Tumor Res. **21**, 1–8 (1986)

Influence of 17 β-Estradiol, Progesterone and Tamoxifen on Direct Cloning of Human Breast Cancer in Soft Agar Culture

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SUMMARY

The action of estrogen, progesterone and tamoxifen on the growth of 22 mammary adenocarcinomas in soft agar was studied using the clonogenic assay described by Hamburger and Salmon. Hormones and tamoxifen were added to a final concentration of 10^{-7} M, and were present in continuous exposure throughout the culture period. Estrogen receptor (ER) and progesterone receptor (PR) levels were measured in the tumor specimens using the dextran-coated charcoal method. For tumor cells exposed to estrogen, no significant difference was noted in clonal growth between ER positive (ER+) and ER negative (ER−) cells, and estrogen did not increase clonal growth. For tumor cells exposed to progesterone, no significant difference occurred in clonal growth between PR positive (PR+) and PR negative (PR−) cells, and progesterone did not reduce clonal growth. No significant difference in clonal growth was seen between ER+ and ER− tumor cells exposed to tamoxifen, but this drug significantly inhibited clonal growth, a finding which confirms the fact that tamoxifen exerts its antineoplastic effect independently of the ER status.

Key words: Breast cancer, Plating efficiency, Soft agar, Hormone receptors, Tamoxifen

INTRODUCTION

Human breast cancer has long been known to be influenced by hormonal factors (7, 14, 17). Use of estrogen receptor (ER) and progesterone receptor (PR)

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assays of normal and neoplastic mammary tissues has led to better classification of breast cancers. Hormone receptors are useful parameters of clinical prognosis(4, 15) and have been utilized to select patients likely to benefit from endocrine therapy (13, 20).

Several types of *in vitro* studies have proven useful in enlarging our knowledge on the effects of hormones on cells. The effect of estrogens, progesterone, androgens and tamoxifen on the growth of breast cancers has been studied on continuous cell lines(12, 16) as well as on cell suspensions prepared from fresh tumor biopsies(9, 19).

Statistical analyses have shown that only 70% of patients with ER+ tumors respond favorably to endocrine therapy(10). Cell culture studies have revealed unexpected effects, requiring further investigations using biological tests(10).

Ever since the studies conducted by Hamburger and Salmon(6), the cloning of cancer cells in soft agar has been used as a test of tumor chemosensitivity. Compared to other tests based on variations in tritiated thymidine incorporation(21) or vital dye exclusion by cells, the method of Salmon has the advantage of reflecting the effect of cytotoxic drugs on a more specific parameter of neoplastic cells, their growth in soft agar.

The present report describes the action of hormones used for therapeutic purposes (estrogen, progesterone, tamoxifen) on 22 mammary adenocarcinomas, as evaluated by study of their clonal growth. ER and PR assays were also performed on fresh tumor biopsies, and the influence of ER and PR status on the clonal growth of the cells exposed to these hormones are discussed.

MATERIALS AND METHODS

Immediately after exeresis, tumor specimens were placed in a culture medium consisting of MacCoy's 5A plus 10% heat-inactivated fetal calf serum, penicillin (400 units/m*l*) and streptomycin (200 μg/m*l*) (Seromed, Biopro, France).

A cell suspension was prepared from the tumor material by mincing with scissors; this suspension was filtered through a 36 micrometer wire mesh gauze and then washed. Assays for tumor colony forming cells were performed as described by Hamburger and Salmon (6), without any conditioned medium or 2-mercapto-ethanol. In brief, 25×10^4 tumor cells were suspended in 1 m*l* of enriched Connaught Medical Research Laboratories Medium 1066 (Grand Island Biological Co.) containing 0.3 (weight/volume) agar on a sublayer of complete MacCoy's 5A medium containing 0.5% agar in a sterile 35 mm dia. Petri dish.

The cell suspensions obtained from the mammary adenocarcinomas each contained only 3×10^6 to 4×10^6 viable cells; this low concentration of viable cells prevented us from studying the effects of various steroid hormone concentrations on

cell growth in soft agar. Hormones were tested only at a final concentration of 10^{-7} M, which is in the range of concentrations that stimulate cloning(12, 16). The final concentration took into account the steroid hormones contained in the calf serum used to enrich the culture medium.

A total of 12 dishes were prepared. Group 1 corresponded to 3 control dishes, Group 2 to 3 dishes containing 10^{-7} M 17β-estradiol, Group 3 to 3 dishes containing 10^{-7} M tamoxifen, and Group 4 to 3 dishes containing 10^{-7} M progesterone.

The hormones and tamoxifen (Sigma) were present continuously throughout the entire culture period. Culture dishes were then put in a humidified incubator at 37℃ in an atmosphere containing 5% CO_2.

The percent increase or decrease in tumor colony forming was defined as the percent increase or decrease in colonies on hormone treated plates as compared to control plates. Cultures were examined using an inverted phase microscope (Olympus) after 15 days. Aggregates of 50 or more cells were considered colonies. The background colony counts on Day 0 were taken into account for the scoring of colonies on plates. Growth was defined as a minimum increase of 5 colonies per control plate.

Plating efficiencies (PE) were calculated by dividing the average number of colonies per dish by the number of cells seeded, and by multiplying the result by 100. Variations in the plating efficiency between dishes containing estradiol, progesterone or tamoxifen and control dishes were noted ΔPE.

Cytosolic estrogen and progesterone receptors

The cytosolic ER and PR levels of all of the tumor specimens were measured using the dextran charcoal method(13, 22). The breast cancer specimens were frozen immediately in liquid nitrogen. Tissues were homogenized in 20 m*l* buffer containing 1 mM EDTA, 0.5 mM dithioerythritol and 10% (vol/vol) glycerol in 10^{-2} M Tris-HCl, pH 7.4. The homogenate was centrifuged at 105,000g for one hour to obtain the supernatant cytosol fraction. Constant amounts of cytosol were then incubated with increasing amounts of (^{3}H)-labelled hormones (Amersham, U.K.). Free hormones were eliminated by the addition of dextran charcoal and centrifugation. The radioactivity of the supernatant was measured with a scintillation counter. Receptor concentrations were expressed in fentomoles per milligram of total proteins.

RESULTS

Twenty of the 22 breast cancers studied gave clones in soft agar. Table 1 gives the mean values (with standard deviations and confidence limits) for the PE in control dishes and dishes containing hormones and tamoxifen.

Table 1 *Plating efficiencies (PE), arithmetic means, standard deviations and geometric means with 95% confidence limits for the four groups of 20 breast cancers.*

Group[a]	PE arithmetic mean	PE standard deviation	PE geometric mean	PE 95% geometric mean confidence limits
1	0.199	0.175	0.131	0.082−0.208
2	0.204	0.285	0.118*	0.068−0.204
3	0.150	0.165	0.080**	0.042−0.149
4	0.195	0.209	0.104*	0.058−0.187

[a] group 1: control dishes
group 2: dishes containing estradiol
group 3: dishes containing tamoxifen
group 4: dishes containing progesterone

* Not significantly different from PE geometric mean value of group 1 (Student's t-test).

** Significantly different from PE geometric mean value of group 1 (Student's t-test; $p < 0.05$).

Figure 1 shows the distributions of individual PE values for the four groups, which were log-normal, as was the distribution of individual ER values(5). The threshold for consideration as a positive receptor status was 12 fmol/mg cytosol protein for both estrogen and progesterone.

Of the 20 breast cancers that showed successful growth, 3 were both ER− and PR−, 3 were ER− but PR+, and 14 were both ER+ and PR+.

As concerns tumor cells treated with estradiol, 5 of the 6 ER− specimens had a $\Delta PE \leq 0$; 8 of the 14 ER+ specimens had a $\Delta PE > 0$ (Table 2); these differences were not statistically significant.

Table 2 *Comparison of ΔPE[a] with ER status*[b].

Group	ER status	No. with $\Delta PE > 0$*	No. with $\Delta PE \leq 0$*
2	ER−	1/6(17%)	5/6(83%)
	ER+	8/14(57%)	6/14(43%)
3	ER−	2/6(33%)	4/6(67%)
	ER+	3/14(21%)	11/14(79%)

[a] ΔPE = PE differences between cells treated with estradiol (group 2) or tamoxifen (group 3) and control cells.

[b] Estrogen receptor positivity (ER+) defined as a value over 12 fmol/mg cytosol protein.

* No association was found by the chi-square test between the negativity or positivity of ER and the negativity or positivity of ΔPE.

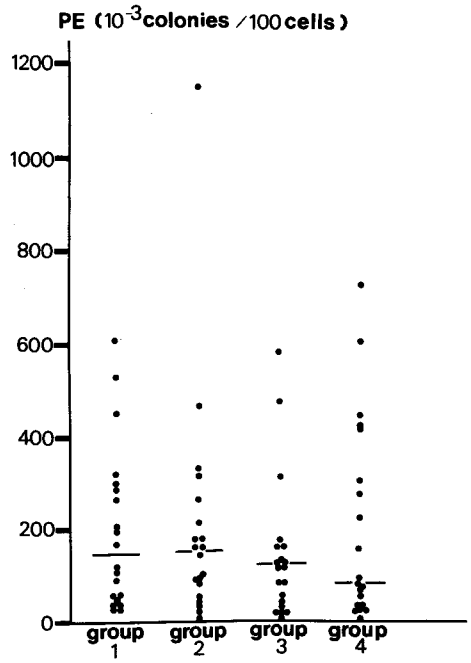

Fig. 1 Distribution of plating efficiencies (PE) in control dishes (group 1) and dishes containing estradiol (group 2), tamoxifen (group 3) and progesterone (group 4). Horizontal lines indicate median values. All four distributions are log-normal, as confirmed by Henry's test and the chi-square test. There are no significant differences between groups 1 and 2 and between groups 1 and 4. Group 3 is significantly different from group 1 (Student's t-test, $p<0.05$).

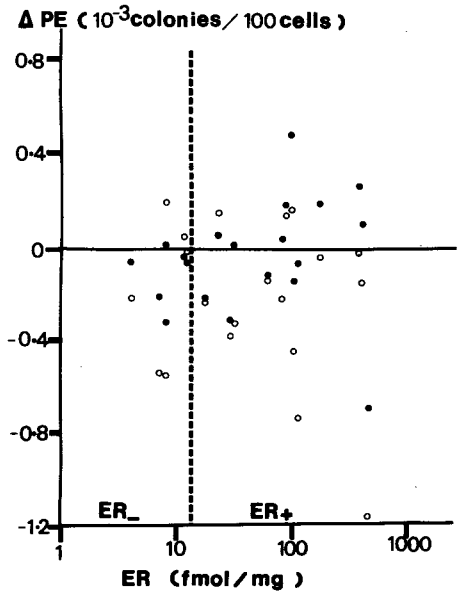

Fig. 2 Plating efficiency variation (ΔPE) between dishes with estradiol (solid dots) or tamoxifen (circles) and control dishes as a function of estrogen receptor values (ER).
ER negative values (≤12 fmol/mg cytosol protein) are noted ER−.
ER positive values (>12 fmol/mg cytosol protein) are noted ER+.
There was no significant differences between ER+ and ER− groups (Mann and Whitney U-test).

For the tumor cells treated with tamoxifen, 4 of the 6 ER− specimens had a $\Delta PE \leq 0$, and 11 of the 14 ER+ specimens had a $\Delta PE \leq 0$ (Table 2); again, these differences were not statistically significant.

Figure 2 shows ΔPE as a function of ER values in groups 2 and 3; the ER status did not appear statistically to influence ΔPE values.

There was no significant difference in the average PE values between groups 1 and 2, but the difference in average PE values between groups 1 and 3 was statistically significant ($p<0.05$). The presence of tamoxifen thus lowered the mean PE value. The correlation coefficient reveals that the PE of control dishes did

not influence the ΔPE of group 3.

For those tumor specimens treated with progesterone, 2 of the 3 PR− specimens had a ΔPE≤0, and 11 of the 17 PR+ specimens had a ΔPE≤0 (Table 3); these differences were not statistically significant.

Figure 3 shows the ΔPE of group 4 as a function of PR values. The PR status did not statistically appear to influence ΔPE values. The difference in the average PE value between groups 1 and 4 was not significant.

Table 3 *Comparison of ΔPE[a] with PR status[b].*

Group	PR status	No. with ΔPE>0*	No. with ΔPE≤0*
4	PR−	1/3(33%)	2/3(67%)
	PR+	6/17(35%)	11/17(65%)

[a] ΔPE: PE differences between cells treated with progesterone (group 4) and control cells.

[b] Progesterone receptor positivity (PR+) defined as a value over 12 fmol/mg cytosol protein.

* No association was found by the chi-square test between the negativity or positivity of PR and the negativity or positivity of ΔPE.

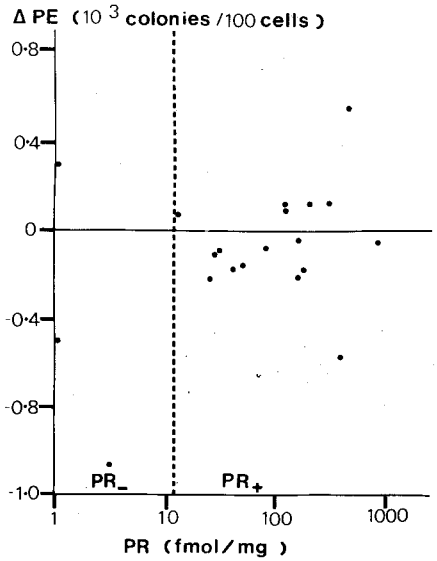

Fig. 3 Variation in plating efficiency (ΔPE) for dishes containing progesterone and control dishes as a function of progesterone receptor values (PR).
PR negative values (≤12 fmol/mg protein) are noted PR−.
PR positive values (>12 fmol/mg protein) are noted PR+.
There was no significant difference between PR+ and PR− groups (Mann and Whitney U-test).

DISCUSSION

Tamoxifen has been used for many years as an adjuvant treatment for breast cancer; in addition to prolonging the disease-free interval and reducing the death rate in patients with mammary carcinomas(14), this drug has the advantage of causing minimal toxicity.

The action of tamoxifen depends on the presence of estrogen receptors in the cytoplasm of cancer cells. Tamoxifen inhibits the estrogen stimulation of cancer cells, as shown by various clinical trials and thorough *in vitro* studies(11, 12, 16). However, tamoxifen did not have any benefit for 30% of patients with ER+ and PR+ tumors for whom antiestrogens might have been useful(15). Likewise, tamoxifen can prove beneficial effect for certain patients with ER− and PR− tumors(2). Like other investigators(9, 10), we conducted our study to try and explain the limitations on the action of tamoxifen.

In vitro studies on cell lines have modified the concepts on the hormone dependency of breast cancers(10), and investigations on hormone dependency are no longer limited to biochemical ER and PR assays. The expression of ER within a given tumor is heterogeneous, and both ER and PR status are associated with the grade of differentiation and cellularity of breast cancers(9, 10). ER and PR concentrations also change with time, and ER and PR levels cannot be used to characterize a patient.

The present study on the effects of hormones and tamoxifen on the growth of breast cancer cells in soft agar, using fresh breast cancer specimens, gave results comparable to those obtained by *in vitro* studies. The soft agar culture system is known to be selective for tumor growth(18). As cell suspensions obtained from breast adenocarcinoma biopsies generally contain a low number of viable cells, only a few breast cancer cells give rise to colonies.

Due to the above limitations, only 22 breast cancers were entered in our study, yet our conclusions are consistent with previously published results(2, 9, 10). Our findings suggest that the ER status does not influence the clonal growth of breast cancer cells exposed to estrogen or tamoxifen, and that the PR status does not influence the clonal growth of breast cancer cells exposed to progesterone. Moreover, our data suggest that estrogen does not increase, and that progesterone does not decrease clonal growth of breast cancer cells. We found that tamoxifen significantly inhibits the clonal growth of breast cancer cells, and this benefit appears to be independent of the ER status. These findings confirm the cytostatic properties of tamoxifen when used for the adjuvant treatment of breast cancers.

ACKNOWLEDGMENT

The authors wish to thank Nancy Rameau for translation and preparation of the manuscript.

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