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Effect of Estrogen and Progesterone on Cellular Replication of Human Breast Tumors[1]

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ABSTRACT

A total of 54 infiltrating carcinomas of the breast were studied for estrogen receptor concentration in the tumor cytosol and thymidine-labeling indexes. The results showed that there was no significant difference in the level of thymidine-labeling index between 22 primary and 32 metastatic breast cancers. No significant association between the levels of thymidine-labeling index and the presence or absence of estrogen receptors was observed. The effect of "physiological" doses of estrogen and progesterone on cell proliferative activity was studied by the level of thymidine-labeling indexes in the tumor cells in 10 patients with multiple skin and s.c. metastases. Tumor biopsies were performed for labeling indexes both before and after hormonal treatment. The results showed that physiological doses of estrogen and progesterone induced a significant rise in thymidine-labeling index within 3 days after hormonal treatment in seven of the 10 tumors. Of the seven tumors that showed a rise in thymidine-labeling index, three were estrogen receptor positive and four were estrogen receptor negative. Of the three nonresponsive tumors, one was estrogen receptor positive and two were estrogen receptor negative. The study suggests that estrogen and progesterone can induce cell replication in both estrogen receptor-positive and -negative tumors.

INTRODUCTION

Among the many factors governing the susceptibility of tumor cells to cytotoxic drugs, the growth fraction of the tumor, *i.e.*, the percentage of tumor cells engaged in DNA synthesis at any one point in time, is perhaps the most important. Several investigators have reported that animal tumors with large growth fractions are most susceptible to the cytotoxic effects of many chemotherapeutic agents (2, 17). These experimental findings have been further substantiated by many clinical studies. It has been shown convincingly that neoplasms which are highly responsive to the effects of cytotoxic drugs are often rapid-growing tumors with short doubling times and a large growth fraction. Thus, acute lymphocytic leukemia with a doubling time of 6 to 8 hr responds to chemotherapy with "complete remission," leading to a significant increase in life expectancy. In contrast, breast cancer is slow growing, with a doubling time ranging from 20 to 120 days. Under these circumstances, although chemotherapy may induce tumor regression, it rarely produces a complete remission which can lead to a significant alteration of survival.

Earlier, we reported that rat mammary gland and mammary tumor explants in organ culture proliferate rapidly in the presence of physiological doses of estrogen and progesterone (8). There has been no report of either *in vitro* or *in vivo* investigations to determine whether human mammary tumor cells can be stimulated to proliferate by hormonal agents and their subsequent response to cytotoxic chemotherapeutic agents. In a preliminary report (1), we demonstrated that resting or slowly replicating breast cancer cells could indeed be stimulated to proliferate by estrogen and progesterone in physiological doses. We now report the results of an extended study of the hormonal effects on human breast cancer cell replication. Our investigation shows that proliferative rates of mammary tumor cells are not related to the presence or absence of estrogen receptors in these tumors and that the response to estrogen and progesterone stimulation for cell replication is not dependent on the presence of hormone receptors.

MATERIALS AND METHODS

Tumor Procurement. Tumors from a total of 54 patients with either primary breast cancer (21) or metastatic disease (33) were examined for both thymidine-labeling index and estradiol receptor concentration. Ten patients had multiple cutaneous metastasis and were studied for their response to physiological doses of estrogen and progesterone.

Breast tumors following surgical excision were carefully trimmed of adipose and connective tissues and were divided into 2 parts. One part, a small piece about 150 to 200 mg, was placed in a sterile container with Eagle's medium for thymidine-labeling index study to be performed within 15 min, and the other part, a larger piece about 250 to 300 mg, was placed immediately in liquid nitrogen for estrogen receptor assay.

Thymidine-labeling Index. Tumors were sliced with a single-edged razor blade into small pieces (approximately 4 x 4 x 2 mm). Tumor slices were then incubated in 5 ml Eagle's medium containing [^{3}H]-thymidine (2 μCi/ml). The medium was buffered additionally with 10 mM NaH_2CO_3. This step is essential because the pH of the medium changes during incubation to about 6.4, which is detrimental to the tissue. Incubation was carried out in a Dubnoff metabolic shaker at 37° under constant agitation in an atmosphere of 95% O_2 and 5% CO_2 for 2 hr. At the end of incubation, explants were fixed in Bouin's fixative and sectioned for autoradiography. Each slide contained at least 4 to 5 tumor slices. The slides were coated with Kodak liquid emulsion NTP-3. The thymidine-labeling index represents the percentage of cells that incorporate radioactive label in the cell nuclei. A minimum of 1000 tumor cells (500 in the center and 500 in the periphery) were counted in each tumor slice. Thus, the number of cells counted is between 4000 and 5000 cells for each slide. A total of 10 slides were made for each tumor. The 2-hr incubation period was chosen after experiments with different time periods, including 2, 4, and 24 hr. The results of these experiments showed that a 2-hr incubation period was the optimal time for the study of labeling indexes in these tumors. Since tumors were incubated within 15 to 20 min after surgery, the incorporation of [^{3}H]thymidine occurs only in those cells which were in the process of DNA synthesis or were just entering the S phase. This is

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particularly significant, since the values would then reflect the *in vivo* proliferative pattern of these tumor cells.

Estradiol Receptor Assay. The procedures used for preparation of tumor cytosol and for estradiol receptor assay include sucrose gradient analysis, dextran-coated charcoal analysis, and Scatchard plots for quantitative estimation. The sucrose gradient analysis was carried out by the method described by Jensen *et al.* (6) but modified in our laboratory (13). Briefly, the tumor powder was homogenized and centrifuged at 100,000 × *g*. The aliquots of tumor cytosol were incubated with 2 to 4 nM 17β-[^{3}H]estradiol in the presence and absence of 10,000-fold molar excess of nafoxidine and were layered on 10 to 30% sucrose gradients and centrifuged again at 350,000 × *g* for 16 hr. Specific estrogen-binding components were identified by determining the distribution of radioactivity of the ligand in various regions of the gradients. Quantitation of estrogen-binding capacity was estimated by dextran-coated charcoal assay, a method first described by Horwitz and McGuire (5) and slightly modified by us (13).

Effect of Hormones on Labeling Index. A total of 10 breast cancer patients with multiple skin and soft-tissue metastases were studied.[3] Tumor biopsies were performed for the labeling index and estradiol receptor assay before hormone injection. Estradiol (0.1 mg) and progesterone (1.0 mg) were injected i.m. daily for 3, 6, or 9 days beginning 2 days after the initial biopsy (Table 3). Tumor biopsies were again performed on Days 3, 6, and 9 during the hormone treatment. The possibility that there may be significant variations in thymidine-labeling index among different tumor nodules in the same patient was examined by biopsies of 3 tumor nodules both before and after hormone injections in 2 patients (T. S. and A. C.).

RESULTS

Thymidine-labeling Index. The thymidine-labeling index ranged from 0.1 to 17.1. Tumors from 3 patients had a thymidine-labeling index of less than 1.0, and only one tumor was above 10 (17.1). Table 1 shows the distribution of thymidine-labeling indexes among patients of different age groups. The thymidine-labeling index in this series of patients shows a simple distribution, the log plot of which is a straight line. The data do not show a clear relationship between either the age or menstrual status of the patient and the level of thymidine-labeling index. The labeling index in 22 primary breast tumors ranged from 1.3 to 17.1 with a median of 3.3 and a mean of 3.8 ± 0.7 (S.E.). The thymidine-labeling indexes in 32 metastatic breast tumors ranged from 0.1 to 8.3 with a median of 2.8 and a mean of 3.3 ± 0.3. The results summarized in Table 2 show no significant difference in the levels of thymidine-labeling index between primary and metastatic breast cancers. Our data also fail to disclose an association between the presence or absence of axillary lymph node metastasis and the levels of thymidine-labeling index in primary breast tumors. However, the data are too few to draw any conclusions.

Relationship between Thymidine-labeling Index and Estradiol Receptors. The data summarized in Chart 1 show that the frequency of estradiol receptor-positive tumors is about evenly distributed among groups with different levels of thymidine-labeling index. When the data are examined by dividing the patients into primary and metastatic groups, a similar finding was observed (Table 2). It appears that the frequency of estradiol receptor-positive tumors rises as the thymidine-labeling index of the tumor increases. There is, however, no statistical difference in this small number of tumors.

Effect of Estradiol and Progesterone on Thymidine-labeling Index of the Tumors. The results disclose that the Thymidine-labeling indexes and the labeling indexes of the tumors in these 10 patients ranged from 1.59 to 8.16 with a median of 3.45 and a mean of 3.68. The thymidine-labeling index in 7 tumors rose significantly above the pretreatment levels. It appears that estradiol and progesterone in physiological doses can induce a significant rise in thymidine-labeling index within 3 days of the hormone treatment (Table 3). The results in Table 3 reveal that continued hormonal treatment does not cause a further increase in the Thymidine-labeling indexes and the labeling index, which reaches a "peak" 3 days after the beginning of the injections. Of the 7 tumors that showed a rise in thymidine-labeling index 3 were estradiol receptor positive and 4 were estradiol receptor negative. Estradiol and progesterone failed to induce changes in thymidine-labeling index in the remaining 3 tumors, of which 2 were negative and one positive for specific estradiol binding. It is interesting to note that the hormonal treatment is capable of inducing an increase in cell replication in those tumors that do not contain estradiol receptors. Biopsies were repeated 5 weeks after the last hormone injection in 3 (M. P., T. S., and J. B.) of the 7 patients whose tumors had a rise in thymidine-labeling index after estradiol and progesterone treatment. Studies of the labeling index revealed that the thymidine-labeling index in all these tumors declined to the pretreatment levels. Repeated estradiol receptor assays of these same tumors (one estradiol receptor positive and 2 estradiol receptor negative previously) revealed no change in their specific estradiol binding following the hormone treatment. In 2 patients (A. C. and T. S.), multiple (2 to 3) tumor biopsies were performed both before and after hormone treatment to determine whether significant variations in thymidine-labeling index would occur in different tumors. The results show a small but not significant difference of 3% in thymidine-labeling index in 2 tumors from one patient (A. C.) and a 5% difference among 3 different tumors in the other

Table 1

Relationship of patient age with [^{3}H]thymidine-labeling index

[^{3}H]Thymidine-labeling index	No. of patients				
	30–39 yr	40–49 yr	50–59 yr	60–69 yr	>70 yr
0.0–2.0		4	4	5	2
2.1–3.0		2	4	3	1
3.1–4.0			7	3	2
4.1–6.0	1	3	3	4	1
>6.0	2	1	1		1
Total (54)	3[a]	10[b]	19[c]	15	7

[a] All premenopausal.
[b] Eight were premenopausal, one postcastration, and one perimenopausal.
[c] Seventeen were postmenopausal and 2 perimenopausal.

Table 2

Thymidine-labeling index in patients with primary and metastatic breast carcinoma

Thymidine-labeling index	Primary tumors			Metastatic tumors		
	No. of patients	ER+[a]	ER−	No. of patients	ER+	ER−
0–2.0	5	4	1	10	4	6
2.1–3.0	6	5	1	4	1	3
3.1–4.0	4	2	2	8	4	4
4.1–6.0	6	4	2	6	6	0
>6.0	1	1	0	4	2	2
Totals	22	16	6	32	17	15

[a] ER$^+$, estradiol receptor positive; ER$^-$, estradiol receptor negative.

[3] Patient consent for these studies was obtained prior to biopsy.

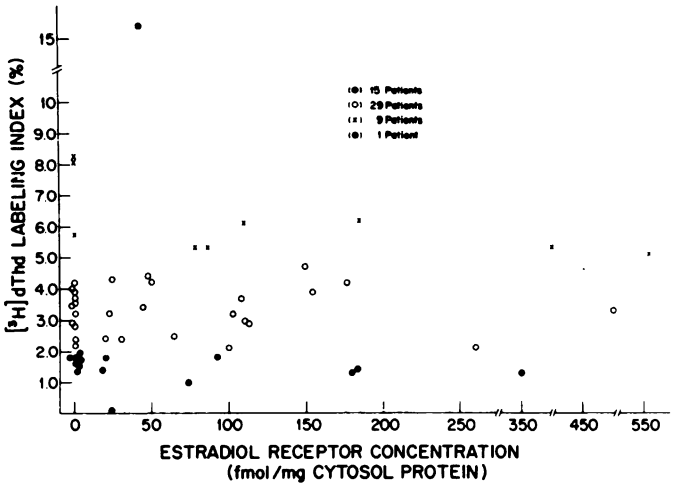

Chart 1. [³H]Thymidine ([³H]dThd)-labeling indexes are plotted against estradiol receptor concentration in the tumor cytosol. ●, patients having a thymidine-labeling index between 0 and 2.0; ⊗, patients having a thymidine-labeling index between 2.1 and 5.0; ×, patients having a thymidine-labeling index between 5.1 and 10.0; ⊗, patients having a thymidine-labeling index above 10.1. A receptor concentration of either 4S or 8S of less than 10 fmol/mg protein was considered to be negative (numbers represent the sum of 4S and 8S receptor concentrations).

Table 3

Effect of estradiol and progesterone on thymidine-labeling index

Patients	Pretreatment thymidine-labeling index	Posttreatment thymidine-labeling index			Estradiol receptor (fmol/mg protein)
		3 days[a]	6 days	9 days	
M. P.	2.8 ± 0.7[b]	6.7 ± 0.7	7.3 ± 0.9	6.5 ± 0.1	Negative
H. E.	3.2 ± 0.2	7.7 ± 0.2	6.9 ± 0.2	7.6 ± 1.0	Negative
T. S.	3.9 ± 0.4	8.9 ± 0.4			76
M. K.	3.4 ± 0.5	6.7 ± 0.6			44
F. W.	3.2 ± 0.3	7.9 ± 0.4	7.3 ± 0.4		101
J. B.	3.7 ± 0.5	8.9 ± 1.4			Negative
D. B.	1.7 ± 0.5	10.1 ± 2.0	10.6 ± 0.7	9.8 ± 0.9	26
A. C.[c]	8.1 ± 0.7	7.1 ± 0.8	5.9 ± 0.6	5.1 ± 0.9	Negative
E. C.[c]	1.5 ± 1.2	1.7 ± 1.0	1.8 ± 0.8		Negative
B. S.[c]	5.4 ± 0.7	5.5 ± 0.9	5.1 ± 1.0	6.0 ± 0.4	567

[a] The increases in thymidine-labeling index on Day 3 after estradiol and progesterone treatment in this column are all significantly higher than the pretreatment levels (range, $p < 0.01$ to $p < 0.001$).

[b] ±S.E. For each patient, the standard error of the labeling indexes was calculated based on the labeling indexes of 10 slides, each containing 4 to 5 tumor slices.

[c] The labeling index in these 3 patients did not respond to estrogen and progesterone.

patient (T. S.). It should be mentioned that there was no clinical evidence of either tumor regression or progression in any of the patients receiving the hormonal treatment for this brief period of time.

DISCUSSION

The thymidine-labeling index of breast cancer, as reported in this paper, is an estimation of the proportion of cells in a population that are in the process of DNA synthesis and are engaged in replication of nuclear DNA during a brief period of exposure to tritiated thymidine. Our results demonstrate conclusively that cellular replication, as estimated by the labeling index, can be significantly stimulated by physiological doses of estradiol and progesterone. This novel observation has significant clinical implications in that a new concept of combination therapy of cytotoxic and hormonal agents can now be enunciated. This concept entails the use of hormones to accelerate tumor cell replication and to enhance the susceptibility of these cells to the lethal effects of the cytotoxic agents. Thus, it differs entirely from the empirical approaches that were based on the assumption that breast cancers consisted of both hormone-independent and hormone-dependent cell populations and that such a mixed cell population could best be eradicated by a combination of hormones and cytotoxic chemotherapeutic agents.

It is of considerable interest to discover that small doses of estradiol and progesterone can accelerate mammary tumor cell replication even in those tumors which apparently do not contain estradiol receptors, as indicated by the usual criteria for positive and negative tumors. Since the hormone responsiveness of mammary cancer is dependent on the presence of specific hormone estradiol receptors, the enhanced cell-replicative activity following the administration of estradiol and progesterone in estrogen receptor-negative tumors is a phenomenon contrary to the concept of hormone receptors. Presently, we do not have an explanation for this observation. It must be pointed out, however, that, although estrogen has been considered to exert a proliferative effect on mammary and connective tissues, this effect on mammary epithelial cells in *in vitro* experiments has not been demonstrated conclusively. In earlier studies, we reported the effect of estrogen and progesterone on rat mammary epithelial cell replication *in vitro* but were unable to show this effect by estrogen alone (8). It is conceivable that the effect of estrogen and progesterone on human breast cancer is the result of an indirect mechanism, for example, the production of some "growth factor" in the host as a result of hormone stimulation. Sirbasku (15) reported evidence that estrogen target tissues contained "growth activity" and that estrogen administration *in vivo* increased the tissue content of these activities. It is suggested that this estrogen-inducible growth factor may act as a "mediator" of estrogen-promoted tumor cell growth (16).

It should also be mentioned that our study does not rule out the possibility that progesterone alone in the absence of estrogen may be effective in stimulating an increase of the thymidine-labeling index, since we have not measured progesterone receptors in all of the 10 patients studied. However, progesterone receptors were measured in 4 patients (M. P., H. E., T. S., and E. C.), and they were all negative.

It is also a plausible suggestion that estrogen and progesterone may induce the synthesis of estradiol receptors in a "negative" tumor, the definition of which is ambiguous at best. Receptor assay methods are mostly concerned with estimation of the concentration of unoccupied receptor proteins, and the question whether there is indeed "receptor-negative" tissue remains. Several reports indicate the presence of a regulatory mechanism of estrogen receptor synthesis by other hormones (3, 9, 14, 18). In the limited studies reported in this paper, we have not observed any changes in the receptor status of these tumors, *e.g.* from estrogen receptor negative to positive in at least 2 tumors after treatment with estradiol and progesterone.

Our data also demonstrate clearly that there is a lack of correlation between labeling index and the presence or absence of estrogen receptors. The results do not agree with the earlier observation of Meyer *et al.* (11), suggesting that rapid rates of cellular replication are associated with the low incidence of estrogen receptors in breast cancers. It is perhaps premature at this time to suggest possible reasons for the

difference between our results and those of Meyer *et al.* (11). In a preliminary report by Rao and Meyer (12), these authors also observed no correlation between thymidine-labeling index and estrogen receptor concentrations in the tumors. They attributed the difference in their results to the lack of tumors with high thymidine-labeling index in the earlier series, since this would be necessary to demonstrate a significant difference. Although there are some differences in the methodologies for both estrogen receptor assay and thymidine-labeling index, it is highly unlikely that this would account for the differences in the data reported by us and by Meyer *et al.* (11). Further investigation is being carried out in our laboratory to resolve this question.

Our present study, in fact, seems to suggest that the incidence of estradiol receptor-positive tumors is higher in tumors having a high thymidine-labeling index. This finding appears to be expected, since receptor-positive tumors should have a faster growth rate under hormonal stimulation. Earlier clinical studies suggesting estradiol receptor-negative tumors respond better to chemotherapy (10) were also not confirmed by others (4, 7). The suggestion that tumors with a high thymidine-labeling index tend to be estradiol receptor negative and therefore more sensitive to chemotherapy is not tenable.

REFERENCES

1. Dao, T. L., Sinha, D. K., and Nemoto, T. Effect of estrogen and progesterone on growth fraction of human breast tumors. Proc. Am. Assoc. Cancer Res., *20:* 105, 1979.
2. DeVita, V. Cell kinetics and the chemotherapy of cancer. Cancer Chemother. Rep., *2:* 23–33, 1971.
3. Gibson, S. L., and Hilf, R. Regulation of estrogen-binding capacity by insulin in 7,12-dimethylbenz(*a*)anthracene-induced mammary tumors in rats. Cancer Res., *40:* 2343–2348, 1980.
4. Hilf, R., Feldstein, M. L., Savlov, E. D., Gibson, S. L., and Seneca, B. The lack of relationship between estrogen receptor status and response to chemotherapy. Cancer (Phila.), *46:* 2797–2800, 1980.
5. Horwitz, K. B., McGuire, W. L., Pearson, O., and Segaloff, A. Predicting response to endocrine therapy in human breast cancer: a hypothesis. Science (Wash. D. C.), *189:* 726, 1975.
6. Jensen, E. V., Block, G. E., Smith, S., Kyser, K., and DeSombre, E. R. Estrogen receptors and breast cancer response to adrenalectomy. Natl. Cancer Inst. Monogr., *34:* 55–79, 1971.
7. Kiang, D. T., Frenning, D. H., Grebman, A. L., Asocusao, V. F., and Kennedy, B. J. Estrogen receptors and response to chemotherapy and hormonal therapy in advanced breast cancer. N. Engl. J. Med., *299:* 1330–1334, 1978.
8. Koyama, H., Sinha, D. K., and Dao, T. L. Effect of hormones and 7,12-dimethylbenz(*a*)anthracene on rat mammary tissue grown in organ culture. J. Natl. Cancer Inst., *48:* 167–168, 1972.
9. Leung, B. S., and Sasaki, G. H. Prolactin and progesterone effect on specific estradiol binding in uterine and mammary tissues *in vitro*. Biochem. Biophys. Res. Commun., *55:* 1180–1187, 1973.
10. Lippmann, M. E., Allegra, J. C., Thompson, E. B., Simon, R., Barlock, A., Green, L., Huff, K. K., Do, H. M. T., Aitken, S. C., Warren, R. The relation between estrogen receptors and response rate to cytotoxic chemotherapy in metastatic breast cancer. N. Engl. J. Med., *298:* 1223–1228, 1978.
11. Meyer, J. S., Rao, B. R., Stevens, S. G., and White, W. Low incidence of estrogen receptors in breast carcinomas with rapid rates of cellular replication. Cancer (Phila.)., *40:* 2290–2298, 1977.
12. Rao, B. R., and Meyer, J. S. Estrogen and progestin receptors in normal and cancer tissue. *In:* W. L. McGuire, E. E. Baulieu, and J. P. Raynaud (eds.), Roussel Workshop on Application of R5020 to the Detection of Progesterone Receptors, pp. 155–169. New York: Raven Press, 1977.
13. Schneider, S. L., and Dao, T. L. Effect of Ca^{2+} and salt on forms of estradiol cytoplasmic receptor in human neoplastic breast tissue. Cancer Res., *37:* 382–387, 1977.
14. Shafie, S., and Brooks, S. C. Effect of prolactin on growth and the estrogen receptor level of human breast cancer cells (MCF-7). Cancer Res., *37:* 792–799, 1977.
15. Sirbasku, D. A. Estrogen-induction of growth factors specific for hormone-responsive mammary, pituitary, and kidney tumor cells. Proc. Natl. Acad. Sci. U. S. A., *75:* 3786–3790, 1978.
16. Sirbasku, D. A., and Benson, R. H. Estrogen-inducible growth factors which may act as mediators (estromedins) of estrogen-promoted tumor cell growth. Cold Spring Harbor Conf. Cell Proliferation, *6:* 477–497, 1979.
17. Skipper, H. E. The cell cycle and chemotherapy in cancer. *In:* Renats Baserga (ed.), The Cell Cycle and Cancer. New York: Marcel Dekker, 1977.
18. Vignon, F., and Rochefort, H. Regulation of estrogen receptors in ovarian-dependent rat mammary tumors. I. Effects of castration and prolactin. Endocrinology, *98:* 722–729, 1976.