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Effects of antioxidants on cancer prevention and neuromotor performance in *Atm* deficient mice

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Abstract

Ataxia telangiectasia (AT) is an autosomal recessive disorder characterized by immunodeficiency, neurodegeneration and cancer. The disease results from bi-allelic mutations in the *AT mutated* (*ATM*) gene involved in cell cycle checkpoint control and repair of DNA double-strand breaks. Evidence has been accumulating that oxidative stress is associated with AT and may be involved in the pathogenesis of the disease. This led to a hypothesis that antioxidants may alleviate the symptoms of AT. Consequently, several studies were conducted in *Atm* deficient mice to examine the role of antioxidants in cancer prevention and/or correction of neuromotor performance. N-acetyl-L-cysteine (NAC), EUK-189, tempol, and 5-carboxy-1,1,3,3-tetramethylisindolin-2-yl oxyl (CTMIO) have been tested in *Atm* deficient mice. In contrast to other antioxidants, NAC has been used in the clinical practice for many decades and is available as a dietary supplement. In this article, we review chemoprevention studies in *Atm* deficient mice and, in more detail, our findings on the effect of NAC. Our short-term study showed that NAC suppressed genome rearrangements linked to cancer. The long-term study demonstrated that NAC reduced the incidence and multiplicity of lymphoma and improved some aspects of motor performance.

Keywords

Atm; mouse; antioxidants; chemoprevention; lymphoma; neuromotor performance

Introduction

Ataxia Telangiectasia (AT) is an autosomal recessive human disorder caused by mutational inactivation of the *AT mutated* (*ATM*) gene. It is a severe pleiotropic disease characterized by progressive neurodegeneration, high incidence of cancer, immunodeficiency,

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oculocutaneous telangiectasias, growth retardation, endocrine abnormalities, infertility, and hypersensitivity to ionizing radiation (Boder, 1985; Boder et al., 1970; Gatti, 2001; Lavin et al., 1997; Meyn, 1999). The most prominent neuropathological manifestation of AT is atrophy of the cerebellar cortex associated with the loss of Purkinje and granule cells. An early sign of neurological degeneration is ataxia characterized by unstable gait and lack of coordination of head and eyes. About 40% of AT patients develop cancer, mostly in the lymphoid organs early in life and solid tumors at later age (Gatti et al., 1989; Taylor et al., 1996; Xu, 1999). AT patients display a variety of lymphoid tumors including non-Hodgkin's lymphoma, Hodgkin's lymphoma and several types of leukemia, most tumors being of T cell origin. AT patients suffer from increased mortality due to malignancy, infections of the respiratory system and various rare complications (Boder, 1975; Crawford et al., 2006). The median survival of AT patients is calculated to be 19 - 25 years (Crawford et al., 2006).

The gene defective in AT, *ATM*, encodes a phosphatidylinositol-3' related kinase that is involved in cell cycle checkpoint and repair responses to DNA double-strand breaks (DSBs) via a series of phosphorylated intermediary proteins including p53, Chk2, Brca1 and Nbs1 (Lavin et al., 2005; Savitsky et al., 1995; Shiloh, 2003). A lack of *ATM* function results in genomic instability characterized by chromosome breaks, chromosome gaps, translocations and aneuploidy (Cohen et al., 1975; Gropp et al., 1967; Stumm et al., 2001). *ATM* deficiency is also associated with elevated oxidative stress. *ATM* deficient cells in culture are more sensitive to oxidative stress than normal cells, cells isolated from AT patients display elevated oxidative damage to lipids and DNA and AT patients have reduced plasma antioxidant concentrations (Reichenbach et al., 2002; Reichenbach et al., 1999; Yi et al., 1990). Further evidence that AT is linked to oxidative stress stems from studies with *Atm* deficient mice. *Atm* deficient mice exhibit elevated levels of reactive oxygen species (ROS), oxidative damage to proteins and DNA, lipid peroxidation and alterations in the levels and function of antioxidative enzymes (Barlow et al., 1999; Ito et al., 2007; Kamsler et al., 2001; Quick et al., 2001; Reliene et al., 2004b). *Atm* deficient mice largely recapitulate the human disease (Barlow et al., 1996; Borghesani et al., 2000; Elson et al., 1996; Xu et al., 1996). Similar to human AT phenotype, *Atm* deficient mice display growth retardation, infertility, immunodeficiency, radiosensitivity and malignant lymphomas (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). Although *Atm* deficient mice do not show the gross cerebellar degeneration that characterizes the human disease, more subtle alterations in the cerebellum have been observed and are consistent with a mild decrease in their motor performance (Barlow et al., 1996; Borghesani et al., 2000; Kuljis et al., 1997).

Since oxidative stress has been evidenced in AT and oxidative stress is linked to neurodegenerative diseases and cancer, it has been suggested that it may contribute to neuropathological and malignant phenotype of AT, while antioxidants might alleviate these symptoms (Barzilai et al., 2002). This hypothesis has been tested in *Atm* deficient mice in several studies that examined the effect of NAC, EUK-189, tempol and CTMIO (Browne et al., 2004; Gueven et al., 2006; Ito et al., 2007; Reliene et al., 2006; Schubert et al., 2004).

NAC

NAC is a low molecular weight thiol-containing molecule that is readily taken up by the cells (Kelly, 1998). It directly inhibits reactive electrophiles and ROS and can enhance the synthesis of glutathione (GSH) as a precursor of cysteine (De Flora et al., 2001). NAC has been used in the clinical practice more than 40 years and has found wide applications (Decramer et al., 2005; Kelly, 1998; Van Schooten et al., 2002). NAC has been used for the treatment of respiratory diseases as a mucolytic agent (Webb, 1962), for acetaminophen overdose, where it rescues from GSH depletion in the liver (Prescott et al., 1977), and is available as an over-the-counter dietary supplement. NAC is most frequently taken orally

and thus, we examined the effect of NAC on *Atm* deficient mice by the oral route (Reliene et al., 2004b; Reliene et al., 2006). We gave NAC supplemented drinking water to *Atm* deficient mice from fertilization throughout their life. In this treatment scenario, *Atm*^{+/-} mice were crossed with each other and dams were given NAC-containing drinking water throughout pregnancy and lactation. After weaning (at about 3 weeks of age) animals continued to receive NAC in their drinking water. The major reason to start antioxidant administration as early as from fertilization was to protect against genome rearrangements that can occur during mouse development and lead to carcinogenesis later in life.

Effect of NAC on cancer prevention

We found that NAC intake significantly increased the lifespan and reduced both the incidence and multiplicity of lymphoma in *Atm* deficient mice (Reliene et al., 2006). The mean survival of NAC treated mice was 68 weeks, while that of untreated mice was only 50 weeks ($p = 0.03$). We completed gross necropsy and histopathological examination to determine a possible cause of death. Consistent with previous studies, the most frequent tumor in *Atm* deficient mice was lymphoma (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). Remarkably, the incidence of lymphoma in NAC treated mice decreased by two-fold (37.5 versus 76.5 %, $p = 0.02$). We examined the lymphoma tissue distribution and found tumors in various organs in both NAC treated and control mice (Fig 1.). However, in NAC treated *Atm* deficient mice, the multiplicity of tumors decreased from 4.6 to 2.8 tumors per mouse ($p = 0.038$). Lymphoma burden was similar in the thymus, spleen and liver, while in other organs, such as lymph nodes, lung, heart, kidney, pancreas, stomach, duodenum and adrenal glands there were fewer or no tumors in the NAC treatment group (Fig. 1).

Subsequently the finding that NAC extends the reduced lifespan of *Atm* deficient mice was reproduced by another group of researchers (Ito et al., 2007). NAC was administered in drinking water and treatment was started from birth. In this study, the mean survival was approximately 20 weeks and 43 weeks in untreated *Atm* deficient mice and NAC treatment group, respectively.

In summary, NAC treatment prolonged the survival of *Atm* deficient mice by 36% in our study and by 2-fold in the subsequent study by Ito et al. (Ito et al., 2007). Although it is highly speculative to extrapolate such a prolongation of life in AT patients, NAC therapy from early age may result in the median survival of about 27 or 40 years, respectively, instead of 20 years (Crawford et al., 2006). This estimate, however, takes into consideration tumorigenesis as a major cause of death as it is in mice. It has been estimated that cancer and complications of lung diseases are two major causes of death in AT patients contributing to death to a similar extent (Crawford et al., 2006).

Possible mechanism of lymphoma prevention by NAC

Several studies examined the molecular action mechanism of NAC in cancer prevention in *Atm* deficient mice. NAC reduced abnormally high DNA synthesis and ROS levels in lymphocytes from *Atm* deficient mice (Ito et al., 2007; Yan et al., 2001). ROS causes oxidative DNA damage, while upregulated DNA synthesis results in a lack of time required for repair of damaged DNA template before it is used for replication. Oxidative DNA damage is often translated into irreversible genome rearrangements during replication (Kuzminov, 2001). We found that NAC suppressed both oxidative DNA damage and DNA deletions in *Atm* deficient mice supporting the interpretation that DNA deletions may be a consequence of abnormal DNA synthesis and oxidative damage (Fig. 2)(Reliene et al., 2004b). These studies showed that NAC may reduce cancer incidence by reducing oxidative stress and genomic instability.

Other studies demonstrated that NAC reduces the number of aberrant V(D)J rearrangements between T cell receptor (TCR) β and γ genes, which can cause lymphoma (Ito et al., 2007; Lista et al., 1997). In fact, tumors in *Atm* deficient mice exhibit abnormal TCR rearrangements suggesting that development of lymphoma may be driven by aberrant V(D)J recombination (Liyanage et al., 2000). NAC reduced the number of defective rearrangements and restored the decreased T cell numbers, which probably accounted for reduced lymphomagenesis (Ito et al., 2007).

ROS have also been proposed to be involved in tumor metastasis, a process that includes epithelial-mesenchymal transition, migration, invasion of the tumor cells and angiogenesis (Nishikawa et al., 2006; Wu, 2006). ROS can oxidize the critical target molecules and thereby play a role in the transcription and expression of genes implicated in tumor progression. NAC can counteract some effects of ROS in tumor progression. NAC has been reported to limit invasion of human bladder cancer cells by inhibiting both the production and activity of matrix metalloproteinase -9 involved in cancer invasion and metastasis (Kawakami et al., 2001). NAC inhibits vascular endothelial growth factor (VEGF) production and growth of angiogenesis-driven Kaposi's sarcoma in nude mice (Albini et al., 2001), promotes anti-angiogenic factor angiostatin production and results in endothelial apoptosis and vascular collapse in an experimental breast cancer assay (Agarwal et al., 2004). We found that NAC reduced the multiplicity of lymphoma in *Atm* deficient mice, which may be explained by NAC's anti-invasive and anti-angiogenic properties (Reliene et al., 2006). The effect was most pronounced in nonlymphoid organs supporting the observation of other studies that NAC exhibits an anti-metastatic effect.

The studies reviewed in this article show that NAC significantly reduces lymphomagenesis in *Atm* deficient mice but these positive effects by no means suggest that NAC administration can replace the missing *Atm* protein. It has been recently reported that ATM prevents cancer progression through detection and response to oncogene-induced DNA replication stress and DNA damage (Bartkova et al., 2005). In this report, NAC had only a marginal effect suggesting that DNA damage caused by hyperproliferative oncogenic stimuli cannot be suppressed by antioxidants. Similarly in our studies the cancer frequency and multiplicity were significantly reduced but still much elevated above wildtype levels.

Effect of NAC on motor function

Unlike AT patients, *Atm* deficient mice do not develop cerebellar degeneration or show dramatic defects in their motor function (Barlow et al., 1996; Borghesani et al., 2000; Kuljis et al., 1997). We tested the effect of oral NAC administration on gait as well as several other motor tests, including, challenging beam traversal and spontaneous activity, which are sensitive to sensorimotor deficits in animal models of basal ganglia dysfunction (Fleming et al., 2004; Fleming et al., 2006; Hwang et al., 2005), to determine whether these could detect additional deficits in the *Atm*^{-/-} mice. In our study, *Atm* deficient mice displayed mild motor impairments consistent with other reports (Table 1)(Barlow et al., 1996; Browne et al., 2004). The gait analysis showed that in *Atm* deficient mice, the stride length and width was shorter by about 10% ($p < 0.05$) compared with controls, confirming the original characterization of these animals (Barlow et al., 1996). The spontaneous activity test showed that grooming in *Atm* deficient mice was significantly reduced, perhaps as a result of a general increase in spontaneous activity as indicated by increased forelimb and hindlimb steps and a tendency to increased rearing. The challenging beam traversal test consisted of a tapered beam with a support platform and ledges and topped with an overlying grid. *Atm* deficient mice made significantly more steps while traversing the beam than control mice. NAC treatment had a positive effect on some aspects of motor function. Spontaneous activity overall returned to control levels with rears and hindlimb steps being significantly

decreased compared to untreated *Atm*^{-/-} mice. NAC treated *Atm* deficient mice made significantly fewer steps on the beam compared to untreated *Atm* deficient mice ($p < 0.01$) and the number of errors were reduced to control level. However, increases in gait length and width were not improved by NAC treatment. Overall, NAC showed a beneficial effect on some but not all motor deficits in the *Atm*^{-/-} mice.

EUK-189

EUK-189, a salen-manganese compound with catalase and superoxide dismutase activities, has been previously shown to be neuroprotective in animal models characterized by oxidative damage (Doctrow et al., 2002; Melov et al., 2001). *Atm* deficient mice were treated with EUK-189 from 40 days of age via an osmotic pump implanted subcutaneously. The EUK-189 treatment improved performance on a rotarod and showed a trend towards prolonged life span ($p = 0.08$) (Browne et al., 2004). When the study was terminated at 5 months, 31% vehicle-treated and 56% EUK-189-treated animals were still alive (Browne et al., 2004).

Tempol

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable nitroxide free radical and superoxide dismutase mimetic (Damiani et al., 2000; Hahn et al., 1994; Mitchell et al., 1990). Tempol detoxifies oxygen metabolites, oxidizes redox-active trace metal ions, reduces quinone radicals and, in biological systems, is itself reduced by GSH and ascorbic acid (Branca et al., 1988; Krishna et al., 1992; Krishna et al., 1996; May et al., 2005). Tempol mixed in a mouse chow was chronically administered to *Atm* deficient mice either from fertilization or from weaning (Schubert et al., 2004). Tempol had no effect, when the treatment was started from fertilization. The intake of tempol food significantly increased the life span (mean survival 62 versus 30 weeks) in the second treatment scenario. Tempol reduced ROS levels, protein oxidation and restored mitochondrial membrane potential in thymocytes implying that chemoprevention by tempol is associated with its antioxidant activity. However, tempol treatment also reduced cell number in the thymus and decreased weight gain in *Atm* deficient mice. These effects were explained by tempol anti-proliferative activity and unknown effects, respectively. A possibility of caloric restriction was ruled out, mainly because no decrease in the intake of food containing tempol was observed.

CTMIO

Like tempol, CTMIO belongs to a class of stable nitroxide free radicals (Damiani et al., 2000; Hahn et al., 1994; Mitchell et al., 1990). The effect of CTMIO intake through drinking water was recently examined in *Atm* deficient mice (Gueven et al., 2006). The treatment was started immediately after weaning. CTMIO prolonged the survival of *Atm* deficient mice resulting in the median survival of 54 weeks versus 16 weeks. CTMIO chemoprevention mechanism does not appear to involve apoptosis, as tumors from CTMIO treated mice did not show higher levels of apoptosis compared to tumors from untreated *Atm* deficient mice. However, it was not shown whether CTMIO induced apoptosis in mice without tumors. This study also examined the effect of CTMIO on neuromotor performance by employing a multiparameter test based on performance on a narrow beam in which a cumulative score of performance using all individual parameters from 10 independent experiments was calculated (Gueven et al., 2006). Cumulative scores were subjected to several models of statistical analysis (univariate-linear, univariate-linear-mixed, and multi-linear-mixed). CTMIO treatment significantly improved overall motor performance and reduced oxidative damage to proteins in Purkinje cells (determined by 3-nitrotyrosination of protein) suggesting that CTMIO corrects neurobehavioral phenotype by its antioxidative activity.

Summary

The effect of NAC, EUK-189, tempol and CTMIO was studied in *Atm* deficient mice to understand whether antioxidant therapy has a potential in the management of AT. All the described compounds had some beneficial effects, particularly, in extending the life span and reducing lymphomagenesis. Of the tested antioxidants, only NAC has a long history of safety and efficacy in the clinical settings. Therefore, NAC has a strong potential to emerge as a dietary supplement against high risk of cancer in AT and possibly other oxidative stress linked disorders. At present there is an ongoing clinical trial in pediatric AT patients, where a cocktail of antioxidants including NAC, is employed (personal communication with Dr. G. Berry, Thomas Jefferson University Medical College, also see <http://www.treat-at.org>). The trial is being conducted in Philadelphia, PA, and is a result of the combined efforts of the national organization to Treat-AT, Dr. Gerald Berry and his colleagues at DuPont Children's and Children's Hospital of Philadelphia, and SHS International Ltd (Liverpool, UK). The aim of this study is to determine whether lymphocytes from AT patients show abnormal levels of ROS and increased apoptosis and whether chronic broad antioxidant therapy retards development of lymphocyte and cerebellar dysfunction or arrest destruction of these tissues.

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Abbreviations

AT	ataxia telangiectasia
Atm	ataxia telangiectasia mutated
CTMIO	5-carboxy-1,1,3,3-tetramethylisindolin-2-yloxyl
DSBs	double-strand breaks
HPLC	high performance liquid chromatography
GSH	glutathione
NAC	N-acetyl-L-cysteine
<i>p</i>	<i>pink-eyed dilution</i> gene

p^{un}	<i>pink-eyed unstable</i> locus
ROS	reactive oxygen species
TCR	T cell receptor

Fig. 1. Lymphoma tissue distribution in untreated and NAC treated *Atm* deficient mice
Only mice that had lymphoma are included in the calculation. Black bars depict untreated mice, gray bars show NAC treated mice. Lymph nodes affected were mesenteric and/or peripheral, thoracic and perirenal. Lymphoma in the heart was seen in epicardium and/or pericardium. Taken from (Reliene et al., 2007).

Fig. 2. The correlation between oxidative DNA damage and the frequency of DNA deletions
Oxidative DNA damage was determined as the number of oxidized guanine residues per 10⁶ guanine residues (8-OHdG/10⁶ dG) using HPLC. The frequency of DNA deletions was determined as the number of eye-spots in the retinal pigment epithelium (RPE) of the eye. The eye-spots are derived from 70 kb DNA deletions at the *pink-eyed unstable* (p^{un}) locus of the *pink-eyed dilution* (p) gene, which result in black pigment accumulation in the affected cells (Reliene et al., 2004a). Data for untreated mice are shown by a black triangle; results for NAC treated mice are shown by a gray rectangle. Taken from (Reliene et al., 2007).

Table 1
The effect of oral NAC treatment on motor function in *Atm* deficient mice

Behavioral Test		Control	Untreated <i>Atm</i> -/-	NAC treated <i>Atm</i> -/-
Gait Analysis	Length (cm)	6.61 ± 0.12	6.13 ± 0.13*	5.93 ± 0.16**
	Width (cm)	4.21 ± 0.09	3.81 ± 0.11*	3.78 ± 0.11**
	Maximum Difference	2.98 ± 0.19	3.51 ± 0.41	2.94 ± 0.31
Spontaneous Activity	Rears	21.88 ± 1.51	24.47 ± 2.50	16.87 ± 2.26 ^Δ
	Forelimb Steps	140.53 ± 4.68	168.12 ± 14.36*	152.47 ± 7.20
	Hindlimb Steps	83.58 ± 5.09	111.00 ± 10.40**	82.47 ± 8.55 ^Δ
	Grooming (sec)	12.40 ± 1.06	8.88 ± 0.94*	10.00 ± 0.76
Challenging Beam	Steps	20.56 ± 0.37	22.94 ± 0.47**	20.63 ± 0.28 ^{ΔΔ}
	Time (sec)	18.24 ± 1.06	22.88 ± 1.72*	23.15 ± 1.41*
	Errors	0.11 ± 0.01	0.14 ± 0.02	0.11 ± 0.01

Untreated *Atm* deficient (n=17) and wildtype mice (n=32) and NAC treated *Atm* deficient (n=15) and wildtype (n=8) mice at 2-6 months of age were tested on classical gait analysis, spontaneous activity measured in a clear cylinder, and a challenging beam traversal test that consisted of a tapered beam with a support platform and ledges and topped with an overlying grid {Fleming, 2004 #108} {Fleming, 2006 #109} {Hwang, 2005 #110}. Treatment group mice received 40 mM NAC in their drinking water from conception throughout life. Male and female mice did not differ (Fisher's LSD, $p > 0.05$) in any of the behavioral tests and were pooled for statistical analysis. Similarly, untreated and NAC treated wildtype mice did not differ behaviorally ($p > 0.05$) and were pooled (referred to as "control"). *, ** indicates significant differences from control mice ($p < 0.05$, $p < 0.01$, respectively). ^Δ, ^{ΔΔ} indicates significant differences from untreated *Atm* deficient mice ($p < 0.05$, $p < 0.01$, respectively).