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Abstract. Effects of vorozole, a potent and specific non-steroidal aromatase inhibitor, were evaluated on female Sprague-Dawley (SD) rats with 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors. Vorozole at a dose of 0.25, 1.0 and 4.0 mg/kg was orally administered once a day for 28 consecutive days. A significant regression in tumor size was observed in each treated group at 1,2,3 and 4 weeks after the start of treatment compared with control group. Tissue insulin-like growth factor I (IGF-I) in the DMBA-induced tumors in each treated group significantly decreased in a dose dependent fashion compared with control group. These results show the mechanism of vorozole in DMBA-induced rat mammary tumors.

Introduction
Breast cancer is frequently estrogen-responsive (1) since this cancer contains estrogen receptors and its growth is stimulated by estrogen. Autocrine and paracrine mechanisms are thought to be involved in the estrogen stimulation of breast cancer cell proliferation (2). Estrogens regulate production of specific growth factors in hormone-dependent human breast cancer (3-7). However the mechanisms of estrogen responsiveness are not fully understood.

The insulin-like growth factors (IGFs) have important roles in normal cellular growth and development. The IGFs have also been implicated in regulation of tumor cell growth (8). Two ligands, IGF-I and IGF-II, have been identified that are homologous to each other and to proinsulin (9,10). They interact with at least two specific cell surface receptors. The type I IGF receptor has a high affinity for IGF-I, a somewhat lower affinity for IGF-II, and a low affinity for insulin. The type II IGF receptor has a high affinity for IGF-II and a moderate affinity for IGF-I (11). The major mitogenic effects of the IGFs are thought to be mediated through the type I IGF receptor (12). Approximately 10-fold higher concentrations of IGF-II than IGF-I were required to stimulate MC3T3-E1 cell proliferation (13). Since IGF-I is a potent mitogen for these cells, local stromal production of IGF-I causes growth of breast cancer by paracrine stimulation (12).

Aromatase is a cytochrome P450 enzyme that catalyzes the conversion of androstenedione to estrone and testosterone to estradiol (22-24). The major sites of estrogen biosynthesis are the ovarian granulosa cells in premenopausal women and adipose tissue in postmenopausal women (25). Some authors found aromatase activities in approximately two thirds of human breast cancer (26,27). Inhibition of aromatase has been found to be of therapeutic value in the endocrine treatment of breast cancer in postmenopausal women (28-30). Previous experiments also showed that binding of IGF-I to the type I IGF receptor and type 1 IGF receptor mRNA levels are stimulated by estradiol (13).

Vorozole (R3842) is a competitive aromatase enzyme that acts specifically to lower production of estrogens and does not compete with either estrogen or androgen receptors (31-33). This new triazole derivative reduces the aromatase activity in rat and human ovarian granulosa cells, and human stromal cells from adipose tissue (34,35).

We have examined whether vorozole alters local production of IGF-I in DMBA-induced mammary cancers of rats as a premenopausal breast cancer model.

Materials and methods

Test compound. Vorozole (8-ethyl-1(2H)-1,2,4-triazole-4(3H)-[1H]-1-methyl-1H-benzotriazole) (R3842) was dissolved in 20% polyethylene glycol for oral administration.

DMBA-induced rat mammary cancers. DMBA-induced rat mammary cancers were obtained as previously described (36). Rats developing solid mammary tumors within 6 - 10 weeks after the DMBA administration were divided into 4 groups.
every week for treatment. If a rat bore several tumors at the start of treatment, only the largest tumor was evaluated. The animals were housed in air-conditioned quarters illuminated from 6 a.m. to 6 p.m. and had free access to pellets (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water.

Treatment groups of rats. The rats were randomized into four groups with no significant difference of tumor size or average body weight among groups (15 rats per group). Vorozole was given by gavage at a dose of 0.25 (V0.25), 1.0 (V1.0), and 4.0 (V4.0) mg/kg body weight once a day for consecutive 4 weeks. Control group (C) received 1 ml of 20% polyethylene glycol daily for the same period. The long and perpendicular axes of tumors were measured conventionally once a week with calipers. The percentage change of tumor size was calculated by product of the long and perpendicular axes. At the end of treatment for 4 weeks all the animals were sacrificed under other anesthesia. The tumors were excised for assays, and sterile and ovarian wet weights were recorded.

Media and chemicals. IGF-I enzyme immunoassay (EIA) was performed using IGF-I EIA kit, and pre-treatment before IGF-I EIA was performed using pre-treatment reagent (acid-ethanol) from Fujisawa Pharmaceutical Co., Ltd (Tokyo, Japan). ER-EIA was performed using Abbott ER-EIA monoclonal antibodies from Abbott Laboratories, Abbott Park, IL. The Bio-Rad protein assay dye reagent was purchased from Nippon Bio-Rad (Tokyo, Japan). The other reagents were of the highest grade available.

Sample preparation. Before assay, the specimens were cleared of fat and debris. The specimens had a wet weight of 100-300 mg. The extraction buffer contained 10 mM Tris- 
HCl, pH 7.5, 0.25 M NaCl, 5 mM EDTA, 50 mM NaCl, 100 g/L sodium pyrophosphate, 50 mM sodium fluoride, 2 mg/ml leupeptin, Phosphomonoisoflouride (PMSF), 1 mM) was added just before use. Samples were homogenized in four volumes of extraction buffer using a micro-homogenizer NS-310E (Nihon-On, Tokyo, Japan) on ice. All debris and nuclei were removed by centrifugation at 900 × g at 4°C for 10 min and supernatant was used as whole cell lysate. IGF-binding protein was dissolved by acid-ethanol method from this lysate before EIA. The supernatant obtained was centrifuged at 105,000 × g at 4°C for 60 min and used as the cytosol fraction. ER-EIA was performed using this fraction. EIA for IGF-I EIA was assayed by ER and IGF-I kit respectively according to the instructions of the manufacturers. Each assay was carried out in duplicate.

Protein assays. Protein concentrations were determined with bovine serum albumin as the reference standard using Bio-Rad protein assay dye reagent (37).

Statistical analysis. Statistical analysis was performed using StatView 4.0 software (Abacus Concepts, Inc). The data are expressed as means±SEM. We examined parameters among groups by using analysis of variance and Fisher's PLSD test as Post-Hoc test. A p-value less than 0.05 was considered to be significant.

Results

Effects on DMBA-induced rat mammary cancer. A significant suppression of tumor growth was observed in each group 1, 2, 3 and 4 weeks after the start of treatment (p<0.0001 vs control). There were no significant differences among vorozole administration groups in the tumor regression (Fig.1).

IGF-I in rat mammary cancer Tissue IGF-I were 0.370±0.022 ng/mg protein in control, 0.308±0.019 ng/mg protein in V0.25, 0.275±0.021 ng/mg protein in V1.0, and 0.266±0.022 ng/mg protein in V4.0 group, respectively. A significant decrease of tissue IGF-I was observed dose-dependently in the groups treated with vorozole (p<0.05 V0.25 vs control, p<0.01 V1.0 and V4.0 vs control).
probable resulted to be due to the shorter duration of aromatase inhibition (34).

Since other studies have shown that vorozole administration lowers aromatase activities and estrogens in serum or tissue (18,19), we have not measured these activities and estrogens. In the present study, the weights of the various mammary gland groups increased significantly compared with those in control group. And then V4.0 group showed remarkable body weight gain. The body weight gain which was observed in the group treated with aromatase inhibitor can be explained to have caused by estrogen deprivation (39). Further, doses of 0.25, 1.0, 4.0 mg/kg per day increased significantly uterine wet weights. These data inferred that vorozole lowered circulating estrogens in female SD rats.

We showed that tissue IGF-I levels significantly decreased in the rats treated with vorozole. Serum IGF-I level is strongly influenced by hepatic production of IGF-I. Growth hormone is the chief regulator of IGF-I gene transcription in the liver (14,15). On the other hand, in local tissues IGF-I gene is expressed by stromal cells of primary human breast cancers (16). Stromal cells of breast cancers are capable of producing IGF-I not only in response to various physiological signals (17-21), but also in response to pathological signals originating from neighbouring tumor cells (16). DMBA-induced rat mammary tumors have been reported to be type I IGF receptor positive (44). It is logical to propose that IGF-I receptor-positive breast cancer cells are influenced by tissue IGF-I bioactivity in their microenvironment rather than by serum IGF-I concentration. So, quantitative measurements of tissue IGF-I levels in regression DMBA-induced rat mammary cancer may elucidate paracrine mechanisms of cell proliferation.

Pekonen et al reported that there was a significant positive correlation between type I IGF receptor and ER in human breast cancer tissue (45). We also observed that ER in DMBA-induced rat mammary tumors treated with vorozole were positive similarly to those in control group. But, ER levels in V1.0 and V4.0 group seemed to be slightly upregulated because estrogen levels were decreased by vorozole. Therefore it is likely that estrogenic effects of vorozole are influenced by both IGF-I and estrogens.

Ovarian wet weights in vorozole groups showed no difference compared with those in control group. This fact is consistent with the report by DeCoster and co-authors that after vorozole treatment the rise in gonadotropins was less pronounced than after ovariectomy (33). On the other hand, after treatment with, for instance, tretinoid, aromatase inhibitors (tamoxifen, CSE 16495A), serum gonadotropins levels and ovarian wet weights increased (42,43). It is likely that vorozole has a different action to genistein.

We conclude that an inhibitory effect on hormone-dependent tumor growth, the body weight gain of rats, the reversible effect on uterine growth, and the inhibitory effect on tissue IGF-I levels observed in the present study following vorozole administration were caused by an aromatase inhibitory action of the drug and that vorozole is an effective agent for premenopausal as well as post- menopausal patients extrapolated from the data obtained in the present animal model.

References
