Chemoprevention of DMBA-Induced Mammary Tumors in Rats by a Combined Regimen of Alpha-Tocopherol, Selenium, and Ascorbic Acid

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■ **Abstract:** This experimental study was designed to evaluate the efficacy of associated naturally occuring antioxidants in the prevention of chemically induced breast cancer using DMBA in virgin female Wistar rats. Rats were randomly allocated to three groups: control group (CG; $n = 20$), induction group (IG; $n = 100$), and prevention group (PG; $n = 70$). A single dose (65 mg/kg) of DMBA was administered in the IG and PG animals at 50 days of age. PG animals also received a single dose of alpha-tocopherol (200 mg/rat) 1 hour after DMBA administration and an association of selenium (p-XSC, 40 ppm/day/rat) and ascorbic acid (540 mg/day/rat) in drinking water, daily, from carcinogenic induction until necropsy. Macroscopic study and pathology revealed a significantly lower development of neoplasms in the PG animals ($p < 0.05$); the number of rats with mammary tumors, breast cancer incidence, and the number of malignant breast tumors per rat as well as per tumor-bearing rat were significantly decreased in the PG animals. Other types of primary neoplasms existing in the IG animals totally disappeared in the PG animals. Immunostaining to hormone steroid receptors (ER and PR) and cathepsin D was similar in both groups. Overexpression of p53 and metallothioneine was significantly increased in the PG animals ($p < 0.05$) and immunostaining to bromodeoxiuridin and Ki-67 was also stronger in the remaining tumors in the PG animals. These data thus add to the accumulating evidence that those micronutrients in combination seem to be effective in reducing the incidence of malignant tumors. Nevertheless, remaining tumors seem to present more aggressive behavior and characteristics of drug resistance. ■

Key Words: antioxidants, breast cancer, chemoprevention, micronutrients

In recent years the incidence of breast cancer has increased almost everywhere. Alternatives to therapy need to be developed for breast cancer control. Toward this end, chemoprevention constitutes a valuable approach. It is mandatory to expand our efforts in identifying synthetic or naturally occuring agents that can inhibit the preneoplastic events preceding the occurence of clinically detectable cancers (1,2). Several agents have been characterized as effective in cancer chemoprevention, but studies are not yet conclusive (3). Chemoprevention properties of vitamins and other micronutrients as single agents or complex mixtures (4–22), fatty acids and related substances (23–27), monoterpens (28), hormones, and related chemical agents (1–3,29) have been

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studied in experimental models. To assess the efficacy of potential chemopreventive agents it is important to develop sensitive and selective methods for determining molecular, metabolic, and cellular transformations that are relevant to the process of carcinogenesis in mammary tissue.

Mammary carcinomas induced in rats by means of chemical carcinogens, mainly DMBA, seem to provide a good model to the understanding of mechanisms of susceptibility to carcinogens (30). Mammary tumors thus induced are hormone-dependent adenocarcinomas arising from terminal end buds (TEBs) on incompletely differentiated glands (31). These tumors bear a close resemblance to human breast cancer in their histologic and hormone-response patterns (29,32); nevertheless, immunostaining and molecular biomarkers have not been clearly defined in those experimental carcinomas (33–35).

In this study we investigated whether combined naturally occuring micronutrients with antioxidant molecular activity inhibit the incidence of DMBA-induced mammary tumors in Wistar rats. We also describe the characteristics of aggressiveness and the drug resistance of remaining tumors using immunostaining for breast cancer biochemical markers.

MATERIALS AND METHODS

One hundred ninety outbred virgin female Wistar rats were obtained from the Instituto Gulbenkian de Ciência (Lisboa, Portugal). Animals were housed four in each cage in a temperature- (23 ± 2°C) and humidity- (50–55%) controlled facility on a 12-hour light, 12-hour dark cycle and fed a standard laboratory chow. Food and water were available ad libitum and mean water consumption was calculated every week.

At 50 days of age, rats were randomly allocated to three groups: control group (CG; $n = 20$), induction group (IG; $n = 100$), and prevention group (PG; $n = 70$). Biopsies of the right cervical mammary gland were performed in all animals under general anesthesia (ketamine intramuscularly, 5 mg/100 g). CG animals received no more drugs until the end of the experience. IG and PG rats were given a single dose (65 mg/kg) of 7,12-dimethylbenz[a]anthracene (DMBA) in olive oil via an intragastric tube. PG rats also received a single dose of alpha-tocopherol (200 mg/rat) 1 hour after DMBA administration via an intragastric tube and a solution of selenium (p-XSC, 40 ppm/day/rat) and ascorbic acid (540 mg/day/rat) in drinking water, daily, from carcinogenic induction until necropsy.

All animals were kept under previously described environmental conditions and they were palpated weekly to determine the appearance, size, and location of tumors. They were weighed monthly.

Twenty-four weeks after carcinogenic induction rats were killed by cervical dislocation 1 hour after intravenous bromodeoxiuridin (BrdUr) administration (50 mg/kg). The rats that died before 24 weeks were excluded from this study.

Macroscopic tumors were excised and the size, measured in two perpendicular dimensions, was estimated from the mean diameter. Mammary glands free of nonpalpable tumors were also removed and biopsies of the following organs were performed: trachea, heart, lungs, esophagus, liver, spleen, pancreas, bowel, bladder, and internal genital organs.

Histologic Evaluation

Tissue fragments were fixed in 10% formalin and a 5 μm section was obtained from the paraffin block and stained with hematoxylin and eosin for histologic examination. Breast disease pathology and histologic type were evaluated by application of the same pathologic criteria used for the classification of human tumors (36). Breast cancer grading was performed according to the Nottingham modification of the Bloom and Richardson system.

Assessment of Staining

Estrogen receptors (ERs, clone 1D5, Dako) and progesterone receptors (PRs, A009850, Dako) were immunostained on frozen sections. Tumors with cytoplasmic ER and PR greater than 5 fmol/mg of protein were considered positive.

Immunohistochemistry was performed for p53 (M700101, Dako), pS2 (A009501, Dako), c-*erb*B-2 (A0485, Dako), cathepsin D (A056101, Dako), metallothioneine (M063901, Dako), Ki-67 (A004701, Dako), and bromodeoxiuridin (BrdUr, clone BU20a, Boehringer Mannheim). Immunohistochemical analyses were performed according to previously published methods (37).

Statistical Analysis

Differences in tumor incidence and immunostaining between groups were determined using the Fisher's test. Differences in rat body weight and tumor size were statistically evaluated by one-way analysis of variance (ANOVA) followed by chi-square tests. Values of $p < 0.05$ were considered significant.

RESULTS

Mammary gland biopsies performed at 50 days of age exhibited similar characteristics of mammary developmental stage: terminal end buds (TEBs) composed of three to six layers of medium-size epithelial cells with a fairly constant mitotic activity as predominantly mammary structures. All rats presented the same mammary gland developmental stage by the time of carcinogenic induction.

Total body weight did not change significantly among the groups of rats throughout the experimental period. The PG animals' body weight 24 weeks after DMBA administration was less than that for the CG and IG animals, but the difference was not significant. No statistically significant difference was observed when comparing the body weight of rats with breast cancer to that of healthy IG and PG rats.

CG rats were all healthy and alive at the end of the experience. Ten rats in each of the other groups died before necropsy and were excluded from the study. IG rats displayed 35 (39%) benign breast disorders and 56 (62%) breast carcinomas. Macroscopic study and histopathologic examination of IG animals showed 7 primary neoplasms and 14 benign lesions of other tissues and organs, mainly benign ovarian disorders. PG rats presented no other tumors but breast tumors: 17 (28%) benign breast disorders and 9 (15%) breast carcinomas (Table 1).

Statistical analysis comparing breast disorders demonstrates that rat body weight and the average diameter of macroscopic breast tumors did not differ significantly in IG and PG rats. Nevertheless, the total number of rats with malignant tumors, breast cancer incidence, and the total number of malignant breast carcinomas were significantly decreased in PG. Moreover, the number of malignant breast tumors per rat as well as per tumor-bearing rat were significantly decreased in animals receiving alpha-tocopherol, selenium, and ascorbic acid (Table 2).

PG rats never presented preinvasive ductal carcinoma in situ or atypical hyperplasias. Benign breast disorders were composed of proliferative lesions without atypia in PG animals (Table 3). No significant difference was reported in histologic grade and microscopic pathology. No breast cancer presented vascular invasion and no metastatic lesions were found in all biopsies performed (Table 4). ER (43% IG and 33% PG) and PR (18% IG and 22% PG) contents of the tumors were not significantly different (Table 5).

Concerning immunohistochemical staining, 29% breast cancer IG rats presented positivity for p53 overexpression; nevertheless, 78% of breast carcinomas in PG rats displayed p53 accumulation, and this difference was statistically significant. All breast carcinomas were c-*erb*B-2 negative and only one IG breast cancer was considered pS2 positive. Immunostaining for cathepsin D was higher in IG than in PG rats, although the difference wasn't significant.

Metallothioneine overexpression was found in 67% of PG breast cancers and occasionally (10%) in IG breast carcinomas; MT-positive tumor cells were significantly increased in PG rats. Finally, considering immunohistochemical Ki-67 and BrdUr expression, staining intensity was significantly stronger in PG breast carcinomas.

DISCUSSION

We have to enhance our understanding of the role of naturally occuring antioxidants in cancer prevention and their possible use in intervention trials for the prevention of cancer in humans. Selenium alone seems to inhibit both the initiation and postinitiation phases of carcinogenesis in animal models (21), and in vitro studies have been performed to investigate the antiproliferative action of selenium (38). The mechanisms and forms

Table 1. Benign Disorders and Malignant Tumors in IG and PG Rats

	IG (n = 90)		PG (n = 60)	
Number of lesions	BEN	MAL	BEN	MAL
Breast	35	56	17	9
Ovary	6	—	—	—
Salivary gland	4	—	—	—
ORL	1	3	—	—
Lymphoma	—	2	—	—
Lungs	—	1	—	—
Liver	2	—	—	—
Pancreas	—	1	—	—
Bowel	1	—	—	—

Table 2. Pathology of Breast Tumors in IG and PG Rats

	N = 90		N = 60	
	n	(%)	n	(%)
Malignant breast tumors	IG (n = 56)		PG (n = 9)	
DCIS	3	(5.4)	—	
Invasive ductal carcinoma	53	(94.6)	9	(100)
Benign breast disorders	IG (n = 35)		PG (n = 9)	
Glandular hyperplasia	24	(68.6)	13	(76.5)
Atypical hyperplasia	7	(20)	—	
Fibroadenoma	3	(8.6)	4	(23.5)
Papillary lesions	1	(2.8)	—	

Table 3. Pathologic Features and Aggressiveness of Malignant Breast Tumors in IG and PG Rats

	N = 90	*N* = 60	
	IG (*n* = 53)	PG (*n* = 9)	Fisher ($p < 0.05$)
GI	49 (93%)	9 (100%)	NS (0.4839)
GII	4 (7%)	—	
GIII	—	—	
Invasive ductal carcinoma (no other specification)	50 (94%)	9 (100%)	NS (0.5485)
Invasive tubular carcinoma	2 (4%)	—	
Invasive papillary carcinoma	1 (2%)	—	
Lymphoplasmacytic infiltrate	13 (25%)	3 (33%)	NS (0.1637)
Fibrosis	29 (55%)	6 (67%)	NS (0.1426)
Necrosis	2 (4%)	—	
Vascular invasion	—	—	
Metastatic lesions	—	—	

of selenium that are responsible for chemoprevention in laboratory animals need to be elucidated (15,20,21).

Previous articles reported the anticarcinogenic activity of aliphatic selenocyanates, suggesting that the anticarcinogenic potency of those compounds is correlated with their ability to inhibit DMBA-DNA adduct formation in mammary cells (10,22). Other studies revealed that selenium supplementation clearly inhibits DMBA-induced mammary tumorigenesis in rats and mice (5–11). Epidemiologic reports show a higher cancer incidence in geographic areas of low selenium intake (39).

Combined chemopreventive effects of alpha-tocopherol and selenium in rodent tumors seem to be more promising than that of selenium or alpha-tocopherol alone (3,13,14,18,19). On the other hand, the effect of ascorbic acid alone in some tumor model systems is not consistent or appears to be weak (17). The combined effect of selenium and vitamin C probably depends on the chemical form of the selenium compound (12).

This experimental study was designed to evaluate the combined effect of the referred three micronutrients. Results point to an effective chemopreventive action of this mixture in reducing the incidence and preventing the development of malignant tumors in this animal model.

Table 4. Estrogen Receptor (ER) and Progesterone Receptor (PR) Contents of Malignant Breast Tumors

	IG	PG	Fisher ($p < 0.05$)
ER+			
n	24/56	3/9	NS (0.0817)
%	43	33	
PR+			
n	10/56	2/9	NS (0.3938)
%	18	22	

After carcinogenic induction, only a few mammary tumors developed and no other primary neoplasms were observed in animals receiving selenium, alpha-tocopherol, and ascorbic acid. Breast cancer incidence, total number of malignant tumors, and the number of breast carcinomas per rat as well as per tumor-bearing rat were significantly decreased in PG animals. So the use of selenium, alpha-tocopherol, and ascorbic acid in a combined regimen seems to be useful as a suppressive chemopreventive regimen for breast cancer and other neoplasms in this particular animal model. Daily intake of those micronutrients beginning immediately after carcinogenic induction causes a significantly lower development of malignancies.

Nevertheless, these naturally occuring antioxidants displayed no inhibitory effect on tumor growth in established tumors. Finally, this remaining group of tumors seems to be associated with a more aggressive behavior and with resistance against anticancer drugs, since they exhibited higher levels of p53 and metallothioneine. Mammary tumors induced in the presence of those antioxidants possess characteristics distinct from the tumors

Table 5. Molecular Tumor Markers: Results of Immunohistochemistry in Malignant Tumors

	IG (*N* = 56)	PG (*N* = 9)	
	n (%)	*n* (%)	Fisher ($p < 0.05$)
$p53^+$	16 (29)	7 (78)	0.0001
$pS2^+$	1 (2)	—	
c-*erb*B-2^+	—	—	
Cathepsin D^+	31 (55)	4 (44)	NS (0.0973)
Metallothioneine$^+$	5 (10)	6 (67)	0.0001
Ki 67 > 50%	10 (18)	4 (44)	0.001
BrdUr > 50%	16 (29)	4 (44)	0.0237

occuring in their absence. Moreover, the appearance of new tumors during micronutrient intake suggests that there is a different class of tumors not susceptible to prevention in this way.

Selection of chemopreventive agents need not to be limited to naturally occuring compounds, and combined effects of synthetic and/or naturally occuring agents with different modes of action should be examined (1) and selected on the basis of efficacy versus toxicity considering the need of long-term intake.

Finally, the availability of increasingly sophisticated biochemical tools in molecular epidemiology in recent years provides a better understanding which undoubtedly offers opportunities for defining and modulating breast cancer risk by means of agents that alter critical steps in the multistage carcinogenic process.

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