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Suppression of Breast Cancer Cell Growth with Grape Juice

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ABSTRACT

Aromatase (CYP19) converts androgens to estrogens. By synthesizing estrogens, aromatase (especially tumor aromatase) is thought to play an important role in promoting breast cancer growth in postmenopausal women. By inhibiting estrogen biosynthesis, aromatase inhibitors, including phytochemicals, are potential chemopreventive agents for breast cancer. Our recent aromatase inhibition experiments have revealed that grape juice contains compounds that inhibit aromatase. Inhibition kinetic analysis indicates that the active components in grape juice inhibit aromatase by competing for the binding of the substrate androstenedione. Results from cell culture experiments suggest that chemicals in grape juice can act as weak agonists/antagonists of estrogen receptor and as aromatase inhibitors. Finally, the breast cancer-protective action of grape juice was demonstrated with a nude mouse model using MCF-7aro, an aromatase-transfected MCF-7 cell line. It was found that the tumor size in mice fed (by gavage) with 0.5 ml of grape juice/day for 5 weeks is reduced 70% by comparing to the tumor size in the animals not fed with grape juice. Our finding suggests that grape juice may be useful in breast cancer prevention by inhibiting in situ *aromatase/estrogen biosynthesis.*

INTRODUCTION

In estrogen-dependent breast tumors, estrogens induce the expression of peptide growth factors that are responsible for the proliferative responses of cancer cells (Lippman et al., 1986; Bates et al., 1988). Aromatase, a cytochrome P450, is the enzyme synthesizing estrogens by catalyzing three consecutive hydroxylation reactions converting C19 androgens to aromatic C18 estrogenic steroids (see Scheme 1). Since aromatase is the enzyme responsible for the synthesis of estrogens, and estrogens have a major effect in the development of breast cancer, an abnormal expression of aromatase in breast cancer cells and/or surrounding adipose stromal cells may have a significant influence in breast tumor maintenance and growth in breast cancer patients. Cell culture and nude mouse studies from our and other laboratories using aromatase transfected MCF-7 and T-47D cells (Santner et al., 1993; Yue et al., 1994; Sun et al., 1997) have demonstrated that tumor aromatase can play a role in breast cancer maintenance and progression. It has also been suggested that *in situ* produced estrogen plays a more important role than circulating estradiol in breast tumor promotion (Tekmal et al., 1996; Yue et al., 1997). Estrogen synthesized by the tumor aromatase was shown to stimulate breast tumor growth in both an autocrine and a paracrine manner (Sun et al., 1997). Complete or partial tumor regression has been reported for postmenopausal patients treated with aromatase inhibitors, such as aminoglutethimide or 4-hydroxyandrostenedione (e.g., Coombes et al., 1987; Miller & O'Neill, 1987). Aromatase-inhibitor therapy is a second-line treatment for those who fail anti-estrogen therapy. Twenty to thirty percent of the patients who fail anti-estrogen treatment respond to aromatase-inhibitor treatment. Furthermore, using a *N*-methyl-*N*-nitrosourea (NMU)-induced rat mammary cancer model, vorozole, an aromatase inhibitor, has been shown to be a more effective chemopreventive agent against mammary cancer than 9-*cis*-retinoic acid, *N*-(4-hydroxyphenyl)-retinamide (4-HPR), and dehydroepiandosterone (DHEA) (Lubet et al., 1997; Steele et al., 1997). Vorozole at 0.08 mg/kg body weight/day (by gavage) reduced the number of mammary tumors/rat by 73%. NMU-induced tumor incidence is estrogen dependent (Kuman et al., 1990), and vorozole is thought to act as a chemopreventive agent by suppressing aromatase activity in the animal.

As pointed out in 1993 (Henderson, 1993), "One of the most dramatic features of breast cancer is the

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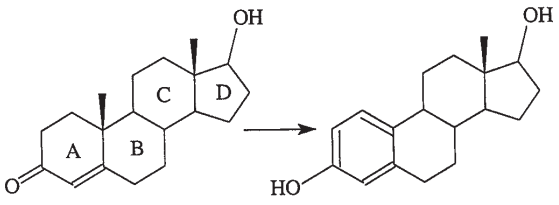


Testosterone Estradiol

Scheme 1. Aromatase converts androgens to estrogens.

disparity in incidence rates between highly westernized and non-westernized countries. Women born and raised in the United States are at least five times as likely to get breast cancer as women born and raised in Japan. But Japanese women increase their risk if they live in rapidly westernizing Japanese cities or emigrate and live in the United States. As is true with coronary heart disease, differences in diet are thought to be a major underlying factor in the different incidence rates of breast cancer, particularly among postmenopausal women." The hypothesis that differences in diet can change breast cancer and heart disease incidence in postmenopausal women is, at least in part, proposed based on results generated from studies on phytoestrogens. Phytoestrogens are plant chemicals that bind to the estrogen receptor and induce many components of estrogen action. The best known phytoestrogens are diphenolic chemicals, belonging to the classes of flavonoids, isoflavonoids, and lignans (Kartin et al., 1978; Kawaga, 1978; Verdeal & Ryan, 1979; London & Willet, 1989; Kaldas & Hughes, 1989; Howe et al., 1990; Adlercreutz et al., 1991; Whitlen & Naphtolin, 1991; Block et al., 1992). These compounds are thought to play a beneficial role in preventing breast cancer. They may function as antiestrogens or weak estrogens by competing with estrogens for binding to estrogen receptor (ER). However, we feel that it is also possible that some of these compounds may act in an indirect fashion by inhibiting aromatase activity, resulting in a decrease in the level of estrogen in women. This aspect may have been overlooked.

Phytoestrogens have been reported to be inhibitors of aromatase (Kellis & Vickery, 1984; Ibrahim & Abul-Hajj, 1990; Adlercreutz et al., 1993; Campbell & Kurzer, 1993; Wang et al., 1994); therefore, these chemicals may function as chemopreventive agents by inhibiting aromatase/estrogen biosynthesis in postmenopausal women. In our laboratory, we have examined the interaction of a number of flavone and isoflavone phytoestrogens with human aromatase and its mutants by inhibition kinetic analysis and computer modeling (Chen et al., 1997; Kao et al., 1998). Some of these compounds, such as chyrsin (5,7-dihydroxyflavone), inhibit aromatase with K_i values similar to that of aminoglutethimide, an aromatase inhibitor used clinically to treat breast cancer (Kao et al., 1998). Flavones have been found to suppress the androgen-dependent growth of aromatase transfected MCF-7 cells (i.e., MCF-7aro) (unpublished results). Since flavones have been shown to be effective inhibitors of aromatase, it is hypothesized that by inhibiting aromatase/estrogen biosynthesis, fruit juices which contain flavones are chemopreventive agents for breast cancer. We have evaluated the hypothesis by determining whether fruit juices repress aromatase activity. Our studies have revealed that grape juice can suppress breast tumor growth in nude mice by inhibiting *in situ* estrogen biosynthesis.

MATERIALS AND METHODS

Materials

Androstenedione, testosterone, tamoxifen, and estradiol were obtained from Sigma (St. Louis, MO). [1β-^{3}H]Androstenedione was purchased from NEN Dupont (Boston, MA). Fruit juices, including grape juices, were prepared by grinding fresh fruit in a blender, followed by a 30-min centrifugation at 3500 g to remove debris. Typically, 900 ml of grape juice can be produced from 1 kg of grape. For cell culture and animal studies, grape juice was autoclaved before use.

Aromatase inhibition studies were performed using human placental microsomal preparations which contain aromatase. Microsomes were prepared using a published procedure (Kadohama et al., 1992). MCF-7 and MCF-7aro cell lines were used in cell culture and animal studies. The MCF-7 cell line, an estrogen receptor-positive human breast adenocarcinoma cell line, was obtained from ATCC. The MCF-7aro cell line was prepared by human aromatase cDNA transfection of the MCF-7 cell line. The detailed procedure for the generation of MCF-7aro cell line was described by Sun et al. (1997).

Aromatase Assay

The aromatase assay was performed in the following manner. The substrate, androst-4-ene-3,17-dione [1β-^{3}H(N)] (specific activity, 24.7 Ci/mmol), was dissolved in serum free cell culture medium, filter-sterilized, and

then added to the assay mixture. The assay mixture (500 µl) contained placental microsomes (20 µg), ^{3}H-androstenedione (100 nM), progesterone (10 µM), BSA (0.1%) and potassium phosphate (67 mM, pH 7.4). Progestrone inhibited the 5α-reductase in the cell homogenates. After a 10 min incubation at room temperature, 50 µl of NADPH (12 mM) was introduced. The assay was continued by incubation at 37°C for 10 min, and stopped by the addition of 500 µl of 5% trichloroacetic acid. After a 5 min centrifugation, 1 ml of chloroform was added to the supernatant to extract unused substrate, followed by further mixing with dextran treated charcoal. Charcoal was removed by a brief centrifugation, and the supernatant containing the product, tritiated water, was counted.

Cell Culture Experiments

MCF-7 and MCF-7aro cells were grown in Eagle's MEM (phenol red-free) with non-essential amino acids, sodium pyruvate and Earle's salts, in the presence of 10% fetal calf serum (dextran-coated charcoal treated). Cells were plated at 2×10^5 cells/60 mm dish. The culture volume was 5 ml and the culture medium was changed on days 3 and 6. On day 8, the culture medium was removed from the dishes, and the cells were solublized with 0.5 N NaOH. The cell growth was evaluated by determining the total protein amounts of the control and treated samples. Protein concentration was determined using the method of Bradford (1976).

Nude Mouse Studies

Eight-week old sexually mature female nude mice were used in these experiments. The animals were injected with 1×10^7 MCF-7aro cells (or MCF-7 cells) in 0.2 ml of media in the subcuties of their flanks (two sites per animals). Normally, within 7 to 11 days after injection of cells, tumor nodules start appearing and reach an approximate size of 0.5 cm in diameter by 14–17 days post-inoculation. The treated animals were fed (by gavage) with 0.5 ml of green seedless grape juice daily, starting on the same day that MCF-7aro cells were injected.

RESULTS AND DISCUSSION

Aromatase Inhibition Studies

Our experiments revealed that among seven fruit juices tested, red seedless grape juice was the most effective in inhibiting the activity of human placental aromatase (see Fig. 1). A 50% inhibition could be achieved with 12 µl of red seedless grape juice (in a

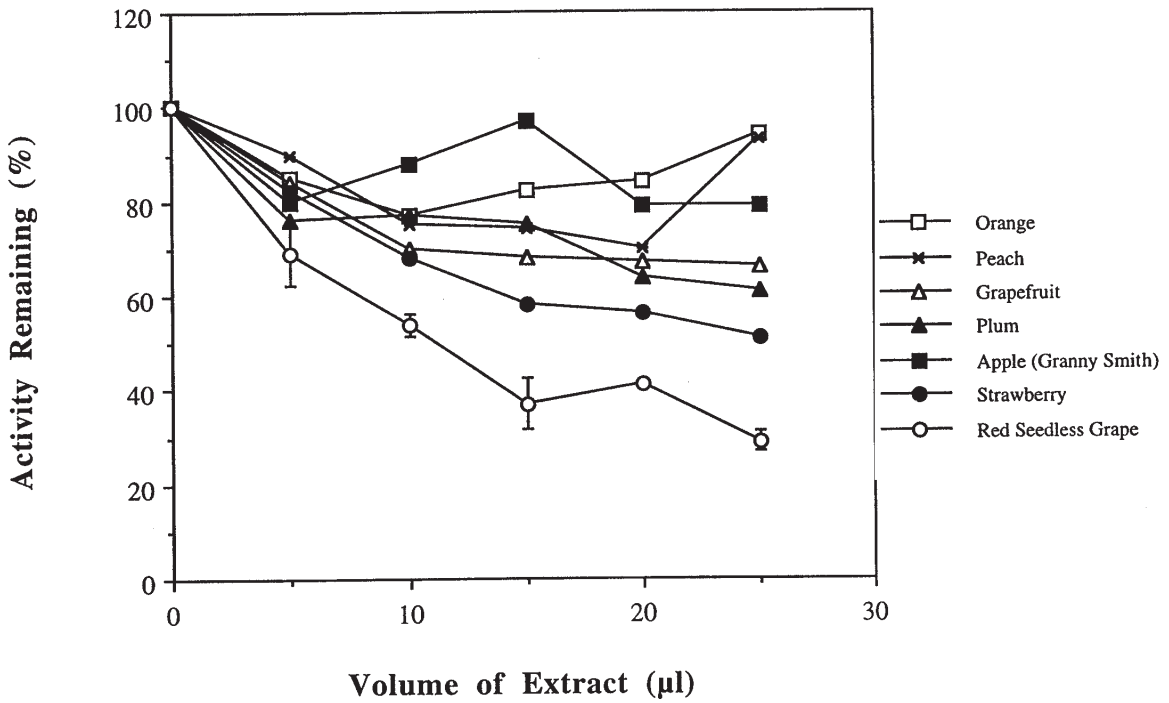


Fig. 1. Inhibition of aromatase by fruit juices. Human placental aromatase activity was measured in the presence of various amounts of fruit juices. The activity of untreated samples was taken as 100%. The assay conditions are provided in "Materials and Methods". The pH of the assay mixture was found not changed with varying amounts of fruit juice added. The measurements were performed in triplicate. For simplicity, only the standard errors for the inhibition curve of red seedless grape juice are shown.

total assay volume of 0.5 ml). We also found that aromatase was inhibited to different degrees with juices prepared from different types of grapes (see Fig. 2). Among the five kinds of grape juice, Champagne grape juice and Red Globe grape juice were ineffective in inhibiting aromatase. Black grape juice, green seedless grape juice, and red seedless grape juice inhibited aromatase in a dose-dependent manner. Furthermore, the potencies of green seedless grape juice and red seedless grape juice were not significantly different. Green seedless grape juice was used in our cell culture and animal studies. For the cell culture and animal experiments that will be discussed below, grape juice was autoclaved before use. Therefore, the active components in grape juice are thought to be heat-stable chemicals such as phytoestrogens.

Grape seed extract was also found to be capable of inhibiting aromatase. Christmas Rose grapes were used in this experiment. Seeds were removed from 22 grapes, suspended in 25 ml of water, and homogenized. The seed extract inhibited aromatase in a dose-dependent manner (Fig. 3) and a 55% inhibition was observed with 5 µl of the seed extract. Twenty-two grapes produced 94 ml juice and a 50% inhibition resulted when 20 µl of juice were added to the assay mixture. Since we have not yet identified the active components in juice or seed extract that are responsible to the inhibition of aromatase, it is difficult to compare the relative potency between the juice and seed extract.

Since Jang et al. (1997) found that resveratrol, a phytoalexin present in grape, has cancer chemopreventive activity, we were interested in knowing whether this compound is an inhibitor of aromatase. The inhibition profile analysis revealed that resveratrol is a relatively poor inhibitor of aromatase – a 64% inhibition was observed at 0.1 mM of resveratrol. These results suggest that resveratrol is not the component in grape juice that is responsible for the inhibition of aromatase. Such a conclusion was further supported by the fact that resveratrol was barely detected in the green seedless grape juice used for our studies utilizing high performance liquid chromatography (HPLC) with an electrochemical detection (unpublished results).

Inhibition kinetic analysis has revealed that green seedless grape juice inhibits aromatase by competing for the binding of the substrate androstenedione (Fig. 4). Since the preliminary examination of the fractions from reverse phase HPLC suggests the presence of several active components which suppress aromatase activity, the results from kinetic analysis indicate that these compounds inhibit aromatase with similar potencies. While we do not know the concentrations of the active components in grape juice, the secondary plot as shown in the inset in Fig. 4 reveals that they are very effective in inhibiting aromatase; the K_i value should be

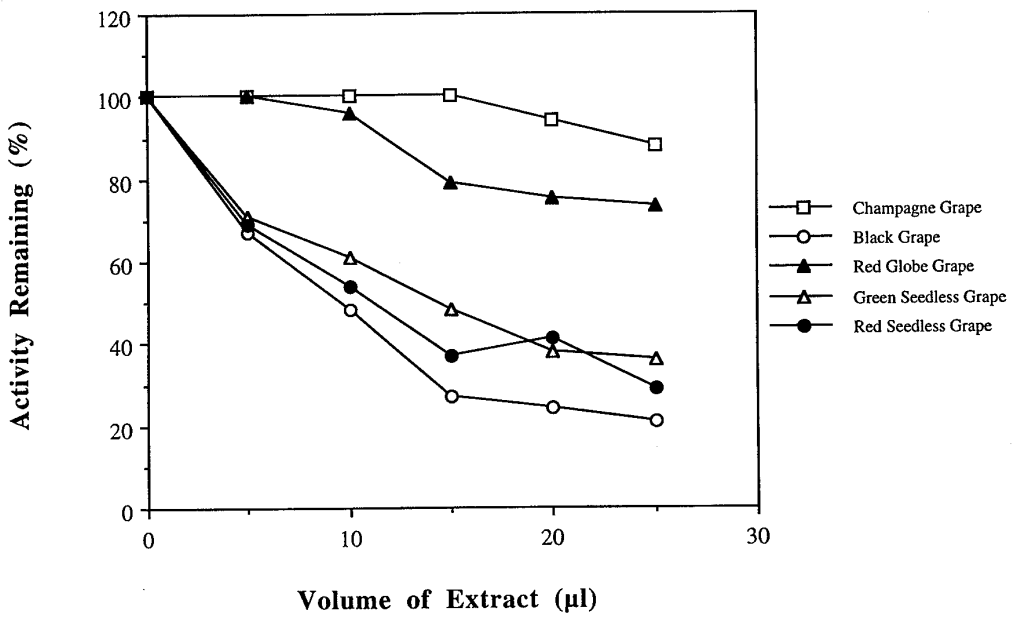


Fig. 2. Inhibition of aromatase by different grape juices. The experiments were performed in the identical manner as those described for Fig. 1, except different grape juices were used.

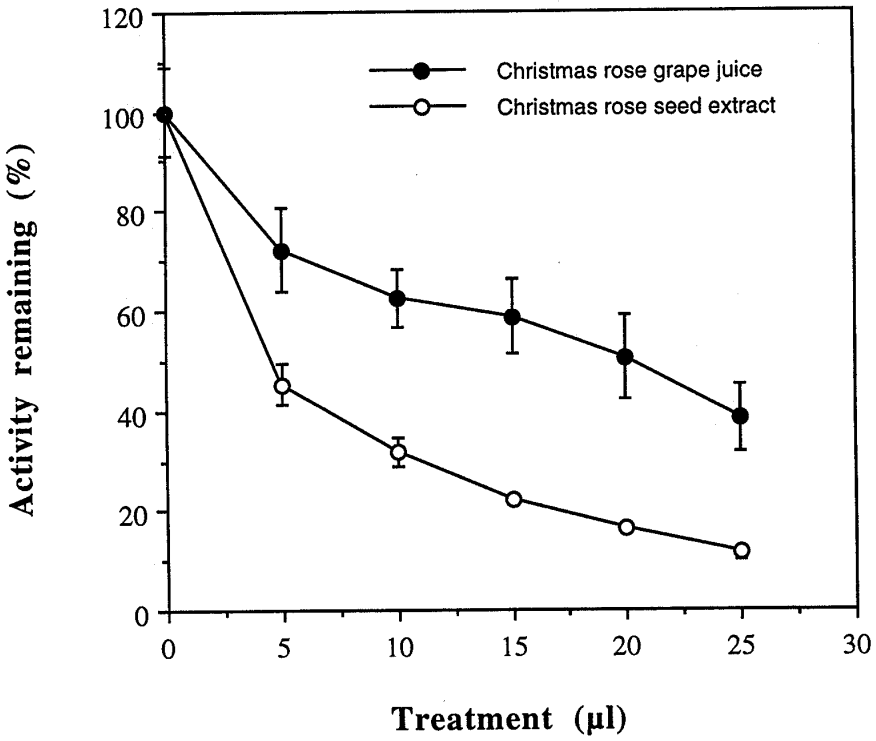


Fig. 3. Inhibition of aromatase by Christmas Rose grape juice and seed extract. The grape juice and seed extract were prepared as described in the text.

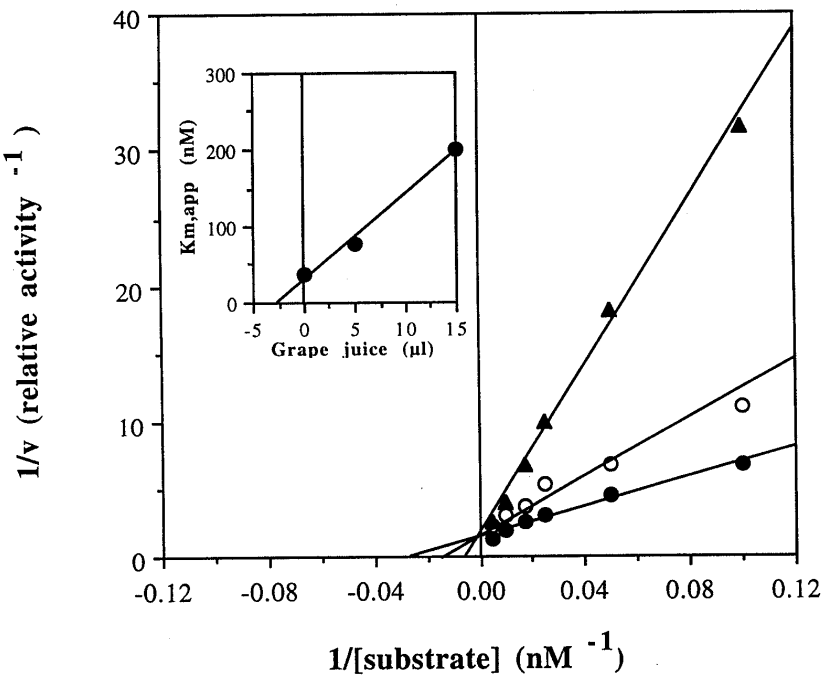
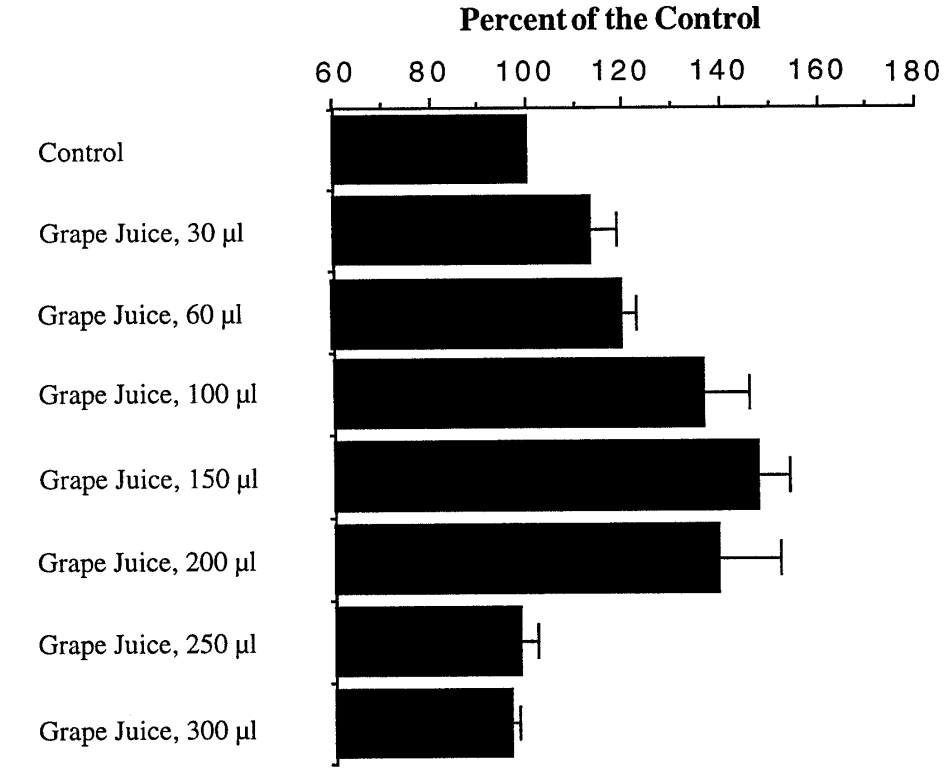


Fig. 4. Competitive inhibition of aromatase with respect to androstenedione by grape juice. The enzyme assays were performed in the presence of 0 (●), 5 µl (○), and 15 µl (▲) green seedless grape juice.



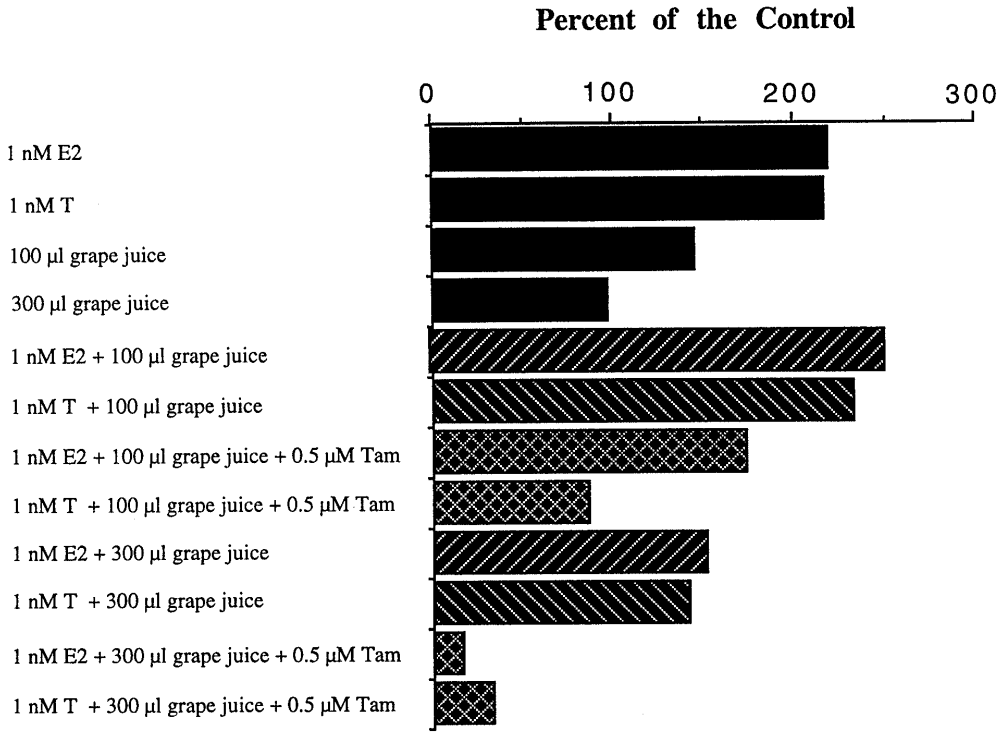


Fig. 5. Effect of grape juice on the growth of MCF-7aro cells. A. The cells were cultured for eight days in the presence of grape juice in the indicated quantities. The cells were lyzed in 0.5 N NaOH. The protein concentration was determined. The total protein amount of the control was taken as 100%. B. The effects of estradiol (E2), testosterone (T), grape juice, and tamoxifen (Tam) on the growth of MCF-7aro cells were examined. The cells were lyzed and the protein concentration was determined after the cells were treated for eight days. The total protein amount of the control was taken as 100%.

the concentration of the components calculated from 3 µl of the grape juice.

In summary, the results from the *in vitro* experiments indicate that grape juice contains compounds which can inhibit aromatase/estrogen synthesis in a potent and selective manner.

Cell Culture Studies

Cell culture experiments were performed to further study the action of grape juice on the growth of aromatase-expressing breast cancer cells. The experiments were performed with the MCF-7aro cell line which is estrogen receptor- and aromatase-positive (Sun et al., 1997). The aromatase activity (V_{max}) in the MCF-7aro cell line was determined to be 73 ± 6 pmol/h/mg. MCF-7aro cells were incubated with grape juice at various amounts for 8 days. Grape juice in a quantity up to 150 µl/5 ml was found to stimulate and a quantity higher than 150 µl/5 ml to suppress the growth of MCF-7aro cells (Fig. 5A).

In order to better understand the action of grape juice, experiments including estrogen, androgen, and tamoxifen were carried out. MCF-7aro cell growth increased to 218 and 216% of the control by incubation with 1 nM estradiol and 1 nM testosterone, respectively (see Fig. 5B). Since MCF-7aro cells are estrogen receptor-positive, it is expected that the growth of the cells responds to estradiol treatment. In addition, MCF-7aro cells are androgen-responsive because the expressed aromatase converts androgen (i.e., testosterone) to estrogen (i.e., estradiol). Under the identical culture conditions, the cell amounts were 144 and 96% of the control when incubated with 100 µl and 300 µl grape juice, respectively. It is thought that grape juice contains phytoestrogens which behave as partial agonists/antagonists of estrogen receptor. At a low level (i.e., 100 µl/5 ml), phytochemicals in grape juice, acting as weak agonists, stimulated the growth of MCF-7aro cells. An additive effect was found when MCF-7aro cells were treated with 100 µl grape juice plus 1 nM estradiol or 1 nM testosterone (251 or 231%, respectively). The induction could be suppressed by tamoxifen (at 0.5 µM); the growth of the samples that were treated with 0.5 µM tamoxifen, 100 µl grape juice plus 1 nM estradiol or 1 nM testosterone were 172 or 85% of the control, respectively. As a control, grape juice at 100 µl did not affect the growth of MCF-10A cells which are estrogen receptor-negative human breast luminal ductal cells. It is difficult to explain the results that were generated with 300 µl grape juice. In this amount, the growth stimulating effect of grape juice may be overcome by the inhibitory action such as the inhibition of aromatase/estrogen biosynthesis and/or the antagonistic action on estrogen receptor. Such an explanation was supported by the fact that the growth of MCF-7aro cells was reduced to 151 or 141% of the control when the cells were treated with 300 µl grape juice plus 1 nM estradiol or 1 nM testosterone, respectively, when compared to the cell amounts when MCF-7aro cells were only treated with 1 nM estradiol or 1 nM testosterone (i.e., 218 and 216%). Interestingly, it was observed that 0.5 µM tamoxifen, 300 µl grape juice plus 1 nM estradiol or 1 nM testosterone reduced the growth of MCF-7aro to 16 or 32% of the

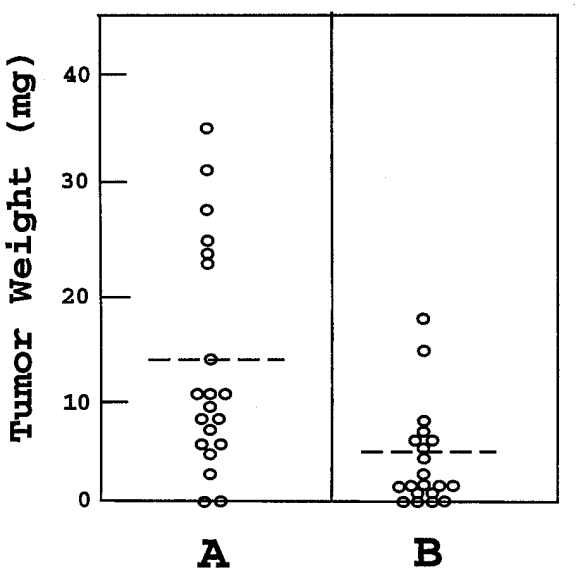


Fig. 6. Suppression of MCF-7aro-induced tumor formation in nude mice by grape juice. The detailed procedure is described in "Materials and Methods". In this study, 10 mice were controls (A) and 10 mice were fed 0.5 ml green seedless grape juice/day for five weeks (B). Grape juice feeding started on the same day that MCF-7aro cells were injected. There were two injection sites per animal, and each site was injected with 4×10^6 cells. At the end of five weeks, mice were euthanized, and tumors were removed and weighed. The average weights of tumors from groups A and B were 14.6 and 4.5 mg, respectively (indicated by dotted lines). The differences between the control and treated group are statistically significant ($p = 0.03$ using a Wilcoxon test).

Table 1. Body weights before and after treatments.

Treatment	Body weight (g)	
	Before	After
Control, MCF-7	24.4 ± 2	26.0 ± 1 (107%)
Grape Juice, MCF-7	21.8 ± 2	24.4 ± 3 (112%)
Control, MCF-7aro	23.3 ± 1	25.4 ± 1 (109%)
Grape Juice, MCF-7aro	22.5 ± 2	24.5 ± 2 (109%)

control, respectively, suggesting a synergistic action between tamoxifen and grape juice. The results of our cell culture experiments suggest that grape juice contains chemicals that modulate breast cancer growth by at least two mechanisms. These phytochemicals may behave as aromatase inhibitors or weak agonists/antagonists of estrogen receptor.

Nude Mouse Studies

Our *in vitro* enzyme assay and cell culture studies have demonstrated that grape juice contains chemicals that can modulate the action of aromatase and estrogen receptor in breast cancer cells, leading to a suppression of the breast cancer cell growth. However, these effects could be different *in vivo* due to metabolic conversion processes in liver or in tumors. Therefore, it is critical to perform *in vivo* studies to determine whether grape juice indeed has a protective effect against breast cancer. During the last two years, we have established a nude mouse model using MCF-7aro cells (described in the "Materials and Methods" section). Tumors form within one month after inoculating MCF-7aro cells into eight-week old female nude mice, and tumor formation can be suppressed by injecting the animals with aromatase inhibitors such as 4-hydroxyandrostenedione. This nude mouse model has been used to evaluate the action of grape juice *in vivo*. It was found that the average tumor weight in 10 mice fed (by gavage) with 0.5 ml of green seedless grape juice/day for 5 weeks was 30% of the tumor size in the animals not fed with grape juice (Fig. 6). Interestingly, grape juice had a weaker effect on tumor formation in animals inoculated with untransfected MCF-7 cells than in the animals inoculated with MCF-7aro; the average tumor weight in mice injected with MCF-7 cells and fed with grape juice was 74% that of the animals not fed with grape juice (results not shown). Such results indicate that grape juice suppresses breast tumor growth mainly by inhibiting *in situ* aromatase/estrogen biosynthesis. As indicated in Table 1, the increases in body weights of animals fed with grape juice were similar to those of the control animals.

In summary, by synthesizing estrogens, aromatase (especially tumor aromatase) is thought to play an important role in promoting breast cancer growth in postmenopausal women. By inhibiting estrogen biosynthesis, aromatase inhibitors including phytochemicals are potential chemopreventive agents for breast cancer. Finally, our recent studies have revealed that grape juice may be useful in breast cancer prevention by inhibiting aromatase/estrogen biosynthesis. While estrogen promotes breast cancer growth, it has many beneficial effects in postmenopausal women, such as reduction of risk of coronary heart disease, prevention of bone fractures, and protection against Alzheimer's disease (Davidson, 1996). Our studies have found that grape juice contains not only compounds inhibiting endogeneous estrogen biosynthesis/aromatase, but also chemicals with weak estrogenic action. Therefore, grape juice would be a favored chemopreventive agent in that it inhibits aromatase to prevent undesired effects of estrogen as well as having weak estrogenic action to protect postmenopausal women against heart disease, osteoporosis, and Alzheimer's disease.

While our findings on grape juice are promising, more extensive studies are needed to confirm the chemopreventive effect of grape juice against breast cancer. Animal experiments involving time-course and dose-response studies are being designed to characterize more completely the breast cancer preventive action of grape juice. In addition, experiments are being performed to identify the active components in grape juice that inhibit aromatase and/or bind to estrogen receptor. The latter experiments not only will lead to an understanding of the molecular basis of aromatase inhibition by grape juice, but also allow us to design a potent and selective breast cancer prevention strategy.

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