Dietary N-acetyl-L-cysteine modulates benzo[a]pyrene-induced skin tumors in cancer-prone p53 haploinsufficient Tg.AC (v-Ha-ras) mice

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Epidemiologic studies support the protective role of dietary antioxidants in preventing cancer. However, emerging evidence from clinical trials and laboratory data suggest that in some cases individual antioxidant supplements may actually exacerbate carcinogenesis. Our goal was to explore these paradoxical activities in a rodent model that possesses genotypic characteristics of human cancers. We selected the p53 haploinsufficient Tg.AC (v-Ha-ras) mouse as a model, because it contains an activated, carcinogen-inducible ras oncogene and an inactivated p53 tumor suppressor gene, which are frequent genetic alterations in human cancers. These mice develop chemically induced benign and malignant skin tumors rapidly which can easily be quantified. Mice were fed basal diets with or without 3% N-acetyl-L-cysteine (NAC), a well-recognized antioxidant, prior to, during and after topical application of the carcinogen benzo[a]pyrene (64 µg/mouse) applied twice per week for 7 weeks. Tumor incidence exceeded 90% for both groups, and NAC did not reduce tumor latency. Mice fed NAC displayed a 43% reduction (P < 0.05) in tumor multiplicity and delayed the appearance of lesions (P < 0.05). Dietary NAC also significantly improved group survival by 5 weeks. Total tumor yields were reduced in both dietary groups but malignant spindle cell tumors (SCT) increased by 25% in NAC-fed mice. The v-Ha-ras oncogene and p53 protein products were clearly co-expressed in both benign and malignant lesions from both dietary groups. In summary, dietary supplementation with NAC was chemopreventive, but the marginal increase in SCT suggests a paradoxical effect.

Introduction

The results of previous studies have shown that a number of antioxidants may exhibit both anti-carcinogenic and carcinogenic effects and may exacerbate carcinogenesis with enhanced malignant conversion (1–3). In two large intervention studies, high-risk subjects including smokers and/or asbestos workers supplemented with beta carotene displayed significant, elevated incidences of lung cancer and mortality when compared with subjects on placebo (2). In a study of breast cancer, the larger more aggressive and invasive cancers were associated with an elevated plasma concentration of vitamin E and a lower concentration of free radical by-products (4). Moreover, stromal cells in a tumor microenvironment oxidize ascorbic acid, a key water-soluble antioxidant, to a transportable form for accumulation by leukemia cells, prostate tumors and breast cancer cells (5). Thus, supplemental antioxidants may exacerbate carcinogenesis.

One proposed mechanism of action for antioxidants is modulation of apoptosis. Transgenic mice harboring a mutant T antigen gene were fed diets replete with antioxidants and demonstrated larger brain tumors than those receiving antioxidant-deficient diets (6). Apoptosis, mediated in part by reactive oxygen species, clearly declined from 19.5% in mice fed antioxidant-depleted diets to 3.4% in antioxidant-depleted mice. Moreover, mice receiving antioxidant-replete diets exhibited larger tumors and enhanced total tumor burden. In an in vitro study, LPS-induced apoptosis in splenocytes from p53 haploinsufficient Tg.AC mice displayed significant decreases in apoptosis when supplemented with NAC. Moreover, mitogenesis was concurrently increased (7). The reasons for these paradoxical effects of individual antioxidants on the increased appearance of malignancies are unknown.

Free radical production and subsequent oxidative stress play a role in tumor initiation, promotion and progression (8). Numerous free radical generators have been demonstrated to act as tumor promoters in models of mouse skin carcinogenesis. In fact in two studies, phenolic antioxidants and vitamin E enhanced tumor promotion and elevated oxidative stress in DMBA-treated, TPA-promoted mice when applied topically (9,10). Compounds capable of inducing oxidative stress have clearly been shown to enhance malignant conversion of benign papillomas to carcinomas (11). However, free radical generation is a normal part of oxidative metabolism, signal transduction and apoptosis, all of which may be altered by an excess of individual antioxidants supplemented in the diet. Thus, supplemental antioxidants may quench free radicals necessary for signaling apoptosis in pre-neoplastic cells.

We hypothesized that dietary administration of NAC would modulate either positively or negatively malignant progression in a cancer-prone mouse model. NAC has been used clinically for decades and its role as both an antioxidant and promising chemopreventive agent is extensively documented (12–14). To address the above hypothesis, we explored the impact of antioxidant supplementation on the number and phenotype of carcinogen-induced skin tumors in a transgenic mouse model. We fed p53 haploinsufficient Tg.AC mice basal diet alone or diet containing NAC (3%) while topically applying the complete carcinogen benzo[a]pyrene (B[a]P) to produce palpable and visible skin lesions that could be easily quantified. Dosing was discontinued when 50% of mice within a group developed lesions in order to monitor tumor progression and malignant conversion. We used a complete dosing protocol in this novel bitransgenic model to enhance induction of neoplasia, which would rapidly progress to malignancy (15,16).

The mouse skin model of multistage carcinogenesis is a powerful model in which to study genetic alterations in chemically induced malignant transformation (17–19). We...
selected the p53 haploinsufficient Tg.AC (v-Ha-ras) mouse as a model because the mutation of the p53 tumor suppressor gene and ras oncogene occur frequently in human cancers (20–22). Tg.AC mice carrying the v-Ha-ras oncogene are considered to be pre-initiated, but require the presence of a carcinogen or tumor promoter for induction of tumor formation (22,23). The p53 haploinsufficiency accelerates tumor progression to malignancies by increasing genetic instability (24,25). Thus, this ‘bitransgenic’ cancer model permits the observance of all stages of carcinogenesis within the context of a single experiment and allows increased or decreased modulation of malignant progression.

Materials and methods

**Animal production and care**

Homozygous p53 null FVB/N (N10) male mice were intercrossed with homozygous p53 wild-type inbred FVB/N transgenic female Tg.AC mice hemizygous for the transgene (ζ-globin promoted v-Ha-ras) to produce heterozygous p53-deficient FVB/N progeny hemizygous for the Tg.AC transgene under an NIEHS contract at Taconic Farms (Germanton, NY). The FVB/N hemizygous Tg.AC dams were genotyped from tail biopsy DNA for both presence and integrity of the Tg.AC transgene (26) and phenotyped for papilloma induction (27). Only dams with appropriate genotype and phenotype were used for the intercross with the p53 homozygous null male mice. All first filial (F1) generation progeny were genotyped for the presence and integrity of the Tg.AC transgene. Only F1 mice hemizygous for an intact transgene and heterozygous for the p53 null allele were used in this study.

Male mice were obtained at 10 weeks of age and quarantined for 2 weeks at the AALAC-accredited NIEHS facility prior to commencement of experiments. Mice were singly housed in temperature-controlled rooms and regulated with a 12 h day/night schedule. Mice (n = 13 for each of two dietary groups) were given tap water ad libitum and placed on either basal semi-purified diet containing 20% soy protein or basal diet plus 3% NAC (Research Diets, New Brunswick, NJ) for 2 weeks prior to dosing with BP (CAS 50-32-8; Aldrich, Milwaukee, WI) and continued throughout the 18-week study.

NAC (Sigma, St Louis, MO) was incorporated into pelleted diets by the supplier (Research Diets, New Brunswick, NJ) prior to study initiation. To prevent degradation, diets containing NAC were stored at 4 °C in a tightly closed container in the dark and added as needed at 2-week intervals. All procedures and studies were reviewed and approved by the NIEHS Animal Care and Use Committee.

**Tumor induction**

The interscapular skin of each mouse was depilated one day prior to application of BP and once weekly afterwards until cessation of dosing. Mice were dosed topically twice weekly with BP delivered by pipet in 100 μl acetone to deliver 64 μg/mouse/dose for 7 weeks. The BP/acetone solution was prepared immediately prior to treatment in amber vials from a stock solution kept at −70°C, and the concentration verified by UV spectroscopy. Dosing was discontinued when 50% of mice within a group developed lesions. At the end of 7 weeks, dosing was discontinued and animals were observed weekly for the succeeding 9 weeks for the appearance of neoplasia. The study was terminated 16 weeks after the initiation of dosing at which time animals were killed by CO₂ narcosis.

**Tumor multiplicity, incidence and kinetics of growth**

The number of animals presenting with tumors and number of lesions per animal were recorded weekly. Criteria for removal of animals from the study were presence of tumors exceeding 1 cm in diameter or body weight loss ≥20% of the animal’s maximal weight. Mice were weighed weekly and food intake determined to monitor the onset of morbidity and development of any toxicity effects. All grossly observable lesions were recorded, but only those ≥4 mm were collected and subsequently scored as either benign or malignant.

**Tumor scoring**

At necropsy, dorsal skin was collected and trimmed to expose tumors with a diameter ≥4 mm. Collected lesions were bisected and half was immediately transferred to 10% neutral buffered formalin (pH 7.0) for 24 h followed by transfer to 70% ethanol for 24 h. The fixed tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathological analysis and scoring. Lesions were scored as benign or malignant and characterized based on extent of hyperplasia, neoplasia and invasiveness using commonly accepted pathology criteria for scoring.

**Immunohistochemistry for detection of p53 protein expression**

Five-micron sections were cut onto SuperFrost microscope slides (Fisher Scientific, Norcross, GA) and tissues deparaffinized and rehydrated through graded ethanol to 1× automation buffer (Biomeda, Foster City, CA). Following blocking of endogenous peroxidase activity for 10 min in 3% aqueous hydrogen peroxide, tissues were subjected to antigen retrieval by heating slides in 0.01 M citrate buffer (pH 6.0) in a vegetable steamer (Black and Decker) for 30 min. Detection of p53 expression was then essentially assessed as described elsewhere (28). Non-specific proteins were blocked (1% BSA and 10% NRS, 1% milk, in automation buffer) and tissues were incubated for 1 h with anti-mouse p53 (pCM5, Vector, Burlingame, CA) or rabbit IgG (Vector) at 1:300. Signal detection was accomplished with the Super Sensitive detection kit (BioGenex Laboratories, San Ramon, CA) using diamobenzidine (DAKO, San Diego, CA) as the substrate. Tissues were counterstained with Harris Hematoxylin (Sigma).

**In situ hybridization for v-Ha-ras expression**

Five-micron serial sections of 10% neutral buffered formalin-fixed, paraffin-embedded tissues were cut onto SuperFrost plus microscope slides (Fisher) for in situ hybridization detection of v-Ha-ras transgene expression as described elsewhere (23,29). 35S-labeled sense and antisense riboprobes were prepared from the SV40 region of the transgene construct.

**Statistical analysis**

Differences in tumor endpoints were assessed using generalized linear regression models and MIXED modeling strategies for longitudinal data employing IML and GENMOD software (SAS, Research Triangle Park, NC). Differences in regression and malignant subgroupings were assessed by Fisher’s exact test. Differences in the incidences of spindle cell tumors between the two dietary groups were tested by chi-square analysis. Survival differences were determined by life table analysis. Differences in maximum tumor yield between the two dietary groups were analyzed by ANOVA.

**Results**

**Tumor multiplicity, incidence and growth**

Given the dosing regimen and the invasive nature of some large lesions, we closely monitored food intake and body weight of all animals to determine morbidity and/or toxicity from diet or treatment. There were no differences between the two dietary groups when food intake and body weight were compared throughout the dosing and post-dosing periods (Figure 1A and B) demonstrating that neither NAC nor BP had an adverse impact on the general health of the animals.

Tumor incidence defined as the percentage of animals displaying at least one lesion increased linearly from 5 weeks for both basal and basal + NAC groups peaking at 10 weeks with maximum responses of 100 and 92%, respectively (Figure 2A). There were no apparent significant differences in tumor incidence between the two treatment groups, although a slight lag in new lesion formation was evident between 7–9 weeks in the NAC-fed animals. The endpoint where 50% of mice within a group presented with lesions was reached simultaneously at 7 weeks for both dietary groups (Figure 2A).

Dosing with BP resulted in tumor formation as early as 6 weeks for both basal-fed animals and those receiving basal + NAC (Figure 2B). Animals receiving basal diet alone, demonstrated increased tumor multiplicity to a maximum of 10.4 tumors/mouse at 12 weeks where the number plateaued. There was subsequent regression of papillomas because the multiplicity declined to 8.6 tumors/mouse by the 16-week termination point, a 17% reduction. Animals fed NAC also displayed increased tumor multiplicity at 6 weeks but with a slope that was less steep and a maximum of 5.8 tumors/mouse at 15 weeks, representing a significant 43% reduction in total tumor number compared with basal controls (Figure 2B). The appearance of tumors in basal + NAC animals continued in a linear fashion throughout the test period compared with an early plateau for the basal group at 11 weeks, suggesting NAC-induced modulation of tumor growth kinetics.
Fig. 1. Body weight and food intake are unaltered by B[a]P dosing and NAC consumption in p53 haploinsufficient Tg.AC (v-Ha-ras) transgenic mice. Animals were acclimated for 2 weeks to basal diet alone or containing 3% NAC prior to dosing topically with B[a]P at 64 µg/application given twice per week for 7 weeks. (A) Animals were weighed weekly to determine onset of morbidity and/or toxicity. (B) Food intake was measured concurrently with body weight. Data are expressed as either body weights or grams of food consumed per animal per day and represent the mean ± SEM of all animals within a dietary group. There were no significant differences between the two dietary groups on either parameter. The experimental design is indicated on the x-axis.

Animals receiving basal diet displayed decreased survival commencing at 10 weeks after initiation of BP dosing. Survival continued to decrease sharply and linearly to 54% at termination of the study at 16 weeks (Figure 3). In contrast, NAC substantially improved survival, which remained at 100% until 14 weeks. At 16 weeks, survival had decreased marginally to 95%.

Histopathology of skin lesions
Lesions less than 4 mm were counted and grouped as gross lesions but not excised (Table I). Tumor multiplicity at the termination of the study, i.e. 16 weeks, was reduced from 9.3 in basal-fed animals to 4.6 tumors/mouse for those receiving NAC resulting in a 50% reduction. There were 135 total tumors in basal-fed animals compared with 85 in the basal + NAC group. Overall reduction in multiplicity was a result of the reduction in smaller tumors. Excised lesions (≥4 mm) were scored as either benign or malignant. Malignancies were identified as squamous cell carcinomas, spindle cell tumors and a single hemangiosarcoma. There were seven malignancies in the basal group versus 12 in the basal + NAC group representing 7 and 19%, respectively, of total tumor numbers.

Fig. 2. Tumor incidence and multiplicity in transgenic mice treated topically with B[a]P and fed either basal diet alone or containing NAC. (A) Animals acclimated to test diets were dosed topically with B[a]P (64 µg/application given twice per week) until half of animals within one of the two dietary groups developed palpable and/or visible lesions. This 50% dosing cessation endpoint occurred simultaneously for both dietary groups after 7 weeks of dosing as indicated in the figure. This experimental design was implemented to critically evaluate the conversion of benign lesions to malignancies in this cancer-prone p53 haploinsufficient Tg.AC model. (B) Tumor multiplicity was determined weekly and expressed as the number of tumors appearing per mouse remaining in the study at the time of tabulation. Data are expressed as tumors/per mouse and represented by the mean ± SEM. *Indicates a significant (P < 0.05) NAC-induced reduction in tumor multiplicity.

Fig. 3. Survival is improved by dietary NAC in B[a]P-dosed transgenic mice. Animals were fed either basal diet alone or containing 3% NAC and dosed with B[a]P as described above. Survival was significantly (P < 0.05) decreased in basal-fed mice compared with mice-fed NAC.
Table I. Incidences and scoring of B[a]P-induced lesions as benign or malignant in transgenic mice fed basal diet alone or containing NAC

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Lesions</th>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Gross&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Keratoacanthoma</td>
</tr>
<tr>
<td>Basal Total lesions</td>
<td>135</td>
<td>121</td>
<td>7</td>
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<tr>
<td>Animals at risk</td>
<td>13</td>
<td>13</td>
<td>7</td>
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<tr>
<td>Lesion/animal at risk</td>
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<td>9.3</td>
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<td>NAC Total lesions</td>
<td>85</td>
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<tr>
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<td>Lesion/animal at risk</td>
<td>6.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.1</td>
</tr>
</tbody>
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<sup>a</sup>Total lesions = (gross + benign + malignant).

<sup>b</sup>Gross lesions = lesions counted but not collected (<4 mm).

<sup>c</sup>Statistically significant (P < 0.05).

Malignant spindle cell tumors were noted in 25% of NAC-fed mice, but not in basal controls.

Benign tumors were limited to keratoacanthomas and the total numbers in basal and NAC-fed animals were 7 and 13, respectively, representing 7 and 21% of the total tumor yields. The number of lesions per animal at risk did not differ between the dietary groups.

**p53 protein expression and expression of the v-Ha-ras transgene**

Both p53 and ras expression may be altered in tumorigenesis. Thus, we next examined expression of the ras transgene and p53 protein in tumor tissue. Histologic examination of lesions revealed numerous focal areas of neoplasia in both benign keratoacanthomas and malignant squamous cell carcinomas as shown in Figure 4A and D. In benign keratoacanthomas, expression of p53 protein was confined to the nucleus of the basal cells (Figure 4B), whereas, p53 protein was diffusely expressed in all neoplastic cells of the squamous cell carcinomas (Figure 4E). Similarly, v-Ha-ras transgene expression was limited to the basal cells in the keratoacanthoma in a pattern that was superimposable with p53 expression from the same lesion (Figure 4B and C). Expression of the v-Ha-ras transgene was diffuse in the squamous cell carcinoma in a manner analogous to p53 expression in a serial section of the same lesion (Figure 4E and F). There were no differences in expression pattern in lesions from animals fed basal or basal plus NAC diets.

**Discussion**

This study demonstrates that dietary administration of NAC prior to, during and after carcinogen application in pre-initiated test animals exhibited beneficial effects. However, the data also suggest an adverse effect on skin lesion formation. Dietary NAC was clearly protective because it reduced tumor multiplicity, slowed tumor appearance and improved survival; however, the observation of increased spindle cell tumor formation suggests that NAC may have paradoxically exacerbated malignant conversion. This suggests that dietary supplementation with individual antioxidants by high-risk individuals that are pre-initiated, i.e. smokers, may place them at higher risk for developing malignancies than those individuals not at high-risk. The present study clearly demonstrates that oral supplementation with NAC is chemoprotective in the skin.

Overwhelmingly, most studies have focused on topical application of antioxidant mixtures to prevent radiation and carcinogen-induced papillomagenesis (30–33). For example,
topical application of glutathione to DMBA-initiated, TPA-promoted skin reduced papilloma formation and delayed malignant conversion in one murine multistage carcinogenesis model although NAC, a precursor of glutathione, did not affect malignant progression (11). In other studies, NAC reduced malignant conversion in mouse skin when applied topically prior to carcinogen exposure (11,34). Thus, topically applied antioxidants may protect against carcinogenesis.

The selection of this specific model was predicated on the presence and potential function of the products of two specific genetic lesions associated with human cancer. The expected pathologies were p53-mediated enhancement of malignant conversion and ras-mediated induction of rapidly growing lesions. We clearly demonstrate expression of both the ras transgene and p53 protein in both benign and malignant tumors as predicted although there were no significant differences between dietary groups. As multiple tandem copies of inserted v-Ha-ras are located distal (near centromere) to the wild-type p53 allele on chromosome 11, loss of p53 through aneuploidy would result in concomitant loss of mutant v-Ha-ras transgene. Thus, both p53 and ras gene products are expressed in this model and probably participate in the experimental outcome. The expression of ras was limited to areas of neoplasia as expected (29) and not expressed in surrounding tissue (35). Expression of ras in benign keratoacanthomas appeared more intense than in squamous cell carcinomas and spindle cell tumors, which is consistent with the observations of others (36,37).

Progression of squamous cell carcinomas to spindle cell tumors in mouse skin has been associated with ras mutations and imbalances of c-Ha-ras alleles on chromosome 7 (38). However, we found no evidence of c-Ha-ras mutation in B[a]P-treated mice (data not shown) consistent with results from other laboratories (29,35). Trisomy 7 has not been observed in Tg.AC mice using alternate dosing protocols in our laboratory (39). Although devoid of c-ras mutations, p53 mutations are likely as studies in mouse squamous cell carcinoma lines using complete B[a]P dosing protocols demonstrate characteristic mutations (40).

The predominance of large keratoacanthomas (>1 cm in diameter) as benign lesions is unusual and infrequent in Tg.AC mice and suggests a genotypic interaction between the cancer-prone genotype, NAC supplementation, and carcino-gen (B[a]P)-induced neoplasia. Studies by others using transgenic models with targeted expression of ras oncogene in epidermis produce progeny that developed multiple, well-differentiated keratoacanthomas (36,41). Some lesions progress rapidly to carcinomas supporting a role for ras in hyperproliferation of both benign and malignant lesions. Malignant conversion of keratoacanthomas has been reported which is consistent with the close histological similarity to squamous cell carcinomas (42,43).

Accumulation of mutant p53 protein enhances malignant conversion (21). Although not statistically significant, the enhanced incidence of spindle cell tumors, i.e. 25%, in the presence of reduced total tumor burden supports a role for NAC and nuclear p53 mutant accumulation (42). Our ongoing studies demonstrate that cyclin D1, a downstream effector that is suppressed by p53, remains upregulated in tumors and a cyclin-dependent kinase inhibitor-induced by p53, viz., p21, is not expressed. This supports the contention that p53 protein is mutated and dysfunctional (44,45).

Supplementation with dietary antioxidants in humans may protect against neoplasia, but growing evidence suggests numerous adverse effects on cancer. As a result, an important public health issue has arisen and has stimulated growing concern in the cancer research community as a result of the lack of scientific data regarding the potential adverse effects of antioxidants on cells. Clearly, supplemental NAC is chemoprotective in this transgenic model of skin carcinogenesis. However, the marginal increase in malignant, poorly differentiated spindle cell tumors suggests potential paradoxical action. Thus, future studies should be designed to specifically address this ‘double-edged sword’ action of dietary antioxidants in the context of human cancer.

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