Influence of Estradiol and Tamoxifen on the Growth of N-Nitrosomethylurea-induced Rat Mammary Tumor Cells in Soft Agar

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ABSTRACT

N-Nitrosomethylurea-induced rat mammary tumors were grown in vitro using the clonogenic soft agar technique. Cells from all tumors (n = 46) formed colonies in vitro. Tamoxifen (10⁻⁷, 10⁻⁶ M) inhibited colony formation to 72 and 53% of control values, respectively. The inhibitory effect of tamoxifen could be reversed with the addition of estradiol (10⁻¹⁰ to 10⁻⁶ M) to the medium. Estradiol (10⁻¹⁰ to 10⁻⁸ M), on the other hand, added alone to serum-containing medium did not influence the number of colonies formed in vitro. We conclude that the soft agar clonogenic assay is a feasible technique to study the influence of hormones and antihormones in vitro. The effects of tamoxifen and estradiol noted in vitro were similar to the known in vivo effects of these agents.

INTRODUCTION

Hormone dependency in the NMU-induced rat mammary tumors has been well established (1, 2, 12). These experimentally induced tumors appear to be suitable models to study hormone-dependent breast cancer. Estrogen, progesterone, and growth hormone have been shown to influence the growth of these tumors in vivo (1, 12).

The introduction of the stem cell clonogenic assay provided a new approach to study a variety of human tumors in vitro (5). Using this technique, a single cell suspension added to the soft agar can form colonies, and the inhibitory effects of drugs added can be assessed by a decrease in colony formation. Most of the data in the literature have dealt with the effect of chemotherapeutic agents on clones of human cancer cells (13, 17). Few data are available, however, on the effects of hormones or antihormones on colony formation from tumor cells (4, 8, 9). In a recent report, Manni and Wright (8) have demonstrated an inhibitory action of tamoxifen on colony formation from cells of NMU-induced rat mammary tumors.

In the present experiments, we studied the growth of NMU-induced mammary tumors using the soft agar technique. We evaluated the inhibitory action of tamoxifen at different concentrations and also the reversibility of that effect by the addition of different concentrations of estradiol.

MATERIALS AND METHODS

Tumor Induction. Mammary tumors were induced in 50-day-old female Sprague-Dawley rats (Zivic Miller) by 2 i.v. injections of NMU (5 mg/100 g body weight) given 1 week apart. Mammary tumors appeared 5 to 20 weeks after the first injection of NMU was given. All tumors used in this experiment were palpable and measured more than 1 x 1 cm in the greatest dimensions. Tumors were freshly excised using sterile techniques and placed in sterile Hanks' balanced salt solution just before plating.

Soft Agar Cell Culture Technique. The method used was modified from that described by Hamberger and Salmon (5) and Von Hoff et al. (17) and has been described recently in detail (3). Briefly, tumor specimens were trimmed and cut into 1-mm pieces using sharp sterile blades. The small tumor pieces were placed on a stainless steel mesh in a collector apparatus (Bellico Glass, Inc., Vineland, NJ) and pushed gently through the mesh into a sterile Petri dish. The resultant suspension in the Petri dish was aspirated through a 26-gauge needle, washed twice with Hanks' balanced salt solution, and allowed to settle undisturbed in a test tube for 7 to 10 min. This procedure allowed big clumps to settle to the bottom and produced significantly fewer clumps in the upper portion to be plated. The latter portion was aspirated and suspended in enriched CMRL medium (Grand Island Biological Co., Grand Island, NY), while the cell count and viability were determined in a hemocytometer using the trypan blue exclusion test.

The underlayer medium consisted of McCoy's medium (Grand Island Biological Co.) enriched with horse serum (5%) and fetal calf serum (10%), penicillin, streptomycin, Fungizone, and several other nutrients (3). A 3% suspension of hot agar was then added to the enriched McCoy's medium to make a final concentration of 0.5% agar. One ml of the resulting suspension was then dispensed immediately into a 25-mm Petri dish and allowed to cool. The upper layer consisted of CMRL medium enriched with horse serum (15%), antibiotics, Fungizone, and other nutrients as described (3). A 3% agar suspension was added to the enriched CMRL medium to give an agar concentration of 0.6%. Cell plating was performed by aspirating 0.5 ml of the cell suspension, followed by 0.5 ml of the upper layer suspension, and dispensing the whole 1 ml on top of the lower layer. The cell suspension was mixed thoroughly every time before aspirating the required volume for plating. The Petri dishes were then incubated at 37°C in a humidified atmosphere with 5% CO₂.

Compounds Tested. Tamoxifen (kindly supplied by ICI, Wilmington, DE) was initially dissolved in absolute ethanol and diluted with distilled water to a concentration of 10⁻⁴ M and used as a stock solution. Similarly, 17β-estradiol (Sigma Chemical Co., St. Louis, MO) was initially dissolved in absolute ethanol and diluted with distilled water to 10⁻⁸ M and used as a stock solution. Both tamoxifen and 17β-estradiol were added to the upper layer medium to give the appropriate concentration in each experiment. The volume of distribution considered in determining the final concentration of either drug was that of the upper layer. The final concentrations of ethanol in the stock solutions were 0.1 and 0.01% (v/v) for both tamoxifen and 17β-estradiol, respectively.

Colony Counting. Using an inverted-phase microscope, the Petri dishes were inspected and counted between 2 and 4 hr after plating and then at Days 6 and 10. Each time, the whole Petri dish was inspected and counted using a crossed grid over the stage of the microscope. The
total number of colonies (>40 cells, each approximately 75 μm in diameter) was recorded in each Petri dish on each occasion. The presence of clumps at the time of plating may be confused with colonies. For that reason, each Petri dish was counted within 2 to 4 hr of plating. This count served as the base-line number and was subtracted from the final count at Days 6 and 10. The number of clumps/Petri dish seen in the first few hr ranged between 5 and 15.

From each tumor, a number of Petri dishes were obtained and were assigned randomly to treatments so that between 3 and 7 Petri dishes were assigned to each treatment. Because of the inherent differences between tumors, the experimental design consisted of a block design with the tumors serving as the blocks. An analysis of variance test for unbalanced complete block design using Statistical Analysis System-General Linear Model procedure was applied for each of the 3 experiments. In all cases, after the analysis of variance test yielded a p value in support of rejecting the null hypothesis (that all treatment means are not different from each other), the Duncan multiple-range test was used to determine differences between the means (14). In addition, the data on the influences of treatments (tamoxifen and/or 17β-estradiol) in these experiments were expressed as a percentage of the respective control in each tumor. These data were then analyzed using the one-way analysis of variance to identify significant differences between various groups.

RESULTS

Cells from all tumors formed colonies in soft agar (n = 46 tumors). The peak number of colonies in control Petri dishes occurred almost equally on either Day 6 or Day 10. We used the time of peak growth in the control Petri dishes and evaluated the effects of tamoxifen and 17β-estradiol at that time. The peak number of colonies formed in the control experiments ranged between 11 and 166 with a mean of 30.5 ± 4.5 (S.E.) colonies/plate. The plating efficiency ranged between 0.005 and 0.07%.

Of the 46 tumors plated, 8 were used to evaluate the effect of several doses of tamoxifen (Chart 1; Table 1), and 9 tumors were used to study variable doses of 17β-estradiol in the presence or absence of tamoxifen (Chart 2; Table 2). The remaining 29 tumors were used for a subsequent experiment after the optimal doses of the drugs were assessed (Table 3).

Tamoxifen inhibited colony formation (Chart 1; Table 1) at concentrations of 10^-6, 10^-7 (p < 0.01), and minimally at 10^-8 M. Reversal of the tamoxifen effect was achieved (Chart 1; Table 1) with 17β-estradiol concentration of 10^-6 M. This concentration of 17β-estradiol reversed the inhibitory action of tamoxifen at 10^-6 and 10^-7 M.

The effect of 17β-estradiol was studied at different molar concentrations (Chart 2; Table 2) either alone or in combination with a known concentration of tamoxifen (5 × 10^-7 M). It is clear that 17β-estradiol by itself, when added at concentrations ranging between 10^-8 and 10^-10 M, had no significant effect on colony formation. However, at concentrations of 10^-10 M or higher, it reversed the inhibitory action of tamoxifen (Chart 2; Table 2).

After the appropriate concentrations of drugs became known, we studied their effects in a larger number of tumors (n = 29 tumors). We studied the effects of these agents, given a known molar concentration (17β-estradiol, 10^-8 M; tamoxifen, 5 × 10^-7 M). In 25 of 29 tumors, tamoxifen inhibited colony formation by >25%. In the remaining 4 tumors, the inhibition ranged between 9 and 15%. The number of colonies in the control plates of this group ranged between 15 and 166 with a mean of 28.8/Petri dish (Table 3). Tamoxifen-treated Petri dishes had significantly fewer colonies (P < 0.001) than either the control or the tamoxifen-17β-estradiol groups. The degree of inhibition of colony formation by tamoxifen in this experiment, where a large number
of tumors (n = 29) were studied, was similar to that seen in the second experiment, where 9 tumors were studied.

**DISCUSSION**

Our data show clearly the feasibility of growing cells of NMU-induced rat mammary tumors in soft agar. In addition, our data also show the inhibitory action of tamoxifen on colony formation and the reversibility of that effect with the addition of 17β-estradiol. Thus, the soft agar in vitro cell culture technique can be successfully used to investigate the effects of hormones or antihormones on the clonogenic potential of tumor cells.

17β-Estradiol has been shown to stimulate the growth of NMU-induced rat mammary tumors in vivo (1). However, we were unable to demonstrate any stimulatory effect of 17β-estradiol when added alone (i.e., without tamoxifen) to the culture medium. The reason behind that is not clear at the present time. However, one possible explanation is the fact that the sera added to the culture media have adequate concentrations of 17β-estradiol such that the addition of extra 17β-estradiol may not result in any further stimulation of growth. The inhibitory effect of tamoxifen and the reversal of that effect by concurrent addition of 17β-estradiol (10⁻⁸ M) were noted when charcoal-treated serum instead of regular serum was used in the medium (data not shown). However, further studies using 17β-estradiol under different experimental conditions, such as using serum-free medium, are needed to evaluate the individual effect of 17β-estradiol addition to the medium on colony formation.

Both tamoxifen and 17β-estradiol were added to the upper layer of agar, where the cells were also added. It is conceivable that diffusion of drugs may have occurred between layers, and therefore, the actual concentration of the drugs at the cellular level might be different from the stated concentrations. However, we are not aware of any studies where this issue was addressed.

In evaluating the influence of drugs on colony formation in this technique, most investigators have used a short exposure time, where the cells are incubated for 45 to 60 min in a solution containing the study drug, following which the cells are washed and then plated. However, we and others (3, 6-9, 16) have added the drugs to the medium and thus allowed continuous exposure of the cells to the drugs. In a very recent report, Ludwig et al. (7) studied the inhibitory action of several chemotherapeutic agents on colony formation of primary human tumors and human tumor cell lines. They compared the inhibitory effect of these drugs at different concentrations when added to the medium (continuous exposure) to that obtained with the conventional limited (1 hr) exposure. They found that there was no difference in the dose-survival curves obtained using either method when studying non-cycle-specific drugs. However, using continuous exposure of cycle-specific drugs, an exponential reduction in colony formation was obtained in contrast to plateau-type dose-response curves obtained with short-term exposure (7). Tamoxifen inhibits breast cancer cells by invoking a transition delay early in the G1 phase of the cell cycle (10) and is thus considered cycle specific. Thus, continuous exposure of the cells to tamoxifen is probably preferable to the limited 1-hr exposure. However, in 4 tumors studied using the 1-hr exposure method (data not shown), tamoxifen inhibited colony formation by 30 to 40%, and the addition of 17β-estradiol reversed that effect.

The inhibition of colony formation demonstrated by tamoxifen in vitro is reminiscent of the in vivo effect of this drug on NMU rat mammary tumors in vivo (11). The inhibition of tumor growth by tamoxifen in vivo can be partial or complete. The inhibition in vitro was not complete. Whether this is due to the presence of a heterogeneous cell population in each tumor or related to the fact that other hormones, such as prolactin and growth hormone, are also involved in the growth of these experimental mammary tumors (1, 12) is not apparent at the present time. Further studies are needed to evaluate this aspect.

In these experiments, we did not consistently evaluate the size of colonies formed. However, in a limited number of tumors (n = 4) where that effect was studied, neither tamoxifen nor 17β-estradiol appears to influence the colony sizes. This is in agreement with the data of Manni and Wright (8), who did not show any significant changes in colony sizes in tamoxifen-treated plates.

Despite recent criticism of the clonogenic cell assay (15), we believe that this technique provides a new, feasible, and potentially valuable approach to investigate the growth of various tumors in vitro.

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