Effects of Hormones on Tumor Growth and Immunoreactive Insulin-like Growth Factor-1 of Estrogen Receptor-positive Human Breast Cancer (MCF-7) Transplanted in Nude Mice

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The effects of estrogens and tamoxifen were analyzed on estrogen receptor-positive human breast cancer (MCF-7) transplanted into athymic nude mice. It was found that (1) the tumor growth and the proportion of ³H-thymidine-labeled cells were significantly increased in the 17 β -estradiol dipropionate (E₂) group, but significantly decreased in the tamoxifen (TAM) group with respect to the control group, and (2) the tumor content of insulin-like growth factor-1 (IGF-1) and the rate of IGF-1-positive cells were significantly lower in the E₂ group, but significantly higher in the TAM group than in the control group. It was concluded that the tumor content of IGF-1 and the proportion of IGF-1-positive cells were inversely correlated to the tumor growth and the ³H-thymidine labeling index *in vivo*.

Key words: Hormones — Insulin-like growth factor-1 — MCF-7

Recently, it has been hypothesized that the hormonal control of breast cancer is mediated by autocrine and paracrine growth factors, such as epidermal growth factor (EGF), transforming growth factor alpha (TGF- α) and insulin-like growth factor 1 (IGF-1).¹⁾ It has been shown that IGF-1 stimulates the growth of breast cancer cells such as MCF-7 cell line *in vitro*,²⁻⁴⁾ and that cancer cells are composed of heterogeneous tumor cell populations with varying IGF-1 levels in a study using IGF-1 immunocytochemical staining.⁵⁾ In a recent *in vitro* study, however, it has been shown that stromal tissue primarily produces IGF-1, which in turn stimulates the growth of cancer cells in a paracrine manner.⁶⁾ Moreover, serum IGF-1 levels are closely controlled by growth hormone *in vivo*. It has been shown in postmenopausal patients that serum IGF-1 decreases with ethinylestradiol treatment,^{7,8)} and that the estrogen inhibits the tendency for IGF-1 to rise toward the pretreatment level with the addition of tamoxifen.⁷⁾ The roles of estrogens and tamoxifen in modulating the serum levels and tumor contents of IGF-1 are complex. Thus, it was of interest to analyze IGF-1 function in breast cancer *in vivo*. In this paper, the effects of hormonal agents on MCF-7 transplanted into athymic nude mice were analyzed in terms of tumor growth, cell kinetics and IGF-1 status.

Approximately 2-mm³ tissue fragments of MCF-7 tumor were transplanted into the back of six- to seven-week-old, female nude mice (BALB/c nu/nu), weighing 18–20 g. Each of the mice then received 0.1 ml of E.P. Hormone Depot (Teikoku Hormone Manufacturing Co., Tokyo), composed of 1 mg/ml 17 β -estradiol dipropionate and 50 mg/ml hydroxyprogesterone caproate,

intramuscularly once weekly. After the tumor began to grow, the mice were divided into the following 3 groups, and examined for the effects of the hormones: (1) 17 β -estradiol dipropionate (E₂) group in which the mice were injected with 5 mg/kg E₂, dissolved in sesame oil, once weekly; (2) tamoxifen (TAM) group in which the mice were intramuscularly injected with 5 mg/kg TAM, dissolved in sesame oil, twice weekly; (3) control group in which the mice were given no hormones. During the endocrine treatments, the tumors were measured in 2 perpendicular dimensions with a vernier caliper, and the measurements were recorded 3 times weekly. The endocrine treatments were continued for 3 weeks, and each group was divided into two subgroups. One of the subgroups was injected intraperitoneally with 50 μ Ci/kg of ³H-thymidine (specific activity: 23.6 Ci/mmol; radioactivity concentration: 1.0 mCi/ml; Amersham Co., Buckinghamshire, England). One hour later, blood samples were taken from the mice given no ³H-thymidine, and immediately stored at –80°C for IGF-1 assay. All the mice were then killed, and their tumors were removed, and weighed. Tumors from the mice given no ³H-thymidine were immediately stored at –80°C for use in radioimmunoassay (RIA) for IGF-1. The tumors from the group of mice given ³H-thymidine were fixed in 10% formaldehyde and phosphate-buffered saline (PBS), and embedded in paraffin for immunocytochemical staining (ICA) of the IGF-1 with or without ³H-thymidine autoradiography.

Estimated tumor weight (ETW) was calculated by using the following equation: ETW = largest diameter $\times$ smallest diameter²/2 (mg). Tumor doubling time (Dd)

was calculated by using the following equation: $Dd = t \ln_{1/2} (\ln Vt - \ln V0)$, where t is the time period of endocrine treatment; Vt is ETW at the termination of endocrine treatment, and $V0$ is ETW at the initiation of endocrine treatment. The serum levels of IGF-1 were measured by double antibody radioimmunoassay. On the other hand, tumor tissues were cut into small pieces, homogenized at 0°C, and centrifuged at 10,000 rpm in a refrigerated centrifuge for 30 min. The supernatant IGF-1 was then determined with a double antibody radioimmunoassay kit available from Nichols Institute Diagnostics (San Juan Capistrano, CA).⁹⁾ Polyclonal human IGF-1 antibody (amino acids 1 to 70, IGF-1) (KabiGen AB, Stockholm, Sweden) was used at a 1:1000 dilution. The formalin-fixed, paraffin-embedded tissue specimens were sectioned at 4 μ m. After deparaffinization, the endogenous peroxidase activity in the sections was abolished with a 0.3% solution of H_2O_2 . The specimens were then immunostained by the avidin-biotin-peroxidase complex method using a Vectastain ABC kit (Vector Lab., Burlingame, CA). The cells were classed on the basis of staining intensity into two types: IGF-1-positive, and IGF-1-negative. The rate of IGF-1-positive cells was determined by counting the number of IGF-1-positive cells per 1000 cells. The IGF-1 immunohistochemically stained sections were mounted on slides, and dried. In a darkroom, the dried sections were dipped in Konica NR-M2 Nuclear Track Emulsion (Konica Co., Tokyo) and stored at 4°C for exposure. After 4 weeks of exposure, the tissues were photographically processed, stained with hematoxylin and eosin, and permanently mounted. Then, a cell was taken as labeled if the number of silver grains over each nucleus exceeded 10. The labeling index was determined by counting the number of labeled cells per 1000 cells.

Table I show the effects of the hormonal agents on tumor growth and cell kinetics. The wet tumor weight was significantly smaller in the TAM group ($P < 0.01$), but significantly greater in the E_2 group ($P < 0.01$) than in the control group. The tumor doubling time was

significantly shorter in the E_2 group ($P < 0.01$), but significantly longer in the TAM group ($P < 0.01$) than in the control group. The labeling index was significantly higher in the E_2 group ($P < 0.05$), but significantly lower in TAM group ($P < 0.05$) than in the control group. Table I also shows the effects of hormonal agents on serum level and tumor content of IGF-1 and the proportion of IGF-1-positive cells. The serum level of IGF-1 was significantly lower in the E_2 group ($P < 0.01$) than in the control group, while there was no significant difference in this parameter between the TAM and control groups. The tumor content of IGF-1 was significantly smaller in the E_2 group ($P < 0.01$), but significantly greater in the TAM group ($P < 0.01$) than in the control group. On the other hand, staining for IGF-1 was found in the cytoplasm, with varying intensity. The average rate of IGF-1-positive cells was significantly lower in the E_2

Fig. 1. IGF-1-immunocytochemically stained 3H -thymidine autoradiography. The tumor is composed of IGF-1-positive cells with 3H -thymidine uptake (arrow A), IGF-1-positive cells without 3H -thymidine uptake, IGF-1-negative cells with 3H -thymidine uptake (arrow B), and IGF-1-negative cells without 3H -thymidine uptake (original magnification: $\times 400$).

Table I. Effects of Hormonal Agents on Tumor Weight, Tumor Doubling Time, Cell Kinetics, IGF-1 Level in Serum and Tumors and IGF-1-positive Cell Rate

Agents	Wet tumor weight (mg)	Tumor doubling time (days)	Labeling index (%)	IGF-1 in serum (U/ml)	IGF-1 in tumor (U/g)	IGF-1-positive cells (%)
E_2	615 $\pm$ 214 (36) ^{a)}	10 $\pm$ 3 (36) ^{a)}	31 $\pm$ 4 (8) ^{c)}	1.2 $\pm$ 0.1 (8) ^{a)}	0.7 $\pm$ 0.1 (18) ^{a)}	8 $\pm$ 8 (8) ^{a)}
TAM	269 $\pm$ 102 (36) ^{b)}	23 $\pm$ 14 (36) ^{b)}	15 $\pm$ 5 (9) ^{d)}	1.5 $\pm$ 0.2 (8)	1.4 $\pm$ 0.4 (24) ^{b)}	70 $\pm$ 10 (9) ^{b)}
Control	419 $\pm$ 161 (28)	13 $\pm$ 4 (28)	22 $\pm$ 5 (7)	1.5 $\pm$ 0.2 (9)	1.1 $\pm$ 0.4 (14)	26 $\pm$ 10 (7)

a) $P < 0.01$, c) $P < 0.05$ in E_2 group versus control group.

b) $P < 0.01$, d) $P < 0.05$ in TAM group versus control group.

Fig. 2. Relationship between ^3H -thymidine-labeled cells and IGF-1-positive cell rate. $\square$: E2 group (n=8); $\bullet$: TAM group (n=9); $\circ$: control group (n=7).

group ($P < 0.01$), but significantly higher in the TAM group ($P < 0.01$) than in the control group (Table I). The nuclear uptake and retention of radiolabeled ^3H -thymidine were observed in the IGF-1-immunocytochemically stained cells. The ^3H -thymidine uptake was more frequent in IGF-1-negative cells than in IGF-1-positive cells (Fig. 1). The labeling index and the IGF-1-positive cell rate for each tumor are two-dimensionally plotted in Fig. 2, where the Y axis denotes the ^3H -thymidine-labeling index, and the X axis, the IGF-1-positive cell rate. There is a statistically significant inverse correlation (Fig. 2).

IGF-1 is produced in the liver and various peripheral tissues, and its levels are closely controlled by growth hormone *in vivo*.¹⁰⁻¹² In the present study, the serum concentration of IGF-1 was significantly lower in the E2 group than in the control group. It is very likely in view of the simultaneously increased release of growth hormone that estrogens exert a direct inhibitory effect on IGF-1 production.^{7,13} Moreover, it is possible that not

only the local stromal production of IGF-1 but also the serum concentration of IGF-1 may affect the uptake of IGF-1 and the proliferation of MCF-7 breast cancer cells *in vivo*. In the present study, the tumor content of IGF-1 and the rate of IGF-1-positive cells were inversely correlated to the tumor growth and the labeling index. Since it has been well demonstrated that IGF-I stimulates the growth of many cell types, it appears paradoxical that the tumor content of IGF-1 and the rate of IGF-1-positive cells were inversely correlated to the tumor growth and the ^3H -thymidine labeling index. There are several possible explanations of the results. Firstly, there may be a discrepancy in cell cycle between the IGF-1-staining of cell membrane and the nuclear uptake of ^3H -thymidine. IGF-1, a late G_1 growth factor, primarily affects the rate of transition of cells from G_1 to S phase.¹⁴ The S phase is not a predominant period of IGF-1 receptor synthesis. Secondly, several paradoxical phenomena have been reported in *in vitro* and *in vivo* studies. Osborne *et al.*¹⁵ demonstrated that the growth factors from estrogen receptor (ER)-negative MDA-231 human breast cancer cells are not capable of replacing estrogens in *in vitro* growth stimulation of MCF-7 cells, while the MDA-231 cells have been shown to secrete high concentrations of several growth factors, including IGF-1. Arteaga *et al.*¹⁶ reported that the antibody-induced blockade of IGF-1 receptors rather promoted the growth of MCF-7 tumors transplanted in nude mice. It has further been reported that the IGF-1 receptor correlated positively with ER and progesterone receptor in human breast cancer,^{17,18} and that patients with IGF-1 receptor-positive tumors had a better prognosis than patients with negative tumors.¹⁹ Since the regulation of tumor growth *in vivo* most probably involves a complex interplay of tumor and host-derived growth stimulators and growth inhibitors, further studies are necessary to elucidate the *in vivo* roles of immunoreactive IGF-1 in ER-positive breast cancer.

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