

Topical and oral administration of the natural water-soluble antioxidant from spinach reduces the multiplicity of papillomas in the Tg.AC mouse model

Abraham Nyska ^{a,*}, Liat Lomnitski ^d, Judson Spalding ^b, David B. Dunson ^c,
Thomas L. Goldsworthy ^c, Shlomo Grossman ^d, Margalit Bergman ^d,
Gary Boorman ^a

^a *Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC 27709, USA*

^b *Laboratory of Environmental Carcinogenesis/Mutagenesis, National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC 27709, USA*

^c *Biostatistics Branch, National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC 27709, USA*

^d *Bar-Ilan University, Ramat-Gan, 52900, Israel*

^e *ILS, Inc., PO Box 13501, Research Triangle Park, NC 27709, USA*

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Abstract

The Tg.AC mouse carrying the v-Ha-ras structural gene is a useful model for the study of chemical carcinogens, especially those acting via non-genotoxic mechanisms. This study evaluated the efficacy of the non-toxic, water-soluble antioxidant from spinach, natural antioxidant (NAO), in reducing skin papilloma induction in female hemizygous Tg.AC mice treated dermally five times over 2.5 weeks with 2.5 µg 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The TPA-only group was considered as a control; the other two groups received, additionally, NAO topically (2 mg) or orally (100 mg/kg), 5 days/week for 5 weeks. Papilloma counts made macroscopically during the clinical observations showed a significant decrease in multiplicity ($P < 0.01$) in the NAO topically treated group. According to histological criteria, papilloma multiplicity were lower in both topical-NAO and oral-NAO groups, but significantly so only in the oral-NAO mice ($P < 0.01$). The beneficial effect of NAO in the Tg.AC mouse is reported. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

* Corresponding author. Tel.: +1-919-5414282; fax: +1-919-5417666.

E-mail address: nyska@niehs.nih.gov (A. Nyska).

Studies with many chemical carcinogens have shown that their effects in the initiation, promo-

tion, and/or progression stages of carcinogenesis are exerted via the generation of oxygen and other organic, free-radical intermediates (Trush and Kensler, 1991). The Tg.AC mouse carrying the *v-Ha-ras* structural gene linked to a ζ -globulin promoter is proving a useful model for the study of chemical carcinogenic potential (Trempus et al., 1998). Mice carrying this oncogene readily exhibit epithelial proliferation when challenged with classical tumor promoters like 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Tennant et al., 1995). Initiation is highly correlated with activation of the *v-Ha-ras* oncogene. Epidermal papillomas induced in the area of topically applied chemical agents within 26 weeks act as a reporter phenotype that defines the activity of the test chemical (Spalding et al., 1999).

The role of the promoter has not been fully elucidated, but the activation of the effect of phorbol esters on protein kinase C (PKC) suggests that promotion might operate through this kinase. The functioning of PKC, among its other actions, induces transcription of an array of genes, including such protooncogenes as *c-jun* (Lee and Lin, 1997; Gopalakrishna and Jaken, 2000).

The reported spontaneous incidence of skin tumors in untreated Tg.AC mice is low. An important characteristic of the Tg.AC model is that, in addition to detecting genotoxic compounds, it is also sensitive to non-genotoxic agents believed to induce tumors via epigenetic mechanisms (Spalding et al., 1999). Biochemical and histological changes described as induced by TPA and considered to be related to its epidermal tumor promotion activity include: skin edema, epidermal hyperplasia, increase of dark basal keratinocytes revealed with proliferating cell nuclear antigen staining, inflammation, induction of epidermal lipoxygenase, cyclooxygenase-dependent metabolism of arachidonic acid, oxidative stress causing increased hydrogen peroxide formation, and enhanced lipid peroxidation (Lahiri-Chatterjee et al., 1999; Reiners and Singh, 1997). Determining which of these or many other effects of phorbol esters are sufficient components of the promotion process is difficult. Agents that reduce inflammation also

reduce the tumor responses in mouse skin carcinogenesis models where TPA is used as a tumor promoter (Lee et al., 1999; Reynolds, 2000).

In recent years the naturally occurring polyphenolic antioxidants have been receiving increased attention as cancer-preventive agents. For example, epigallocatechin gallate is currently being tested in clinical trials for its efficacy in chemoprevention of human skin and prostate cancer. Studies have suggested that factors like structural variation and the number of polyphenolic groups may influence effectiveness in impairing signaling pathways whose involvement in tumor-promoting effects has been suggested (Lahiri-Chatterjee et al., 1999). A recent report from the Institute of Medicine of The National Academy of Sciences recommended, based on epidemiologic evidence suggesting the benefits of antioxidants, a modest increase in dietary antioxidant consumption (Reynolds, 2000).

The objectives of this study were to evaluate the efficacy of a water-soluble antioxidant, NAO, found in spinach leaves, in reducing papilloma development in the Tg.AC model and to test whether inflammatory processes are involved in its effect. NAO is a mixture of mainly aromatic polyphenols and flavonoids, an effective free-radical scavenger, and, specifically, an inhibitor of the lipoxygenase enzyme (Grossman et al., 1994). NAO can easily be used in mice because it is water-soluble and highly stable at high temperatures and lacks toxicity (LD₅₀ of NAO in mice is 1500 mg/kg). NAO is not genotoxic, has a long shelf-life, and demonstrates excellent penetrative properties in the skin. In hairless mice exposed to UV irradiation, NAO was more effective than vitamin E in reducing the level of skin peroxides (Grossman et al., 1994). NAO also reduces lipopolysaccharide (LPS)-induced inflammation and oxidative stress in rat and rabbits (Lomnitski et al., 2000a,b). Based on these properties, the inhibitory effect of NAO on inflammation and skin papilloma levels induced by TPA in Tg.AC mice was investigated; the results indicated a beneficial potential.

2. Methods and materials

2.1. Animals

Sixty-nine female hemizygous Tg.AC mice were divided into three groups (Table 1). The animals aged approximately 32 and 36 weeks of age were received from the animal breeding facilities of the National Institute of Environmental Health Sciences (NIEHS). They were housed in a barrier facility approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and all procedures, maintenance, and treatment of the mice were in accordance with the principles of humane treatment published by the National Academy of Sciences.

After 3 days of acclimatization, animals were randomized and housed four/cage in polycarbonate cages with absorbent hardwood bedding. The temperature and humidity of the rooms were monitored continuously, and the lights were maintained on a 12-h light/12-h dark cycle. Animals were identified by tail tattoo. Pelleted PicoLab diet No. 5058 (PMI Nutrition International, St Louis, MO) and tap water (City of Durham, NC) were provided ad libitum.

Table 1
Experimental design — group designations, dose levels, and number of animals

Group no.	Treatment	Dose levels	No. of animals
1	TPA ^a	2.5 µg	23
2	NAO (dermal) ^b	2 mg	22
	TPA ^c	2.5 µg	
	NAO (gavage) ^d	100 mg/kg	24
3	TPA ^c	2.5 µg	

^a Applied as skin paint from beginning of 2nd week from the beginning of the experiment and continued for 2.5 weeks (two treatments/week).

^b Applied topically 5 days/week, from week 1–5.

^c Administered 1 h after NAO dosing.

^d Administered by oral gavage for 5 days/week, from week 1–5.

2.2. Test articles and administration

2.2.1. Natural anti-oxidant (NAO)

NAO, a water-soluble antioxidant composed of a mixture of natural molecules extracted and purified from spinach leaves was prepared as described previously (Grossman et al., 1994). Spinach leaves were homogenized with an equal amount of H₂O (w/w). The supernatant was collected and chromatographed on a sephadex G-25 column. Fraction A was collected and used as NAO in this study. NAO, a brown powder, was stored dry in a closed desiccator at room temperature and removed from light. The same batch of NAO was used for oral and topical treatment. The dosing solution was prepared at the beginning of the study, dispensed into brown glass vials cleared with nitrogen, and stored at a temperature below –20°C until use. Samples were thawed each day for usage on that day. Selection of dosage was based on previous experience with NAO oral treatment of mice in a methylcholanthrene model in which the effective oral dose of NAO was 0.1% (1 mg/ml) in drinking water (about 200 mg/kg body weight/day) (unpublished data).

For gavage dosing solutions, the appropriate amount of NAO was dissolved in calcium-free phosphate-buffered saline (PBS) to yield a concentration of 20 mg NAO/ml. NAO was administered by oral gavage at a dosing volume of 5 ml/kg, yielding a dose of 100 mg NAO/kg body weight.

For the topical application, a 1% solution of NAO (10 mg/ml) was prepared in 40% acetone (v/v). The NAO solution was prepared at the beginning of the study, dispensed into brown glass vials cleared with nitrogen, and stored at a temperature below –20°C until use. A volume of 200 µl was applied by pipette to the test site of the appropriate animals yielding a dose level of 2 mg NAO/mouse (about 100 mg/kg).

2.2.2. 12-O-tetradecanoylphorbol-13-acetate (TPA)

TPA (Sigma Chemical Co., St. Louis, MO.), a

nongenotoxic skin carcinogenic agent, was used as a positive control. Ten milligrams of TPA were dissolved in 20 ml of acetone. Volumes of 100 μ l of the solution were dispensed into glass vials cleared with nitrogen. The vials were left uncapped until the acetone was completely evaporated and then capped and stored at temperatures below -10°C . As needed for dosing, the contents of each vial were dissolved in 4 ml of acetone to yield a dosing solution of 2.5 μg TPA in 200 μ l of acetone. A volume of 200 μ l was applied by pipette to the test site yielding a dose level of 2.5 μg . TPA at 2.5 or 5 μg has been demonstrated to be consistently tumorigenic in female hemizygous Tg.AC mice (Spalding et al., 1999). The TPA was administered dermally over the back in five doses of 2.5 μg over 3 weeks (Tuesday and Friday of weeks 2 and 3 with the final dose on Tuesday of week 4 of the study). This particular dose regimen induces a reproducible, but not maximum, tumor response. The proportion of animals developing papillomas within 26 weeks at 5 μg has been reported to range from 93–100%, with the average maximum multiplicity ranging from 11 to 19.5 papillomas per mouse. Treatment with 2.5 μg tends to produce a similar incidence but fewer tumors per mouse (four).

2.3. Experimental design

The three group designations, dose levels, and numbers of animals are presented in Table 1. Prior to initiation of treatment, the test site (approximately 12 cm) was shaved on all mice and defined as an area on the back extending from the interscapular to the pelvic region. NAO was dosed in groups 2 and 3, for 5 days/week, from week 1–5 of the study. TPA was administered to groups 1–3, beginning on the second week of the experiment and continuing for 2.5 weeks (two treatments/week). The dosing volume of NAO given by gavage was adjusted weekly, based on the most recent group mean body weights. During the TPA dosing period, TPA was administered 1 h after NAO dosing. The animals of all groups were held for 9 weeks after the end of the TPA dosing.

2.4. Mortality/moribundity checks, clinical observations, and body weights

Mortality and moribundity checks were performed twice daily (at least 6 h apart) during the week and once daily on weekends and holidays. Clinical observations, consisting of a thorough examination including eyes and all orifices and a count of papillomas on the test site were performed on day 1 (prior to dosing) and weekly thereafter. Tumors >1 mm in diameter were included in the cumulative total if they persisted for 3 weeks or longer. Tumors were counted and recorded up to 30 tumors per mouse with higher occurrences recorded as 'greater than 30 tumors.' Body weights of all animals were recorded on day 1, weekly thereafter, and prior to termination.

2.5. Collection of samples for genotyping

During week 1 of treatment, tail samples were collected from all animals, flash-frozen in liquid nitrogen, and stored at -70°C for possible genotyping. Approximately one third of the samples were genotyped. In addition, tail and ear samples were taken from all animals at the terminal sacrifice, flash-frozen in liquid nitrogen, and stored at -70°C , but not genotyped.

2.6. Pathological investigation

Unscheduled necropsies on mice found dead or sacrificed for humane reasons were performed as needed. An interim sacrifice took place 48 h after the last dosing of TPA and included five randomly selected mice from each group. The terminal sacrifice was performed during the 13 weeks after study initiation. At scheduled sacrifices, animals were euthanized by CO_2 asphyxiation and exsanguination. A limited necropsy was performed on all animals. The test site on all animals was carefully examined, and an exact count of all papillomas in the test site was recorded. From the skin test site, in locations uniformly selected in all animals, two anterior samples, approximately 1 cm^2 , were taken parallel to the long axis of the mouse, and one posterior sample, approximately 1 cm^2 , was taken across the long axis (Fig. 1). The

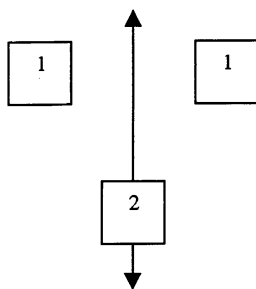


Fig. 1. Method of skin test site tissue collection for histology.

three areas selected for histopathological evaluation represented approximately 25% of the total test area. These sections were pinned on cardboard with the orientation noted (head/tail, left/right). The external features, including all orifices, and the organs of the abdominal and thoracic cavities were examined. Gross lesions were documented and, at the discretion of the project scientist or study director, collected and fixed in 10% neutral buffered formalin (NBF). All tissues collected in 10% NBF were transferred to 70% ethanol within 18–24 h. For histology, a separate slide was prepared for each of the skin samples (i.e., a total of three blocks/animal). Tissues were trimmed along their long axis into approximately 2 mm thick specimens; therefore, each skin sample may have been trimmed into two–three sections.

2.7. Statistical analysis

Body weight data were analyzed by one-way ANOVA and Student *t*-test comparisons using StatMost 32 (PC statistical software). Comparisons were made between treatment groups and between each treatment group and the control group. The skin papilloma response profiles for the different treatment groups were compared by applying the Bayesian approach proposed by Dunson et al. (2000), which distinguishes between differences in tumor latency and multiplicity on the basis of weekly papilloma count data. This approach incorporates all of the information in the weekly clinical observations while also accounting for differences in animal survival and susceptibility. Comparisons of the exact papilloma counts measured by histology were based on an over-dispersed Poisson

regression model, which is a collapsed version of the approach described by Dunson et al. (2000). Group comparisons were based on posterior probabilities, which are Bayesian alternatives to the *P*-values.

3. Results

3.1. Tissue genotyping

Twenty four tail samples taken during the first week of treatment were genotyped using established procedures. All samples were shown to have the transgene.

3.2. Body weights

Mean body weights recorded during the study and presented in Fig. 2 were similar for all groups at study initiation. All groups showed a slight decline in mean body weight through week 4. After week 4, groups 1 and 3 remained relatively stable, while group 2 (TPA + topical NAO) showed a steady increase in mean body weight. Significant differences were seen between the mean body weights of groups 2 and 3 from week 10 through the terminal sacrifice. In addition, the mean body weights of groups 1 and 2 were significantly different from week 12 through the terminal sacrifice. Groups 1 and 3 showed mean body weight losses over the period of the study (1.9 and 2.7 g, respectively), while group 2 showed a mean body weight gain (1.2 g). There was a significant difference between the mean body weight changes of groups 2 and 3.

3.3. Survival at terminal sacrifice

Four deaths occurred during the study (one from group 2 and three from group 3). None of these deaths was considered to be related to treatment.

3.4. Investigation of the test site

3.4.1. Papilloma count during the clinical observation

The incidence (i.e., % animals with tumors),

multiplicity (i.e., number of tumors/animal), and distribution of maximum papillomas per mouse are presented in Table 2 and in Figs. 3 and 4. A statistical analysis was conducted to assess differences between the positive control group and each of the two NAO treatment groups with respect to: (i) the latency time prior to the development of the first papilloma; (ii) the rate of increase in papilloma multiplicity after the development of the initial tumor; and (iii) the overall rate of change in papilloma multiplicity. There were no significant differences between the positive control group and the oral treatment group ($P = 0.184$). However, there was a significant decrease in papilloma multiplicity in the topical treatment group relative to the positive control ($P < 0.01$). There was no evidence of a change in the latency time prior to

appearance of the first papilloma with topical treatment ($P = 0.435$).

3.5. Histopathological evaluation

3.5.1. At the interim sacrifice (2 days following last dosing with TPA)

TPA administration was not associated with an inflammatory reaction in the skin. No difference was noted among the three groups with respect to any histological parameters examined (e.g. epidermal hyperplasia, inflammation of the dermis or subcutis).

3.5.2. At terminal sacrifice

No difference among the three groups was noted with respect to an inflammatory reaction of the skin and malignancy of the tumors. In fact, all

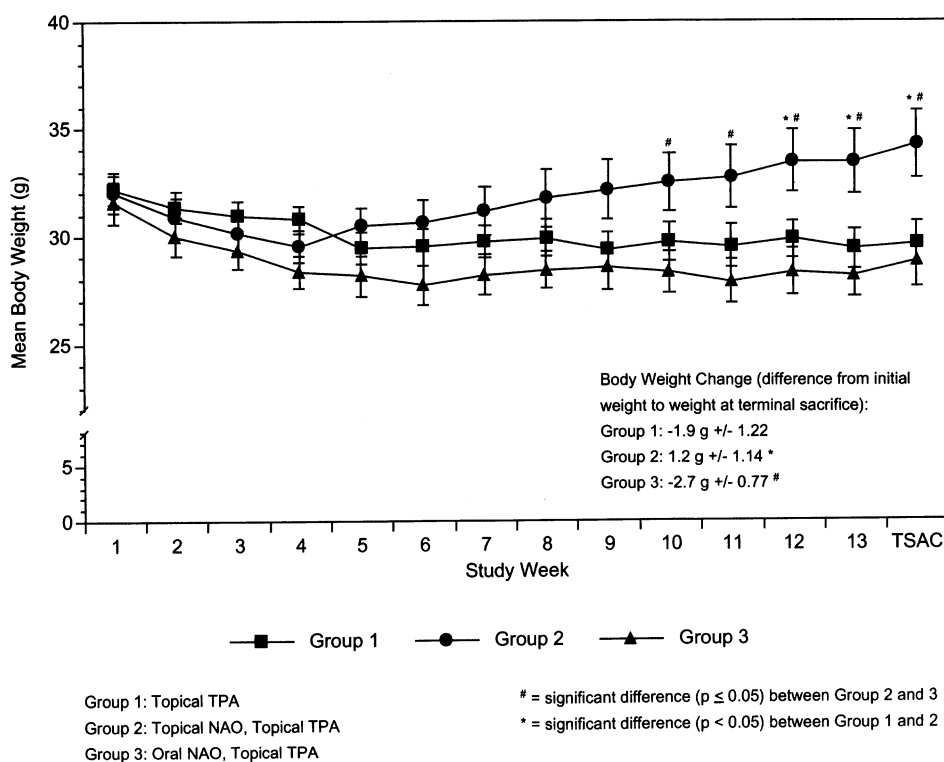


Fig. 2. Mean body weights (g): exposure of female hemizygous Tg.AC mice via the oral and dermal (skin paint) routes to NAO and TPA.

Table 2
Distribution of maximum papillomas per mouse for animals at terminal sacrifice

Group no.	Treatment	No. of mice at TSAC ^c	Maximum number of papillomas per mouse ^a																														Mean				
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	> 30 ^b			
1	TPA	18	1	1		1	1	1			1		1	1	1			2				1					1									5	15.39
2	NAO (dermal)	16	2		3	1			1			1			3					1			1				1	2									10.69
3	/TPA NAO (gavage) /TPA	16	2	4		1		1			1						1		1								1									4	12.00

^a Maximum number of papillomas = maximum number sustained for 3 consecutive weeks.

^b Papilloma counts greater than 30 are recorded as >30 in the raw data.

^c Terminal sacrifice 13 weeks after study initiation.

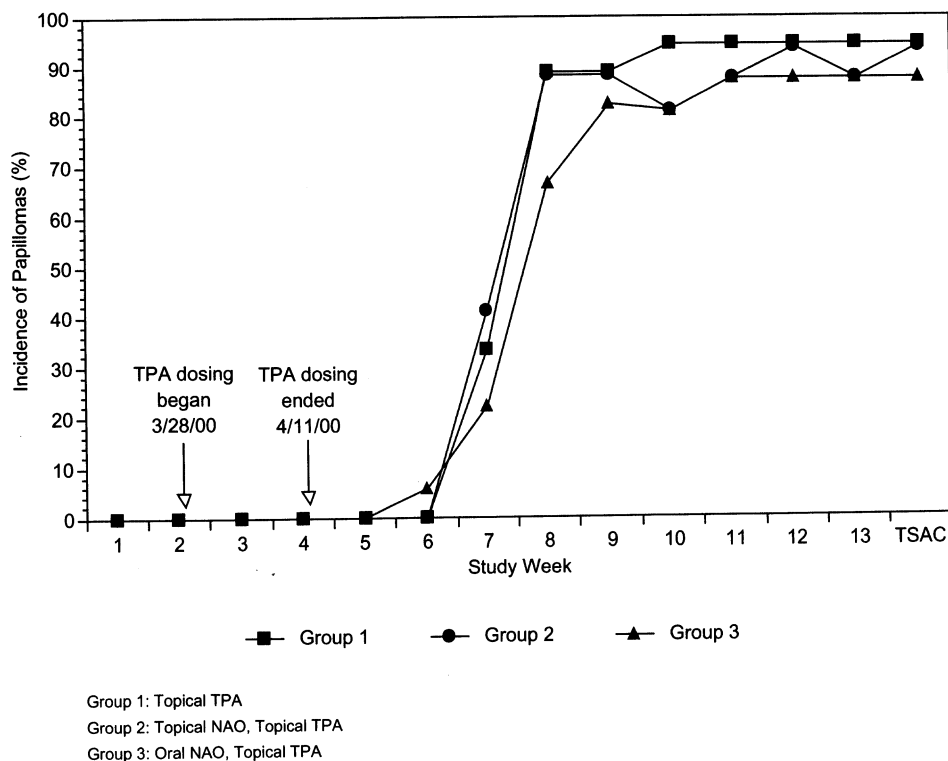


Fig. 3. Incidence of papillomas on test site: exposure of female hemizygous Tg.AC mice via the oral and dermal (skin paint) routes to NAO and TPA.

tumors noted in the three groups were defined as papillomas. The total incidence of papillomas was 75 with a mean of 4.1, 52 with a mean of 3.05, and 44 with a mean of 2.3, respectively, in groups 1, 2, and 3. A significant decrease in papilloma multiplicity was found in the oral group, but not in the NAO topical group. The mean papilloma count was lower in both groups receiving NAO when compared to the control group, with $P = 0.08$ for the topically applied NAO group, and $P < 0.01$ for the group dosed orally with NAO.

4. Discussion

The Tg.AC mouse is a promoter-sensitive system, which provides a powerful and convenient model for the identification of chemical carcinogens and the testing of potential anti-tumor and anti-proliferative agents that might act on the v-Ha-ras

or PKC pathways (Leder et al., 1990). The inflammatory process has been shown to be an important factor in cancer induction in various organs and tissues such as the ovary (Ness and Cottreau, 1999), stomach (Nishiya et al., 2000), lung (Shi et al., 1998; Tsutsumi et al., 2000), and others (Ohshima and Bartsch, 1994) (Seidman et al., 1999).

The results of the present investigation indicate that NAO was effective in reduction of the multiplicity of TPA-induced papillomas in the Tg.AC model. The TPA treatment was not associated with an inflammatory reaction in the skin, and, therefore, effects of NAO on inflammation could not be verified. We do not exclude the possibility that the sampling used for histological evaluation of the skin at the interim sacrifice occurred after the TPA-related inflammation had already subsided.

Papilloma counts made during the clinical observations showed a significant decrease in multiplicity ($P < 0.01$) in the NAO topical treatment

group relative to the positive control. Histopathology of three uniformly selected areas indicated that papilloma multiplicity was lower in both groups treated with NAO compared to the TPA group, but only in the oral NAO group was the effect significant ($P < 0.01$). These apparent differences in the results obtained by the two dosing methods may be explained by the more accurate definition of papillomas by histological criteria in contrast to macroscopic assessment, or by a possible non-homogeneous distribution of papillomas within the area of the treatment site.

The reason for the differences in body weight and, in particular, the findings that, after week 4, groups 1 and 3 remained relatively stable while group 2 (TPA + topical NAO) showed a steady increase in mean body weight, is unknown. In studies conducted in the TRAMP transgenic mice in which the 200 mg/kg/day of NAO was repeatedly administered by gavage (unpublished data),

no decrease in body weight was observed. In experiments in which NAO was repeatedly administered to rats by the intraperitoneal route, no toxic effects were noted, including no effect on body weight (Lomnitski et al., 2000b). Therefore, we suggest that the change in body weight is spurious.

NAO was purified from spinach extract and contains mainly aromatic polyphenols and flavonoids. The antioxidant capacity of spinach flavonoids has been recently determined by the free radical scavenging assay (Gil et al., 1999). In addition, the effect of consumption of spinach products on antioxidant activity in human blood was tested in healthy volunteers. The consumption of spinach resulted in greater erythrocyte glutathione reductase and lower erythrocyte catalase activity (Castenmiller et al., 1999).

Flavonoids and other compounds related to the polyphenol family are known to be potent natural

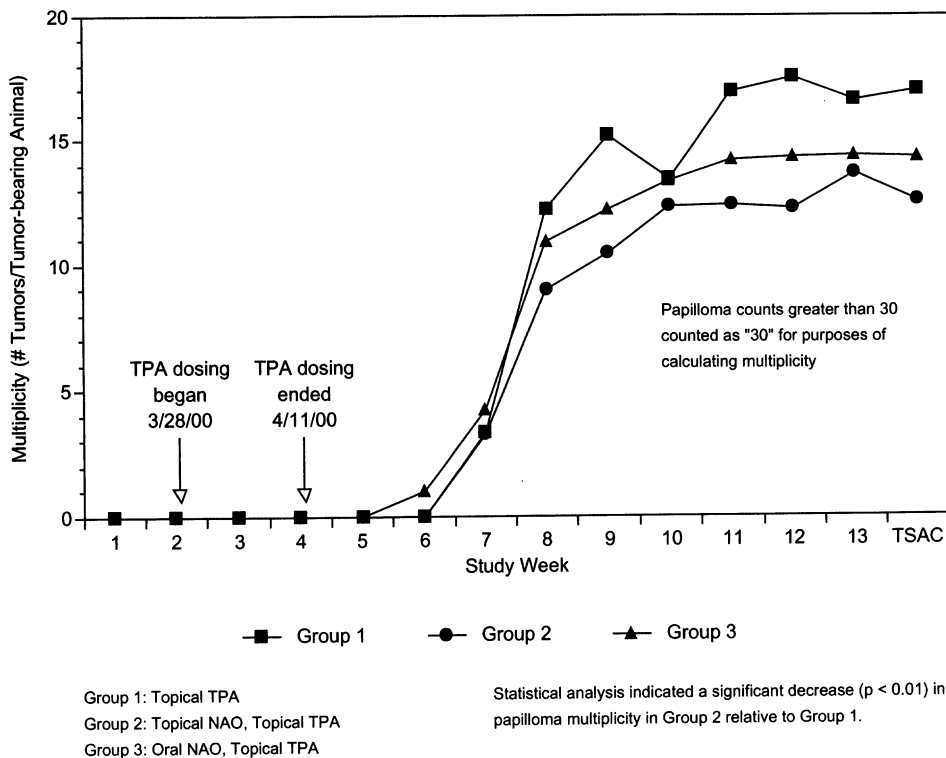


Fig. 4. Multiplicity of papillomas on test site: exposure of female hemizygous Tg.AC mice via the oral and dermal (skin paint) routes to NAO and TPA.

antioxidants having preventive effects in various diseases in which oxidative stress is involved, such as atherosclerosis, carcinogenesis, and endotoxemia (Breinholt, 1999; Kanner, 1999; Wakabayashi, 1999). Mechanistically, compounds related to the polyphenol family are believed to act by SOD-like activity by decreasing the level of superoxide anion radicals. They effectively scavenge hydroxyl and NO \cdot radicals and inhibit peroxyl radical-mediated and heme-catalyzed lipid peroxidation (Kanner, 1999).

The use of another polyphenolic flavonoid antioxidant silymarin in the SENCAR mouse skin tumorigenesis model indicated protective effects by reduction of tumor incidence, multiplicity, and volume as well as inhibition of edema, hyperplasia, proliferation, and oxidation in the epidermal cells (Lahiri-Chatterjee et al., 1999). That oral administration of glucosinolate-containing vegetable, *Brassica oleraceae*, significantly reduced the incidence and multiplicity of epidermal papilloma in DMBA-initiated – TPA-promoted CD-1 mice has been recently shown (Isbir et al., 2000). The level of glutathione (GSH) significantly increased in papillomas of *Brassica*-treated groups compare to control.

Concerning the effect of NAO in the rat septicemic model, our previous investigations indicated that NAO significantly ($P < 0.05$) attenuated lipopolysaccharide-stimulated cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase induction in the liver (Lomnitski et al., 2000a). COX-2 is highly expressed in skin cancer (Buckman et al., 1998; Fosslie, 2000). Celecoxib, a specific COX-2 inhibitor, has shown a dose-dependent reduction in tumor yield in UV-induced skin tumors in hairless mice, emphasizing the role of COX-2 in epidermal cell proliferation (Fischer et al., 1999). That COX-2 is induced by the oncogenes *ras* and *src*, as well as by various factors such as interleukin (IL)-1, epidermal growth factor, transforming growth factor beta, and tumor necrosis factor alpha, has been suggested (Fosslie, 2000). Dexamethasone, antioxidants, and the tumor-suppressor protein p53 suppress COX-2 expression. We would suggest that the protective effect of NAO in the Tg.AC model was, at least in part, through inhibition of prostaglandin synthesis in the skin.

Our recent investigation of the protective mechanism of NAO in the oxidation-stress model of doxorubicin-induced cardiotoxicity indicated that the protective effect of NAO was associated with induction of superoxide dismutase (SOD) activity (Breitbart et al., 1999). Results obtained in the same model have shown that, in addition to cardiomyopathy, doxorubicin injection may cause damage to the immune system and impaired functioning of splenocytes. This possible impairment was characterized by decreases in secretion of IL-2, IL-6, and IL-10 (Breitbart et al., 1999). Treatment with NAO, for 7 days before and 6 days after doxorubicin treatment, caused the splenocytes to secrete almost the same amount of cytokines as the controls.

PKC is an upstream target for both oxidants and chemopreventive antioxidants. Furthermore, modification of PKC contributes to redox-mediated signaling events, including tumor promotion (Gopalakrishna and Jaken, 2000). Polyphenol antioxidants can modify cysteines required for the activation of PKC within its catalytic domain (Gopalakrishna and Jaken, 2000). Curcumin is a polyphenolic compound which possesses anti-tumor-promoting capabilities by inhibition of the expression of the proto-oncogene *c-jun*; however, its free-radical scavenging ability is low (Lee and Lin, 1997). Additionally, dimethyl sulfoxide, a scavenger of hydroxyl radicals, cannot completely inhibit the TPA-induced *c-jun* expression, indicating that TPA-induced *c-jun* expression is only partly derived from hydroxyl radical formation (Lee et al., 1996). These findings support the idea that TPA-induced tumor promotion occurs by more than one pathway and, therefore, free-radical scavenger antioxidants cannot provide complete protection against tumor promotion processes.

We suggest that the effect of NAO in the current study may be related to its ability to detoxify peroxides and free radicals generated by TPA exposure, either directly or indirectly by induction of antioxidant defense enzymes.

According to the results of our present investigation, NAO may be added to a growing list of naturally occurring dietary antioxidants that may be considered a useful strategy against the toxic

effects of dietary mutagens and carcinogens (Ames et al., 1995).

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