia (AT) is a hereditary human disorder resulting in a wide variety of clinical manifestations, including progressive neurodegeneration, immunodeficiency, and high incidence of lymphoid tumors. Cells from patients with AT show genetic instability, hypersensitivity to radiation, and a continuous state of oxidative stress. Oxidative stress and genetic instability, including DNA deletions, are involved in carcinogenesis. We examined the effect of dietary supplementation with the thiol-containing antioxidant N-acetyl-L-cysteine (NAC) on levels of oxidative DNA damage and the frequency of DNA deletions in Atm-deficient (AT-mutated) mice. We confirmed that Atm-deficient mice display an increased frequency of DNA deletions (Bishop et al., Cancer Res 2000;60:395). Furthermore, we found that Atm-deficient mice have significantly increased levels of 8-OH deoxyguanosine, an indication of oxidative DNA damage. Dietary supplementation with NAC significantly reduced 8-OH deoxyguanosine level and the frequency of DNA deletions in Atm-deficient mice. These levels were similar to the levels in wild-type mice. Our findings demonstrate that NAC counteracts genetic instability and suggest that genetic instability may be a consequence of oxidative stress in Atm-deficient mice.

INTRODUCTION
Ataxia telangiectasia (AT) is a severe, progressive, and terminal autosomal recessive disorder caused by a mutation in the ATM (AT-mutated) gene and is characterized by progressive neuromotor dysfunction, immunodeficiency, premature aging, striking elevated incidence of malignancies, and extreme sensitivity to ionizing radiation (1–3). Approximately 40% of patients with AT have increased tumorigenesis, mostly of the lymphoid organs early in life and solid tumors at later age (4). There is no available therapy for the disease, and death from progressive malignancy or neurologic degeneration typically occurs in the second or third decade.

The AT phenotype can be attributed to a defective cellular response to DNA double-strand breaks (DSBs) and/or oxidative stress (5, 6). The ATM protein, a phosphotyrosine 3-like kinase, is considered a primary activator in cellular response to DSBs after initial DSB processing by MRN complex (7, 8). ATM phosphorylates numerous substrates involved in cell cycle checkpoints, stress response, and DSB repair or cell death by apoptosis (5, 6), and it may be involved in sensing oxidative stress or damage (9, 10).

Cells from patients with AT exhibit chromosomal breaks (11, 12) and translocations (13), as well as extreme sensitivity to DSB-inducing agents, such as ionizing radiation (14, 15) and radiomimetic chemicals (16–18). Furthermore, ATM-deficient cells show signs of continuous oxidative stress, namely, elevated sensitivity to oxidants (19–25), constitutive activation of nuclear factor κB (26), reduced levels of exogenous antioxidants (27), and oxidative damage to lipids and DNA (28).

Atm-deficient mice recapitulate most of the phenotypes associated with AT, including high lymphoid tumor incidence, immunodeficiency, radiosensitivity, genetic instability, and oxidative stress (29–32), and spontaneously exhibit high frequencies of DNA deletions (33, 34). Altered activity of antioxidative enzymes (32, 35) and increased levels of oxidatively damaged proteins and lipids (32) demonstrated presence of oxidative stress and damage in Atm-deficient mice. DNA isolated from cells from Atm-deficient mice exhibits mutational signatures suggestive of oxidative stress (36). Selectively elevated reactive oxygen species (ROS) levels are found in Purkinje cells and nigrostriatal dopaminergic cells (37). It recently has been shown that synthetic catalytic antioxidant EUK-189 decreased oxidation of brain fatty acid and improved performance on a rotating rod in Atm-deficient mice (38).

In the current study, we examined the effect of N-acetyl-L-cysteine (NAC) on the frequency of DNA deletions and oxidative DNA damage in Atm-deficient mice. NAC is a thiol-containing compound that nonenzymatically interacts with and detoxifies reactive electrophiles and free radicals. The efficacy and safety of NAC have been established in clinical practice for >40 years as a mucolytic drug and more recently as an antidote for acute acetaminophen poisoning (39, 40). NAC also has emerged as a potent antioxidant and a promising cancer chemopreventive agent (41–46).

Mice were treated during embryo development by administering NAC in drinking water to pregnant dams. Oxidative DNA damage was measured by determining 8-OH deoxyguanosine levels on embryonic DNA. To determine the frequency of DNA deletions, we used an in vivo DNA deletion assay (47, 48), which allows detection of 70-kb deletions occurring at the p<sup>+</sup> allele of the pink-eyed dilute (p) gene (49, 50) in melanocyte precursor cells of developing embryos. The p<sup>+</sup> allele contains a 70-kb internal duplication resulting in a dilute, light gray coat color and pink eyes (51). Intrachromosomal homologous recombination between the 70-kb repeats followed by a deletion of one of the repeats reconstitutes the wild-type p gene, resulting in black patches on the light gray fur (fur spots) or black pigmented cells (eyespots) on the unpigmented retinal pigmented epithelium (RPE). On the C57BL/6dp<sup>+</sup>/<sup>+</sup>p<sup>+</sup> inbred strain background, ~5–10% of the mice spontaneously display fur spots (50, 52) and 4–6 eyespots per RPE (47, 53). The frequency of such deletion events is elevated in mice with mutations in Atm (33, 34), Trp53, Gadd45 (34), and Wnt (54) genes and in wild-type mice exposed to carcinogens in utero (52, 55, 56). In this report, we show that NAC treatment reduced oxidative DNA damage and the frequency of DNA deletions in Atm-deficient mice.

MATERIALS AND METHODS
Mice and NAC Treatment. C57BL/6dp<sup>+</sup>/<sup>+</sup>p<sup>+</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Atm-deficient mice were created previously (29) and crossed into a mixed C57BL/6J-129/Sv background. The ATM mutation was crossed into the C57BL/6dp<sup>+</sup>/<sup>+</sup>p<sup>+</sup> genetic background by six backcrosses. The resulting p<sup>+</sup>/p<sup>+</sup>, Atm<sup>−/−</sup> mice had a genetic background containing 99.992% of C57BL/6J and were morphologically similar to the parental C57BL/6dp<sup>+</sup>/<sup>+</sup>p<sup>+</sup> strain. Genotyping for ATM was carried out by PCR.
as described by Liao et al. (57). Mice were bred in the institutional specific pathogen-free animal facility under standard conditions with a 12-h light/dark cycle and were given standard diet and water ad libitum. Pregnancy was timed by checking for vaginal plugs, with noon of the day of discovery counted as 0.5 days postcoitum (dpc). Similarly, the time of birth of a litter was timed with the noon of discovery counted as 0.5 days postpartum (dpp).

Atm−/− female mice were mated with Atm+/− males, and a group of dams were given free access to drinking water supplemented with 40 mM NAC (Sigma, St. Louis, MO), which was calculated to yield an average dose of 1 g NAC/kg body weight/day. The same intake of NAC was shown to reduce DNA-adduct formation in rats exposed to genotoxic carcinogens and cigarette smoke (58). The control group received regular water. The treatment group received NAC starting at 0.5 dpc until the end of gestation. From these treatments, we obtained four groups of offspring: NAC in utero-treated Atm−/− and Atm+/− mice and control (untreated) Atm−/− and Atm+/− mice. These mice were examined for the frequency of DNA deletions that occurred during embryonic development. To determine oxidative DNA damage, embryos from NAC-treated and control dams were isolated at 17.5 dpc, and 8-OH deoxyguanosine levels were determined on NAC-treated Atm−/− and Atm+/− mouse embryos and control (untreated) Atm−/− and Atm+/− mouse embryos.

**Dissection of the RPE.** Eyes from sacrificed 20-day-old C57BL/6J+/+/p−/+ mice were processed to expose the RPE layer as described previously (47, 48). An eye was removed from its orbit and immersed in fixative [4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4)] for 1 h and then in PBS until dissection. An incision was made at the upper corneoscleral border to allow removal of the cornea and lens. To flatten the eyecup, six to eight incisions were made from the corneoscleral margin toward the centrally positioned optic nerve, and the dissected eyecup was placed on a glass slide with the retina facing up. The retina then was gently removed, and the residual specimen, consisting of sclera, choroid, and RPE, was mounted in 90% glycerol for microscopic analysis.

**Scoring Single Deletion Events, Visualized as Eyespots.** A pigmented cell or a group of adjacent pigmented cells separated from each other by no more than five unpigmented cells was considered as an eyespot that resulted from one deletion/p+ renversion (Refs. 47, 53, 59; Fig. 1B). The number of eyespots in each RPE and the number of cells that comprised each eyespot were counted. Positions of eyespots in the RPE were mapped.

The RPE was scanned with a DC120 digital camera (Eastman Kodak Company, Rochester, NY) mounted on a DMLB microscope (Leica Microsystems, Wetzlar, Germany) using a 2.5 × N-plan objective. The images were assembled and examined in Adobe Photoshop 5.0 (San Jose, CA) on a Macintosh Power Computer (Apple Computer, Cupertino, CA). All of the data were stored and processed with Microsoft Excel 98 (Redmond, WA).

**Distance Analysis of Eyespots from the Optic Nerve.** Eyespots were identified under the microscope and compared with their scanned digital images. Distances were measured with the Adobe Photoshop 5.0 Measurement Tool. Distances were converted from pixels to millimeters by counting the number of pixels/mm on the image of a micron scale reticule scanned at the same optical settings of the RPE. Two distances were measured for each eyespot: the eyespot distance is the distance from the center of the optic nerve head to the most proximal edge of the eyespot, and the RPE distance of an eyespot is the distance from the optic nerve through the eyespot to the outer edge of the RPE. Dividing the eyespot distance by the RPE distance gives the proportional distance of each eyespot from the outer edge of the RPE, or its position. The position of each eyespot was determined in this manner to compensate for minor differences in the size of the eyes.

**Determination of 8-OH Deoxyguanosine.** Mouse embryos at E17.5 were isolated, immediately frozen in liquid nitrogen, and homogenized under liquid nitrogen. DNA was extracted from 100 mg of tissue using the DNA isolation kit from Roche Diagnostics Corporation (Indianapolis, IN). DNA was dissolved in AE buffer (Qiagen, Valencia, CA), and 100 μg were digested as described previously (60). The following incubations were performed: DNase I, 30 min at 37°C; NP1, 60 min at 37°C; AP, 30 min at 37°C; and PDEI and PDEII, 30 min at 37°C. The incubation mixture was filtered through a 0.5 μm ultrafilter centrifugal filter (Millipore, Billerica, MA). The iron chelator DTPA was added to the DNA hydrolysates to prevent artificial oxidation. Twenty μl of hydrolysate were analyzed by high-performance liquid chromatography. The high-performance liquid chromatography with electron capture detection system consisted of an Agilent 1100 binary pump, autosampler, and variable wavelength detector controlled by Chemstation Software 7.01 (Agilent Technologies, Palo Alto, CA); an ESA Coulochem II electrochemical detector (ESA, Inc., Chelmsford, MA); a C18 Alltima guard column, 7.5 × 4.6 mm, particle size of 5 μm (Alltech, Deerfield, IL); and a YMC ODS-AQ column, 4.6 × 15 cm, 120 Å, 5-Å (Waters Corporation, Milford, MA). The mobile phase consisted of 8% aqueous methanol containing 50 mM sodium acetate buffer (pH 5.2). Elution was isocratic at a flow rate of 0.8 ml/min. The deoxyguanosine concentration was monitored based on absorbance (245 nm), and 8-OH deoxyguanosine concentration was based on the electrochemical reading (400 mV). Levels were quantified using the standard curves of each compound. The degree of DNA damage was expressed as 8-OH deoxyguanosine per 106 deoxyguanosine.

**Statistical Analysis.** Comparison between numbers of events was done by Student’s t test or a contingency χ² test.

**RESULTS**

**Atm-Deficient Mice Showed Elevated Frequencies of DNA Deletions.** The frequency of DNA deletions was examined as the number of eyespots in the RPE (Fig. 1). The RPE of Atm−/− mice had 6.27 ± 2.13 (average ± SD; n = 30) eyespots, and Atm+/− mice had 4.24 ± 1.57 (n = 29) eyespots. The frequency of eyespots in the two groups comprised overlapping normal distributions but were highly statistically significant (P < 0.005; Fig. 2A). The average number of eyespots in the mutants versus controls differed by 32%. This finding was consistent with previous reports in which frequencies of fur spots (33) or eyespots (34) were determined.

**NAC Treatment Reduced the Frequency of DNA Deletions in Atm-Deficient Mice to the Wild-Type Level.** The RPE of NAC-treated Atm−/− mice had significantly fewer eyespots than untreated mutant mice (P < 0.005; Fig. 2B), with the averages differing by 30% [4.38 ± 2.39 eyespots (n = 24) and 6.27 ± 2.13 eyespots (n = 30), respectively]. This finding demonstrated that NAC oral administration to dams reduces the frequency of DNA deletions in developing Atm-deficient embryos. We next compared the RPE of NAC-treated Atm−/− mice with the NAC-treated Atm+/− littersmates. The two groups comprised overlapping and similar (not statistically different) distributions (Fig. 2C), which implied that NAC treatment not only reduces but also reverts the elevated frequency of deletions in developing Atm-deficient mice to the level of wild-type mice.

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A schematic representation of the dissected RPE. The RPE develops radially away from the optic nerve head. B. photographs of the region of eyespots (80-fold magnification). Eyespots are visualized as black, pigmented cells scattered on the unpigmented background RPE. The RPE is a monolayer consisting of hexagonal-shaped epithelial cells (53). Single pigmented cells or clones thereof, the result of DNA deletion events, are scored (48).

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Fig. 1. Dissected retinal pigment epithelium (RPE) and eyespots. A, schematic representation of the dissected RPE. The RPE develops radially away from the optic nerve head. B, photographs of the region of eyespots (80-fold magnification). Eyespots are visualized as black, pigmented cells scattered on the unpigmented background RPE. The RPE is a monolayer consisting of hexagonal-shaped epithelial cells (53). Single pigmented cells or clones thereof, the result of DNA deletion events, are scored (48).

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The RPE of NAC-treated Atm<sup>−/−</sup> mice had similar numbers of eyespots compared with untreated Atm<sup>−/−</sup> mice: 4.85 ± 1.81 (∼34) and 4.24 ± 1.57 (n = 29), respectively. There was no significant difference in the distribution of eyespots between the groups (Fig. 2D). Thus, NAC had no effect in wild-type mouse embryos, whereas it suppressed DNA deletions specifically in Atm-mutant mouse embryos.

**Effect of NAC Treatment in Atm-Deficient Mice Was Most Pronounced Later in Development.** The mouse RPE forms from the optical nerve head with a pattern of radial edge-biased interstitial growth (Fig. 1A; Ref. 61). The RPE formation begins in the developing eyecup at ∼8.5 dpc (62), reaches the highest rate of mitotic division between 11.5 and 15.5 dpc, and ceases after birth (63). Mapping an eyespot relative position within the RPE allows determining the approximate time during embryo development at which a deletion event occurred (64). We divided the RPE into 10 concentric regions starting at 0.0–0.1′, the concentric region at the edge of the RPE indicating the beginning of RPE development, up to 0.9–1.0′, the concentric region closest to the optical nerve head indicating the beginning of RPE development. The mouse RPE forms from the optical nerve head with a pattern of radial edge-biased interstitial growth (Fig. 1A; Ref. 61). The RPE formation begins in the developing eyecup at ∼8.5 dpc (62), reaches the highest rate of mitotic division between 11.5 and 15.5 dpc, and ceases after birth (63). Mapping an eyespot relative position within the RPE allows determining the approximate time during embryo development at which a deletion event occurred (64). We divided the RPE into 10 concentric regions starting at 0.0–0.1′, the concentric region at the edge of the RPE indicating the beginning of RPE development, up to 0.9–1.0′, the concentric region closest to the optical nerve head indicating the beginning of RPE development.

Positional distribution of eyespots was different in NAC-treated Atm<sup>−/−</sup> mice compared with untreated Atm<sup>−/−</sup> mice, such that the number of eyespots was similar in the first half of the RPE comprising the region 0.0–0.1 to 0.4–0.5 (1.1 compared with 0.97 eyespots/RPE) and 40% lower in the remaining RPE (3.2 compared with 5.3 eyespots/RPE; P < 0.05, Fig. 3B). This observation suggested that NAC suppresses DNA deletions later in development, at the region 0.5–0.6 to 0.9–1.0. This region falls within the time of most intense RPE growth as calibrated after benzo(a)pyrene induction of deletions in these regions (64). The positional eyespot distribution was not statistically different in NAC-treated Atm<sup>−/−</sup> mice compared with untreated Atm<sup>−/−</sup> mice (Fig. 3C). NAC treatment had no effect on positional eyespot distribution in wild-type mice (Fig. 3D).

**NAC Treatment Suppressed Oxidative DNA Damage in Atm-Deficient Mice.** We examined the degree of oxidative DNA damage by measuring the level of 8-OH deoxyguanosine in DNA isolated from NAC-treated and untreated Atm<sup>−/−</sup> mouse embryos and their respective wild-type controls. The level of 8-OH deoxyguanosine was significantly higher in Atm<sup>−/−</sup> mouse embryos (n = 5) than in Atm<sup>+/+</sup> mouse embryos (n = 9; P < 0.001; Fig. 4A). The 8-OH deoxyguanosine level was twofold lower in NAC-treated (n = 3) than in NAC-untreated Atm<sup>−/−</sup> embryos (n = 5; P < 0.005; Fig. 4B). The level was similar in the NAC-treated Atm<sup>−/−</sup> embryos (n = 3) compared with NAC-treated Atm<sup>+/+</sup> controls (n = 5; not significant; Fig. 4C). NAC lowered the 8-OH deoxyguanosine level in wild-type mouse embryos (P < 0.005; Fig. 4D).

**DISCUSSION**

Normal cellular metabolism, mainly oxidative metabolism in mitochondria and pathologic processes such as inflammation, produce ROS. Prooxidative conditions are linked to many diseases, including cancers (65, 66). Increased oxygen pressure induces chromosomal aberrations (67), and prooxidant xenobiotics cause DNA deletions (68, 69). Thus, it is of utmost importance for cellular survival to orchestrate cellular responses to repair oxidative DNA damage. ATM seems to be involved in the cellular response to ROS or oxidative damage (6, 9, 10). In this report, we showed that Atm-deficient mice spontaneously had elevated levels of oxidative DNA damage. We confirmed that Atm-deficient mice displayed an increased frequency of DNA deletions (33, 34). We also showed that dietary supplementation with NAC reduced the level of oxidative DNA damage and the frequency of DNA deletions to wild-type levels.

**Effects of NAC.** Effects of NAC can be ascribed to multiple protective mechanisms, such as antioxidant activity, ability to act as a precursor of intracellular reduced glutathione (GSH) (70), detoxification (45, 71), and modulation of DNA repair processes (72). NAC
may regulate expression of numerous genes by inhibiting activation of c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, redox-sensitive activating protein 1, and nuclear factor κB transcription factor (73). NAC directly modifies the activity of several enzymes (73). NAC appears to restore GSH levels following experimental depletion but does not increase plasma GSH concentration under normal conditions (74). NAC protects against many aspects of radiation-induced oxidative damage by free radical scavenging activity rather than through its conversion to GSH (75). To protect against an increased occurrence of DNA deletions in Atm-deficient mice, NAC could have exerted its effect by several mechanisms, such as scavenging ROS, regulating transcription of genes involved in the maintenance of cellular redox homeostasis, reducing antioxidative enzymes containing redox-sensitive cysteine residues, and/or enhancing intracellular biosynthesis of GSH.

Oxidative DNA Damage Induction of Deletions in Atm-Deficient Mice. We found that the high frequency of DNA deletions in Atm-deficient mice was accompanied by increased oxidative DNA damage. NAC treatment reduced oxidative DNA damage and the frequency of DNA deletions. We postulate that an initiating step in the process of a deletion may have been arrest of DNA replication and/or formation of DNA single-strand breaks (SSBs). 8-OH deoxyguanosine transiently (76) and other oxidative DNA lesions more severely inhibit DNA replication (77, 78). Recombination is a mechanism that resolves stalled replication forks (79, 80) but can result in DNA deletions or other rearrangements. SSBs could be generated through actions of nucleotide excision repair or during base excision repair through removal of the oxidized base by DNA glycosylases and incision by AP-endonuclease (81). SSBs can be directly induced by ROS in naked plasmid DNA in the absence of DNA repair enzymes (82–84).

An SSB represents an end of a contiguous template, and replication to the end of this broken template produces a DSB, a highly recombinogenic substrate. In yeast cells containing a DNA duplication similar to that in our mouse system, artificially generated DSBs induced homologous recombination and gave rise to deletions of a repeat in G1 or G2 arrested cells (85, 86). Other DNA lesions, such as SSBs, or DNA alkylation events were translated into deletions only in dividing cells. DNA replication was required to turn those DNA damages into recombinagenic substrates. NAC in our results had its most powerful effect in suppression of the elevated frequency of DNA deletions during the high rate of mitotic division of the RPE, suggesting that most of the deletions occurred during DNA replication (63). Thus, oxidized bases may have led to stalling of the replication fork,
which is resolved by recombination and/or resulted in SSBs, which during replication were converted to DSBs. DSBs, in turn, resulted in homologous recombination and gave rise to DNA deletions in our mouse assay.

Possible Source of Oxidative Stress in ATM-Deficient Cells. Presently it is not clear whether (a) ATM itself is directly involved in sensing the increase in ROS preventing DNA DSBs; or (b) oxidative stress in ATM-deficient cells is caused by continuously present unrepaired DSBs, which are normally repaired in ATM-proficient cells (6, 87). The molecular mechanism of the ATM function in protection from oxidative stress is not well characterized, but data vaguely supporting both of these hypotheses exist. Although ATM protein in proliferating cells is predominantly present in the nucleus, a significant portion is present in cytoplasmic peroxisomes, the major sites of oxidative metabolism (88, 89). This, together with the similarity of ATM protein COOH-terminus to catalase, suggests a protective role against peroxisome-generated ROS (89). These findings support the first hypothesis. In support of the second hypothesis, persistent DNA DSBs activate poly-ADP ribose polymerase to produce ADP polymers at the DSB sites. This poly-ADP ribose polymerase activation rapidly reduces cellular NAD+ pools (6). NADH and NADPH provide reducing equivalents in reactions that are crucial for the antioxidant capacity of the cell, and NADH can directly act as antioxidant (6, 90). Lower levels of NAD+ were found in brains from Atm-deficient mice (90). Thus, the ROS could be a result of the persistent DSBs in ATM-deficient cells, in support of the second hypothesis.

In this study, antioxidant NAC counteracted oxidative DNA damage and DNA deletion frequency in Atm-mutant mice. Therefore, our findings support the first hypothesis that ATM senses ROS rather than the second hypothesis that ROS is generated because of DSBs (i.e., ATM is involved in maintaining ROS levels low). This prevents oxidative DNA damage and DSBs and thereby, DNA deletions. In summary, NAC dietary supplementation had a fully protective effect against oxidative DNA damage and DNA deletions in ATM-deficient mice. Our data may stimulate further studies to examine whether antioxidants can suppress cancer or neurologic degeneration in AT mice.

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