Timing of Supplementation With the Antioxidant N-Acetyl-L-Cysteine Reduces Tumor Multiplicity in Novel, Cancer-Prone p53 Haploinsufficient Tg.AC (v-Ha-ras) Transgenic Mice but Has No Impact on Malignant Progression

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Abstract: Epidemiological studies support the protective role of dietary antioxidants in preventing cancer. However, emerging evidence suggests that antioxidant supplements may actually exacerbate carcinogenesis. We explored this paradox in a model containing two common genotypic characteristics of human cancers. We selected p53 haploinsufficient Tg.AC (v-Ha-ras) mice as a model, because it contains an activated, carcinogen-inducible ras oncogene and an inactivated p53 tumor suppressor gene. These mice develop chemically induced benign and malignant skin tumors rapidly. Mice were fed basal diet with or without 3% N-acetyl-L-cysteine (NAC) before and after topical application of the carcinogen benzo[a]pyrene (64 µg twice per week for 7 wk) until 50% of mice within a group displayed at least one lesion. Half each of mice fed the basal and the NAC-supplemented diet were then switched to the alternate diet. Mice fed the NAC-supplemented diet or switched from the NAC-supplemented to the basal diet displayed 38% and 26% reductions, respectively, in tumor multiplicity and a 15% reduction if switched from the basal to the NAC-supplemented diet. Although latency was unaffected, NAC induced a lag in tumor incidence, which exceeded 90% at 10 wk for all groups. The timing of NAC supplementation did not affect malignant progression. Thus dietary NAC was chemoprotective by slowing tumorigenesis but did not affect malignant conversion.

Introduction

A growing body of experimental evidence suggests that individuals consuming increased amounts of fruits and vegetables in the diet are protected against a variety of cancers (1,2). The protective effects have been attributed, in part, to the abundance of antioxidants as natural plant constituents, including the carotenoids, flavonoids, and tocopherols (3,4). The capacity of individual antioxidants to quench the generation of reactive oxygen species (ROS) and mitigate subsequent DNA damage associated with carcinogenesis has been documented (5,6). As a result, recent research in the area of cancer chemoprevention has focused on the potential of dietary supplements to prevent the onset or delay the progression of neoplasia (7).

Antioxidants can clearly protect against all stages of cancer, including initiation, promotion, and malignant progression (8–10). However, accumulating evidence suggests that supplementation with dietary antioxidants may have adverse effects on carcinogenesis (11–14). In two large epidemiological studies, supplementation with β-carotene significantly increased the occurrence of lung cancer and mortality (15). The subjects used in these studies were smokers and/or asbestos workers and likely represented a high-risk, pre-initiated population. In other studies, transgenic mice fed antioxidant-replete diets displayed larger brain tumors than mice fed antioxidant-depleted diets (16). In a study of breast cancer, the larger, more aggressive cancers correlated with elevated plasma vitamin E levels and lower concentrations of free radical by-products (17). The mechanism for these paradoxical effects is unknown and represents a conundrum to the nutrition and cancer research community.

One proposed mechanism for the apparent paradox is the timing of antioxidant supplementation (18). Recent discoveries indicate that low-level ROS can modulate intracellular redox status and stimulate signal transduction pathways for processes such as apoptosis (19). Apoptosis is critical for removing preneoplastic, mutated cells. Thus, although antioxidants may prevent ROS-mediated DNA damage, quenching ROS at critical junctures may interrupt cell signaling, which is needed to prevent clonal expansion of neoplastic cells. Our hypothesis is that supplementation with a well-recognized antioxidant before carcinogen exposure will be che-
moprotective by reducing total tumor burden. However, antioxidant supplementation subsequent to toxicant exposure will not be protective and may exacerbate carcinogenesis by enhancing the severity of lesions.

The development and increasing use of transgenic mice have presented a powerful biotechnological tool to investigate the timing of genetic events associated with carcinogenesis, including protooncogene activation and tumor suppressor gene inactivation (20). In this study, we selected the p53 haploinsufficient Tg.AC mouse as a model, because it contains the most prevalent mutated protooncogene and mutated tumor suppressor gene in human cancers. The p53 haploinsufficiency results from inactivation of one of the two p53 alleles, causing p53 heterozygosity and decreased production of functional p53 protein (haploinsufficiency). As a result, these mice are susceptible to enhanced malignant progression via increased genetic instability (21). Tg.AC mice carrying the viral Harvey ras (v-Ha-ras) oncogene exhibit point mutations in codons 12 and 59, facilitating rapid clonal expansion of carcinogen-induced skin lesions (22). Numerous reports have confirmed that v-Ha-ras is transcriptionally silent in normal, nontreated skin but is transcriptionally activated by carcinogens in ras-dependent papillomagenesis. Moreover, mutations to c-Ha-ras in Tg.AC are absent, further enhancing the utility of this model (23,24).

The use of this transgenic model permits modulation positively or negatively of tumorigenesis and malignant conversion within the context of a single experiment. Benzo[a]pyrene (B[a]P) is used as a complete carcinogen to corroborate the results of previous studies, because it initiates and promotes neoplasia (25,26). The experimental design will elucidate any temporal impact of antioxidant supplementation. We placed mice on a basal diet or a diet containing the well-recognized antioxidant N-acetyl-L-cysteine (NAC) before topical dosing with B[a]P (14,25). We monitored the appearance of palpable and visible lesions until at least half of the mice within a group displayed lesions. At this point, we discontinued dosing to all animals, transferred half of the mice fed the basal diet and half of the mice fed the NAC-supplemented diet to the alternate diet, and monitored the animals for an equivalent time during the postcrossover period (Fig. 1).

Materials and Methods

Animal Production and Care

Only first familial (F1) mice hemizygous for an intact transgene and heterozygous for the p53-null allele were used in this study. Mice were produced under a National Institute of Environmental Health Sciences contract at Taconic Farms (Germanton, NY), as described elsewhere (26). Briefly, FVB/N hemizygous Tg.AC dams were produced by intercrossing homozygous p53-null FVB/N (N10) male mice with homozygous p53 wild-type inbred FVB/N female Tg.AC mice hemizygous for the transgene (ζ-globin-pro-
recorded, but only >4-mm-diameter lesions were collected and subsequently scored as benign or malignant. Criteria for removal of animals from the study were presence of >1-cm-diameter tumors or body weight loss >20% of the animal’s maximal weight.

**Tumor Scoring**

At necropsy, >4-mm-diameter lesions were trimmed and collected from dorsal skin. Lesions were immediately transferred to 10% neutral buffered formalin (pH 7.0) overnight and then transferred to 70% ethanol for an equivalent interval. The fixed tissue was embedded in paraffin, sectioned, and stained for histopathological analysis and scoring. Lesions were scored as benign or malignant using commonly accepted pathology criteria (26).

**Statistical Analysis**

Differences in tumor end points 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 maximum tumor yield between the two dietary groups were analyzed by analysis of variance. Differences in the number of malignancies between groups were assessed by $\chi^2$ analysis and the Fisher’s exact test. Survival differences were determined by life table analysis.

**Results**

Mice were supplemented with high-dose NAC and exposed to a systemic, complete carcinogen; thus we monitored body weight (Fig. 2A) and food intake (Fig. 2B) for the

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**Figure 2.** Body weight and food intake of p53 haploinsufficient Tg.AC (v-Ha-ras) transgenic mice fed basal diet alone or basal diet containing NAC and dosed with B[a]P. Animals were acclimated for 2 wk to basal diet alone or basal diet containing 3% NAC before topical administration of 64 µg of B[a]P twice per week for 7 wk. A: animals were weighed weekly to determine onset of morbidity or toxicity. B: food intake was measured concurrently with body weight. Values are means ± SE of all animals within a dietary group. There were no significant differences between dietary groups for either parameter.
appearance of morbidity and toxicity. There were no significant differences in body weight or food intake in mice from any of the four groups (Fig. 2).

Mice were topically dosed with B[a]P until 50% of the animals in a group developed at least one palpable or visible lesion. Lesions began forming at Week 6 and increased sharply for mice fed the basal diet to >90% at Week 10, when the number of lesions plateaued (Fig. 3A). Tumor number in mice initially receiving NAC also began to increase sharply, but cessation of dosing revealed a delayed appearance of lesions by ~2 wk, between Weeks 6 and 8. By Week 12, the numbers had plateaued and were not different from those in mice fed the basal diet.

Similar to the incidence results, mice fed the basal diet demonstrated increased tumor multiplicity in a sharp linear fashion to a maximum of 8.5 tumors per mouse at the end of the study (Fig. 3B). Mice fed the NAC-supplemented diet displayed increased tumor multiplicity beginning at Week 6 but with a less steep slope. Moreover, removal of carcinogen revealed a delay of 3 wk in the appearance of additional lesions (Fig. 3B). In mice fed the NAC-supplemented diet, maximum tumor multiplicity yielded 5.5 tumors per mouse at Week 14. Although not significant, there appeared to be a marginal reduction in lesions when mice were transferred from the basal to the NAC-supplemented diet. Mice switched from the NAC-supplemented to the basal diet demonstrated a slight, but nonsignificant, increase in tumor multiplicity. Differences in tumor multiplicity were significant ($P < 0.05$) between mice fed only the basal or the NAC-supplemented diet throughout the study.

**Figure 3.** Tumor incidence and multiplicity in transgenic mice treated topically with B[a]P and fed basal diet or NAC-supplemented diet. A: animals acclimated to test diets were dosed topically with B[a]P (64 µg applied twice per week) until half of animals within 1 of 2 dietary groups developed palpable and/or visible lesions. At this crossover point, dosing was discontinued for all animals, and half of mice fed basal diet and half of mice fed NAC-supplemented diet were switched to alternate diet. B: tumor multiplicity was determined weekly and expressed as number of tumors appearing per mouse remaining in study at time of tabulation. Values are means ± SE. *, Significant ($P < 0.05$) NAC-induced reduction in tumor multiplicity.
Survival was decreased in mice fed the NAC-supplemented diet at any time during the study compared with control mice fed the basal diet, although the decreases were not statistically significant (Fig. 4). Mice fed the basal diet throughout the study survived 4 wk longer than mice fed the NAC-supplemented diet. In the precrossover period (before Week 7), 21% of the mice fed the NAC-supplemented diet died compared with 5% of mice fed the basal diet ($P = 0.08$). In the postcrossover period (after Week 7), survival was not significantly decreased ($P = 0.23$) when all groups were compared.

Lesions from mice fed only the basal or the NAC-supplemented diet throughout the study were counted and scored as benign or malignant to determine the impact of supplementation on malignant progression. Dietary NAC significantly reduced the number of gross lesions from 7.0 to 3.8 lesions per animal at risk and included all visible and/or palpable lesions (Table 1). A subset of larger (>4-mm-diameter) tumors was collected and scored as benign or malignant. The number of benign keratoacanthomas was marginally reduced from 1.2 to 1.0 per mouse. Malignant squamous cell carcinomas were also marginally reduced by 28% from 1.8 to 1.3 lesions per mouse. No malignant spindle cell tumors or hemangiosarcomas were found in mice fed the NAC-supplemented diet compared with 0.2 lesions per animal at risk for mice fed the basal diet.

**Discussion**

The present work is predicated on previous results in NAC-fed male p53 haploinsufficient Tg.AC (v-Ha-ras) mice, suggesting paradoxical activity on carcinogenesis. NAC was chemoprotective, because it reduced tumor multiplicity and improved survival but increased marginally the

| Table 1. Lesion Spectrum in Female p53 Haploinsufficient Tg.AC (v-Ha-ras) Mice Fed Basal or NAC-Supplemented Diet$^{a,b}$ |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Dietary Group** | **Lesions** | **Benign** | **Malignant** | **Squamous cell carcinoma** | **Hemangiosarcoma** | **Spindle cell tumor** |
| | Total$^c$ | Gross$^d$ | (Keratoacanthoma) | | |
| Basal | 111 | 91 | 7 | 11 | 1 | 1 |
| Animals at risk | 13 | 13 | 6 | 6 | 6 | 6 |
| Lesions/animal at risk | 8.5 | 7.0 | 1.2 | 1.8 | 0.2 | 0.2 |
| NAC | 60 | 42 | 8 | 10 | 0 | 0 |
| Animals at risk | 11 | 11 | 8 | 8 | 8 | 8 |
| Lesions/animal at risk | 5.5* | 3.5* | 1.0 | 1.3 | 0.0 | 0.0 |

*a: Statistical significance is as follows: *, significantly different from basal ($P < 0.05$).

b: NAC, N-acetyl-L-cysteine.

c: Total lesions = gross + benign + malignant.

d: Gross lesions represent lesions (<4 mm) counted but not collected or scored.
appearance of malignant spindle cell tumors (26). Thus we designed the present study to specifically observe 1) modulation positively or negatively of all three major stages of carcinogenesis and 2) the impact of timing of antioxidant provision on skin neoplasia in a novel, cancer-prone transgenic model. Our results suggest that supplemental NAC effectively modulates tumor promotion when fed before B[a]P exposure but when fed subsequent to B[a]P exposure neither attenuates nor exacerbates malignant conversion.

Supplemental NAC fed before B[a]P dosing delayed tumor incidence at the crossover point. Clearly, all four dietary groups began to rapidly display lesions as early as Week 6. However, discontinuing carcinogen application reduced the rate of appearance of subsequent new lesions in mice fed the NAC-supplemented diet, but not in identically treated control mice. As a result, animals fed the NAC-supplemented diet reached the 50% crossover point 10 days later than those fed the basal diet. Mice switched from the two initial diets to the alternate diets at the crossover point displayed responses identical to those of mice receiving the respective initial diets alone. Tumor latency was not affected by any treatment or diet. These data suggest that once carcinogen exposure was discontinued, NAC, when given early, protected the skin against subsequent new lesion formation.

In tumor-bearing mice, NAC reduced the appearance of new lesions by 4 wk, between Weeks 6 and 9. Provision of NAC at the beginning of the study reduced the maximum number of lesions from 8.5 to 5.4 lesions per mouse. Similar to the tumor incidence data, tumor multiplicity increased steeply initially, but cessation of dosing at Week 7 slowed the appearance of additional lesions. In contrast, mice switched to alternate diets displayed intermediate levels of tumor multiplicity, suggesting that NAC is more effective when provided early, although it is marginally effective when provided late. Collectively, the data suggest that NAC is modulating tumor promotion.

Because there were no significant differences between the crossover groups and their respective controls, we examined tumor quality in lesions from mice fed the basal or the NAC-supplemented diet throughout the study. When considered independently, there were no significant differences in the incidences of malignant lesions. Collectively, cumulative malignancies did not differ between mice fed the basal diet (12%) and those fed the NAC-supplemented diet (17%). Previous studies with male bitransgenic mice revealed decreased overall tumor number but suggested a 25% increase in malignant spindle cell tumors (26). NAC did, however, appear to marginally, but not significantly, reduce benign and malignant lesions, which is consistent with results from other laboratories (29,30). For example, although tumor numbers were relatively low, the number of benign keratoacanthomas and malignant squamous cell carcinomas was reduced by 20% and 33%, respectively. There were no malignant hemangiosarcomas or spindle cell tumors in mice fed the NAC-supplemented diet. Other studies have shown that NAC can reduce tumor weight, decrease tumor latency, and inhibit tumor invasiveness in a dose-dependent manner (31). The slight reductions are consistent with a protective effect against malignant progression and may be biologically relevant.

Supplemental NAC was chemoprotective but paradoxically appeared to increase mortality regardless of time given. Survival decreased during the precrossover (\(P = 0.08\)) and postcrossover periods (\(P = 0.23\)), although the differences were not statistically significant. In other studies, toxicity from dietary NAC was not observed at this supplemental level in male bitransgenic mice, suggesting a sex-specific effect (32). Supplementation significantly improved survival in male mice over an identical time interval. In another study, however, high-dose NAC given intravenously has been shown to increase mortality in lipopolysaccharide-dosed rats, but low-dose NAC was protective (33).

Other laboratories have demonstrated that rodents can tolerate dietary NAC at 10 g/kg body wt (14). Our estimates indicate that mice consumed 4 g/kg body wt. Survival declined in mice fed the NAC-supplemented diet, although food intake and body weight were identical for all groups. Supplemental NAC appeared to be marginally toxic in female mice when consumed concomitantly with B[a]P exposure. Cessation of carcinogen dosing improved survival during the postcrossover period compared with the precrossover period. Thus it appears that supplemental NAC contributes to more than one physiological outcome.

B[a]P and NAC may increase p53 expression during different protective responses (34,35). B[a]P damages DNA and subsequently induces p53 gene expression through transcription factor activation (35,36). NAC quenches ROS and prooxidants such as B[a]P directly and modulates ROS-dependent cellular redox status, which induces p53 expression (37,38). However, NAC also functions in a p53-independent manner by modulating phosphorylation of retinoblastoma protein, stimulating downstream effectors (p16\(^{ink4a}\) and p21\(^{waf}\)), or altering glutathione response profiles of cells under oxidative stress (34,39,40). Thus NAC and B[a]P may function via p53-dependent and/or -independent mechanisms.

ROS can stimulate ras expression and be produced as downstream mediators of ras pathways, establishing a potential positive-feedback loop. Antioxidants, such as NAC, can quench normal ROS-dependent cell signaling and alter gene expression of proliferative, i.e., ras, and/or apoptotic pathways. Thus it is likely that a complex genotypic interaction exists between the cancer-prone genotype (p53 and ras), NAC supplementation, and carcinogen (B[a]P)-induced neoplasia. Moreover, it is also likely that chemoprotection by NAC is due to more than one specific mechanism.

The paradoxical results observed in epidemiological studies suggest that supplemental antioxidants can exacerbate cancer. We have found that supplemental NAC given before or subsequent to B[a]P exposure did not affect positively or negatively malignant conversion in female cancer-prone transgenic mice. However, NAC supplementation before carcinogen exposure did significantly delay neoplasia. In mice without preexisting lesions, NAC delayed tumor appearance, but only after carcinogen exposure was discontinued.
The misperception that dietary antioxidants may be a panacea for cancer is tempered by their utility as important tools in cancer prevention (41). Clearly, “high-risk” individuals such as smokers should discontinue practices that prolong the duration of carcinogen exposure. Given the results presented here, this may also confer an additional benefit from dietary supplementation by delaying further proliferation of lesions. However, future studies are needed to elucidate the “double-edge sword” effects of other supplemental antioxidants, particularly because supplementation is a routine and unregulated practice.

Acknowledgments and Notes

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