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# Prevention of Mammary Tumorigenesis in Acatalasemic Mice by Vitamin E Supplementation

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**Adult male and female acatalasemic (C3H/AnLCs$^{b}$Cs$^{b}$), hypocatalasemic (C3H/AnLCs$^{c}$Cs$^{c}$) and normal mice of C3H strain fed on regular laboratory chow for 15 months showed an increased incidence of spontaneous mammary tumor in the decreasing order of female acatalasemic, male acatalasemic, female hypocatalasemic and male hypocatalasemic mice. Normal mice did not develop mammary tumor. We conducted a prospective study with female acatalasemic mice, which showed the highest incidence of mammary tumor, to examine the preventive effect of vitamin E on mammary tumor. Female acatalasemic mice were fed on vitamin E-deficient (28 animals) and vitamin E-supplemented diet (25 animals) for 29 months. The incidence of mammary tumor in mice given the vitamin E-supplemented diet was 47%, while that in mice given vitamin E-deficient diet was 82% ($P < 0.002$). Mammary tumors were apparent after 9 months of vitamin E deprivation and after 14 months of vitamin E supplementation. Female normal mice did not develop mammary tumor during a comparable period of time. The mean catalase activity of mammary gland in acatalasemic mice was 18.8% of that in normal mice. The results indicate that vitamin E protects acatalasemic mice against the development of mammary tumor.**

Key words: Mammary tumorigenesis — Acatalasemic mouse — Vitamin E

Mutant mice, C3H/AnLCs$^{b}$Cs$^{b}$ (acatalasemic) and C3H/AnLCs$^{c}$Cs$^{c}$ (hypocatalasemic) have blood and tissue levels of catalase that are one-tenth and half of those in normal mice (C3H/AnLCs$^{a}$Cs$^{a}$), respectively, so these mice should be of considerable value in studying the possible role of hydrogen peroxide in tumorigenesis and carcinogenesis, as suggested by Warburg *et al.*[1] and by Holman.[2] It was reported by Heston and Vlahakis[3] and Andervont[4] that the C3H strains of mice spontaneously develop mammary tumor, although no such tumor has been reported in C3H/AnLCs$^{b}$Cs$^{b}$ or C3H/AnLCs$^{c}$Cs$^{c}$ mice. Reddy *et al.*[5] found that the incidence of hepatocarcinoma in acatalasemic mice receiving long-term treatment with Nafenopin was significantly higher than that in control mice. Watanabe *et al.*[6] also reported that X-ray-irradiated hypocatalasemic mice readily produce signet ring cell carcinoma. With this background, the present study was carried out in an attempt to see whether the acatalasemic mouse produces spontaneous tumors at a higher rate than the normal mouse and whether the carcinogenesis can be prevented by vitamin E.

The importance of vitamin E in prevention of neoplasms is widely espoused on the basis of the antioxidant effect of vitamin E.[7–10] However, concrete evidence of the protective effect of vitamin E is lacking. The chemopreventive effect of vitamin E on breast cancer has been studied by comparing the dietary intake of vitamin E or its tissue level of vitamin E and the cancer risk. London's group[11] conducted a case-control study and found that a high intake of dietary vitamin E is associated with a reduced risk of breast cancer. However, in the Canadian National Breast Cancer Screening Study,[12] vitamin E intake was not found to be associated with altered risk of breast cancer. In a prospective study by Hunter *et al.*,[13] large intake of vitamin E did not protect women from breast cancer. Thus, the preventive effect of vitamin E on human breast cancer is controversial. A similar study by Lee and Chen[14] on animals has shown some preventive effects of vitamin E against mammary tumors.

In the present study, we attempted to see whether or not vitamin E has any preventive effect on the development of spontaneous mammary tumors in acatalasemic mice by comparing the incidences of mammary tumor between mice given a vitamin E-deficient diet and those given a vitamin E-supplemented diet.

## MATERIALS AND METHODS

**Animals** Three strains of adult male and female C3H mice originally provided by Feinstein *et al.*,[15] normal (C3H/AnLCs$^{a}$Cs$^{a}$, 28 and 23, respectively), acatalasemic (C3H/AnLCs$^{b}$Cs$^{b}$, 32 and 38, respectively), and homozygous hypocatalasemic (C3H/AnLCs$^{c}$Cs$^{c}$, 31 and 22, respectively), were maintained in an air-conditioned room at the Animal Center for Medical Research, Okayama University Medical School. They were fed on

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Oriental MF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum* for 15 months after birth to evaluate the incidence of spontaneous mammary tumor. In the study of the preventive effect of vitamin E on spontaneous mammary tumor development, normal and acatalasemic female mice, four to five weeks of age and weighing 15–22g, were fed on vitamin E-deficient and -supplemented diets provided by Eisai Co., Tokyo, for 29 months. The compositions of the diets used, according to "The Report of the American Institute of Nutrition *Ad Hoc* Committee on Standards for Nutritional Studies"[16] are given in Table I.

**Determination of catalase activities of mammary glands and hemolysates** Catalase activities in mammary gland homogenates of pregnant mice were determined by the Purpald method of Johansson and Borg[17] and expressed as units (U)/mg protein, one unit being defined as 1.0 $\mu$mol of $H_2O_2$ decomposed per min at pH 7.0 and at 20°C. Catalase activities in hemolysates were determined by both the Purpald method and the perborate method,[18] and the activities were expressed, respectively, as U and as perborate units (PU) on the basis of g hemoglobin.

**Determination of protein contents** Protein contents of mammary gland homogenates were determined by the BCA method,[19] using a bicinchoninic acid kit (Sigma Chemical Co., Rockford, Ill.).

**Determination of hemoglobin concentration** Hemoglobin concentrations of hemolysates were determined by the modified method of van Kampen.[20]

**Statistical analysis** The $\chi^2$ test was used to determine the significance of differences in incidence of spontaneous mammary tumor between acatalasemic and hypocatalasemic mice and the log-rank test for analysis of the difference in cumulative incidence of mammary tumor between the vitamin E-deficient and vitamin E-supplemented diet groups of mice.

**Histological examination** Formalin-fixed spontaneous mammary tumors were processed for hematoxylin-eosin (HE) staining and examined under a light microscope.

Table I. Composition of Vitamin E-deficient and -supplemented Diets

| Components | VE deprived | VE supplemented |
|---|---|---|
| Vitamin-free casein | 20 | 20 |
| dl-Methionine | 0.3 | 0.3 |
| Glucose | 25 | 25 |
| Sucrose | 25 | 25 |
| Alpha-starch | 15 | 15 |
| Cellulose powder | 5 | 5 |
| AIN-vitamin (-VE) | 1 | 1 |
| AIN-mineral | 3.5 | 3.5 |
| Choline bitartrate | 0.2 | 0.2 |
| Distilled corn oil | 5 | 5 |
| dl-Alpha-tocopheryl nicotinate | 0 | 50 IU (58.5mg) |
| Total | 100 | 100 |

Compositions are given in percentages unless otherwise indicated. Prepared by Eisai Co. as AIN-76 purified diet.[16]

## RESULTS

Incidences of spontaneous mammary tumor in acatalasemic, hypocatalasemic and normal mice during the 15-month maintenance period on a regular laboratory chow are shown in Table II. Acatalasemic mice showed a higher incidence of spontaneous mammary tumor as compared with hypocatalasemic mice in both male and female groups. Female mice showed a higher incidence of spontaneous mammary tumor as compared with male mice among the acatalasemic and hypocatalasemic

Table II. Incidence of Spontaneous Mammary Tumor in Mice

| Mouse | Male | Female | Total |
|---|---|---|---|
| Acatalasemic | 31.3 (32) | 36.8 (38) | 34.3 (70) |
| Hypocatalasemic | 9.7 (31) | 22.7 (22) | 15.1 (53) |
| Normal | 0 (28) | 0 (23) | 0 (51) |
| $\chi^2$ test | $P<0.002$ | $P<0.004$ | $P<0.00001$ |

Values are given in percentages. ( ), the number of mice.

Table III. Catalase Activities of Blood and Mammary Gland in Mice

| Mouse | Catalase activity (U/mg protein)[a] | | Catalase activity (PU/g Hb)[b] |
|---|---|---|---|
| | Blood | Mammary gland of pregnant mice | Blood |
| Acatalasemic | 6.4±1.9 (16) | 2.2±0.8 (16) | 19.1±4.5 (8) |
| Hypocatalasemic | 19.7±1.7 (3) | 5.8±2.1 (4) | 211.3±80.4 (5) |
| Normal | 32.5±8.3 (16) | 11.7±2.1 (16) | 850.1±70.1 (9) |

*a*) The Purpald method. *b*) The perborate method.

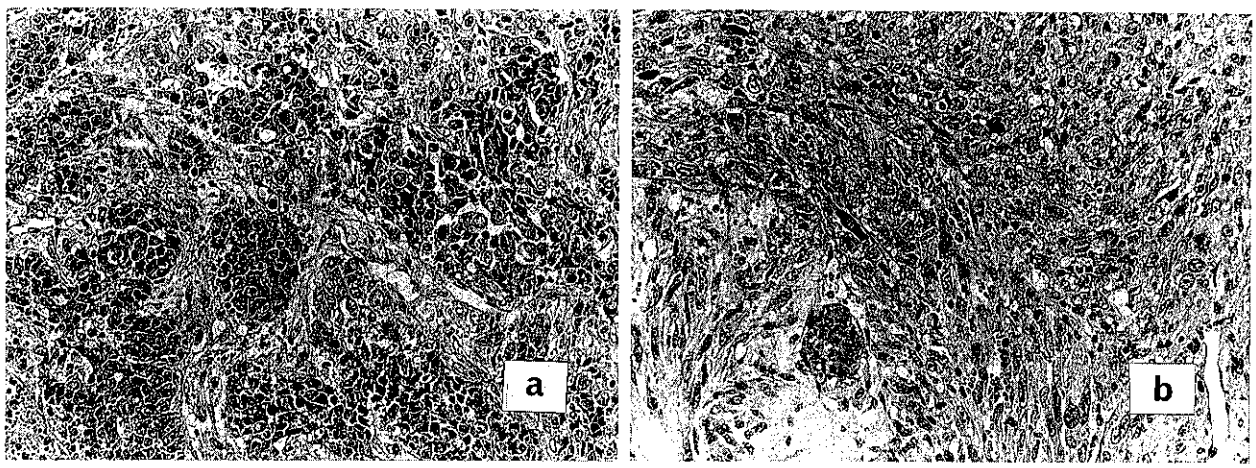


Fig. 1. Histopathology of mammary tumors (HE stain). a: Moderately differentiated papillotubular carcinoma (×200). b: Undifferentiated spindle cell-like carcinoma with severe cell dysplasia, mimicking a sarcoma (×200).

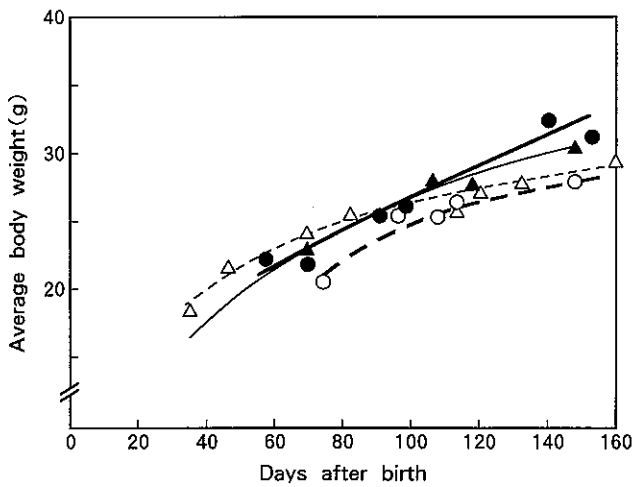


Fig. 2. Average body weights of acatalasemic mice in vitamin E-supplemented and -deficient groups. △, normal mice in vitamin E-deprived group (n=35); ▲, normal mice in vitamin E-supplemented group (n=37); ○, acatalasemic mice in vitamin E-deprived group (n=37); ●, acatalasemic mice in vitamin E-supplemented group (n=30).

groups. Normal mice did not develop mammary tumor during the observation period of 15 months.

The mean catalase activities in mammary gland homogenates of pregnant acatalasemic mice were significantly lower than those of hypocatalasemic and normal mice, and those of hypocatalasemic mice were significantly lower than those of the normal mice. Not only in mammary gland, but also in blood, the mean catalase activity in hypocatalasemic mice was approximately half that of the normal mice and that in acatalasemic mice was less than 20% that of the normal mice. Catalase activities of blood and mammary gland are shown in Table III.

In acatalasemic and hypocatalasemic mice, solitary tumors developed along the lines connecting mammary glands in close association with the skin. Light microscopic views of the tumor tissues are shown in Fig. 1. The extent of tumor dedifferentiation varied widely from well or moderately differentiated papillotubular carcinoma (Fig. 1a) to poorly or undifferentiated spindle cell-like carcinoma mimicking a sarcoma (Fig. 1b). These findings are compatible with mammary carcinomas of varying degrees of differentiation. Eccrine carcinoma and other sweat gland tumors may be excluded by these histological findings.

On the basis of these results, an attempt was made to see whether or not the spontaneous mammary tumor in acatalasemic mice can be prevented by supplementation with vitamin E. Two kinds of diet, vitamin E-deficient and vitamin E-supplemented, were employed. Acatalasemic mice were maintained on the two diets and the incidences of spontaneous mammary tumors in the two dietary groups were compared.

During the maintenance of animals on the diets, an average of 3.5 g of diet per animal per day was consumed. During this period, body weight gain of vitamin E-supplemented mice was slightly greater than that of the vitamin E-deficient mice, although there was no statistically significant difference between them (Fig. 2).

The cumulative incidence of spontaneous mammary tumor in vitamin E-deprived acatalasemic mice was significantly greater than that in vitamin E-supplemented acatalasemic mice, as shown in Fig. 3. The significance level of the difference between the two groups was deter-

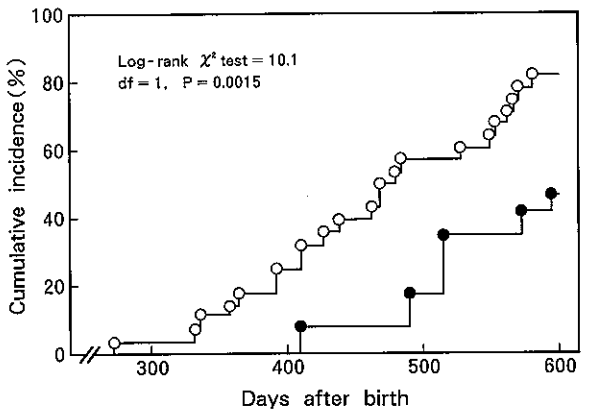


Fig. 3. Cumulative incidence of spontaneous mammary tumor in acatalasemic mice fed on vitamin E-deficient and -supplemented diets. ○, vitamin E-deprived group (n=28); ●, vitamin E-supplemented group (n=25).

mined by the log-rank $\chi^2$ test, and the $P$ value was smaller than 0.002.

Normal mice did not develop mammary tumors either on vitamin E-deficient or -supplemented diet.

## DISCUSSION

In the early part of the present study, we demonstrated increased incidence of mammary tumor in mice with lower catalase activities of the mammary gland, suggesting the involvement of free radicals in mammary carcinogenesis. On the basis of this result, female acatalasemic mice were used as a model animal to study the preventive effect of vitamin E against mammary tumor in mice.

The results of previous studies on the preventive effect of vitamin E on the development of breast cancer are equivocal, including those of animal experiments.[11-14] However, the present study showed a distinct preventive effect on spontaneous mammary tumor in acatalasemic mice. This is probably because the carcinogenesis in acatalasemic mouse, which was used as a model animal, is associated with increased free radical formation, and vitamin E is an antioxidant.

In most of the human studies,[7-13] the intervention period was too short, usually less than 10 years, and also intervention was started late, probably at a premalignant stage. Also, vitamin E deficiency may be essentially absent in most of the human populations studied, and this would obscure the results of trials involving human breast cancer.

The less catalase activity the model mice had in comparison with normal mice, the higher the incidence of mammary tumor in the model mice, indicating that $H_2O_2$ is possibly responsible for the spontaneous mammary tumorigenesis. Further, the incidence of spontaneous mammary tumor in vitamin E-supplemented acatalasemic mice is less than that in vitamin E-deprived acatalasemic mice, again suggesting the possible involvement of free radicals in the mammary tumorigenesis. In other words, ·OH radicals seem to play an important role in mammary tumorigenesis.

Approximately 400 human acatalasemic subjects, who are highly vulnerable to $H_2O_2$, have been found in Japan, and 0.23% of the total Japanese population is hypocatalasemic. The morbidity and mortality of breast cancer in Japan are reported to be increasing, probably because of changes in life style. Thus, subjects with acatalasemia or hypocatalasemia would have a risk factor of breast cancer, and vitamin E should be effective in preventing mammary tumorigenesis in the high-risk group of subjects with lower catalase activity.

It may be concluded, therefore, that vitamin E intrinsically has a protective effect against the development of mammary tumor, and this may apply not only to the acatalasemic mouse, but also to humans.

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