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Effect of Tamoxifen and α-Difluoromethylornithine on Clones of Nitrosomethylurea-induced Rat Mammary Tumor Cells Grown in Soft Agar Culture[1]

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ABSTRACT

N-Nitrosomethylurea-induced mammary tumors were grown in a bilayer soft-agar culture system. The antiestrogen tamoxifen prevented formation of 50% of colonies formed in this *in vitro* system. Possible mediation of these antimitotic effects through the polyamine pathway was suggested by a similar inhibition of colony formation by difluoromethylornithine (DFMO), a suicide inhibitor of ornithine decarboxylase, and the lack of additivity of DFMO and tamoxifen. The cytostatic effect of DFMO was found to be dose dependent. The specificity of the DFMO effect through the polyamine pathway was indicated by the dose-dependent rescue of colony growth with exogenous administration of putrescine, the polyamine distal to the site of inhibition. A lack of alteration of colony size in proliferating clones was uniformly observed. These data indicate that the soft-agar culture technique can be successfully used to investigate the endocrine mechanisms affecting the growth of individual experimental mammary cancers. The data also suggest an important role of polyamines in mediating the growth of this mammary tumor model.

INTRODUCTION

The NMU[3]-induced rat mammary tumor is a well-established model of hormone-responsive breast cancer (12, 13). Estrogen, prolactin, and perhaps growth hormone appear to be the primary hormones involved in tumor growth (1, 9). This and other endocrine-dependent tumors, however, contain heterogeneous subpopulations of hormone-dependent and -independent sublines. Hamburger and Salmon (3) first introduced a soft-agar culture system which enabled them to grow separate cellular clones from a variety of human cancers. A major advantage of this clonogenic method is selective facilitation of the growth of tumor stem cells while inhibiting the growth of common contaminants such as fibroblasts. While not exploited by these original investigators, this system allows assessment of biochemical differences between clones of hormone-dependent and -independent cells. In the present experiments, we used the soft-agar culture system for growing the NMU-induced mammary cancer and studying the effects of tamoxifen administration on formation and size of stem cell-derived colonies. In addition, we explored the role of the polyamine pathway in hormone-mediated tumor growth in this system. A variety of recent experiments indicates that polyamines are involved in the growth of various experimental tumor models both *in vitro* and *in vivo* (5, 7, 11). Specifically, we investigated whether tumor growth could be inhibited by blocking polyamine biosynthesis with DFMO, a suicide inhibitor of ODC, the first enzyme mediating polyamine biosynthesis. We also studied whether the effects of DFMO were additive with those of tamoxifen. The specificity of DFMO effect was investigated by rescuing DFMO-blocked clones with putrescine, a polyamine distal to the site of inhibition. Our results suggest that subpopulations of hormone-dependent and -independent cells respond differently to tamoxifen in the clonogenic assay and that antiestrogen and polyamine-blocking effects are not additive.

MATERIALS AND METHODS

Tumor Induction. Mammary tumors were induced in 50-day-old female Sprague-Dawley rats by 2 i.v. injections of NMU (5 mg/100 g body weight) given 1 week apart. Mammary tumors appeared 5 to 15 weeks following the first NMU injection. All tumors used in these experiments were freshly excised prior to plating.

Cell Culture Technique. Cell suspensions of tumors are prepareo by mechanical dispersion. After being excised the tumor is placed in HBSS and cut into small pieces using 2 sterile scalpels. The tumor pieces are then placed on a fine stainless steel mesh (100-μm pores) in the selector apparatus (Bellco Glass, Inc., Vineland, N. J.). Using a pestle, the tumor tissue is gently pushed through the mesh into a Petri dish. The resultant suspension is washed twice and resuspended in enriched Connaught Medical Research Laboratories media (Grand Island Biological Co., Grand Island, N. Y.). Cell viability was checked using the trypan blue exclusion test prior to plating. The cells were cultured in a bilayer soft-agar system using the techniques described by Hamburger and Salmon (4) and Van Hoff *et al.* (14). Briefly, the bottom layer consists of McCoy's medium (Grand Island Biological Co.) enriched with horse serum (5%), fetal calf serum (10%), sodium pyruvate (0.22 mg/ml), L-serine (42 μg/ml), glutamine (2.9 μg/ml), sodium bicarbonate (4.5 mg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), asparagine (100 μg/ml), DEAE-dextran (375 μg/ml), and tryptic soy broth (7.5 mg/ml). Three % agar is then diluted in the medium to give a final mixture of 0.5% agar. One ml of the suspension is then dispensed immediately into a 35-sq-mm Petri dish. The upper layer consists of Connaught Medical Research Laboratories medium enriched with horse serum (15%), calcium chloride (444 μg/ml), porcine insulin (2 units/ml), ascorbic acid (53 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), DEAE-dextran (250 μg/ml), mercaptoethanol (0.4 μg/ml), and asparagine (66 μg/ml). Three % agar is then added to make a final agar concentration of 0.6%.

Plating is performed by aspirating 0.5 ml of the cell suspension followed by 0.5 ml of the upper layer suspension and dispensing the 1-ml suspension on top of the lower layer. Between 160,000 and 350,000 viable cells were plated per dish. Experiments were performed using quadruplicate dishes for each experimental condition. The plates were incubated at 37° with 5% CO_2 and 100% humidity.

Compounds Tested. Tamoxifen was initially diluted in 100% ethanol (10^{-4} M) and then added to both layers of the media with a final

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[3] The abbreviations used are: NMU, *N*-nitrosomethylurea; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; HBSS, Hank's balanced salt solution.

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concentration of 10^{-7} M. DFMO was initially dissolved in HBSS (10 mM) and then diluted in both layers with a final concentration of 0.8 mM. Similarly, putrescine was initially dissolved in HBSS (50 mM) and then added to both layers with a final concentration of 50 μM. In a separate experiment, the cytostatic effect of DFMO was tested in a dose-response study. DFMO was initially dissolved in HBSS (100 mM) and then diluted in both layers with final concentrations ranging from 0.05 to 10 mM (Chart 1). In an additional experiment, the rescue effect of putrescine was also tested in a dose-response study with concentrations of putrescine ranging from 0 to 2.5 mM (Chart 2).

Colony Counting. The plates were inspected 2, 4, 6, 8, and 10 days after plating. Colonies (>40 cells) were counted using an inverted-phase microscope at ×40. Since maximum colony formation in control dishes was observed on Day 6, this time was selected to evaluate the effect of the compounds tested. Colony size, expressed as mean square diameter in μm, was measured by means of a graduated microscope eyepiece and corrected for the magnification factor.

Statistical analysis was performed using the Newman-Keuls method (15).

RESULTS

The soft-agar culture system provided an efficient means of growing the NMU tumor *in vitro*, since 16 of 16 separate neoplasms grew with an average of 55 ± 10.0 (S.E.) colonies/control dish (Table 1). Approximately 50% of stem cell-derived cells were hormone dependent as reflected by their inhibition with tamoxifen (10^{-7} M). Colony growth could be blocked to a similar degree with DFMO, the suicide inhibitor of ODC (Table 1). This effect was dose dependent with concentrations of DFMO ranging from 0.05 to 10 mM (Chart 1). Furthermore, the colony-blocking action of DFMO was specifically related to its effect on ODC. In fact, exogenous administration of the more distal polyamine, putrescine, reversed in a dose-dependent fashion the cytostatic effect of DFMO (Chart 1; Table 1). The lack of additive effects of DFMO and tamoxifen (Table 1) on colony formation suggests that both compounds might exert their action on similar subpopulations of cells. It was of interest that, in all experiments, clones of cells which grew despite DFMO or tamoxifen achieved a similar colony size.

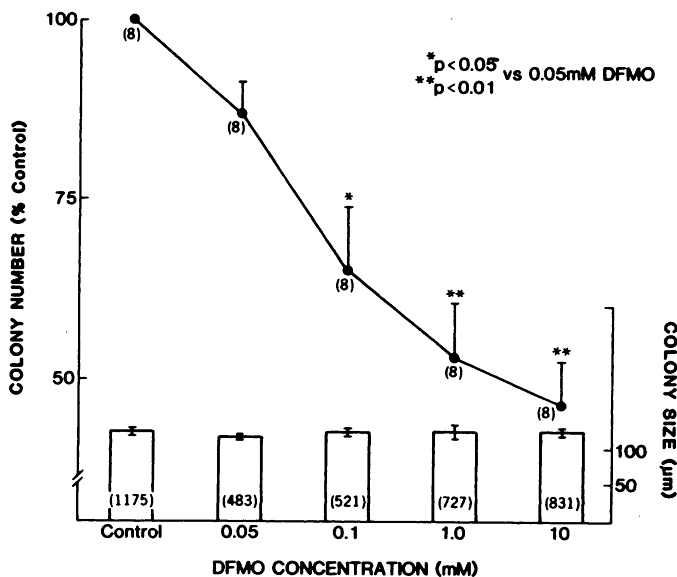

Chart 1. Effect of increasing concentrations of DFMO on the number (●) and size (□) of colonies of NMU-induced mammary tumors grown in soft agar. Data are mean; *bars*, S.E. *Numbers* in *parentheses*, number of tumors in the case of "colony number" and number of colonies measured in case of "colony size."

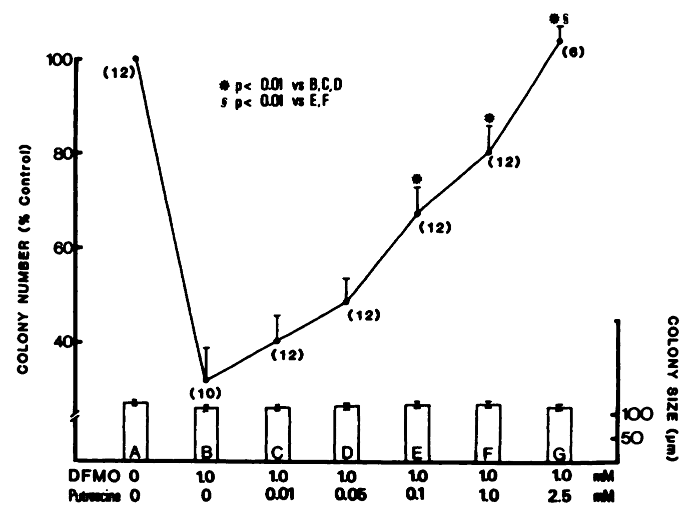

Chart 2. Effect of increasing concentrations of putrescine on the number and size of colonies of NMU-induced mammary tumors grown in soft agar in the presence of DFMO. Data presented as in Chart 1. Number of colonies measured *A*, 1465; *B*, 554; *C*, 610; *D*, 788; *E*, 1144; *F*, 1316; *G*, 480.

Table 1

Effect of the treatments on the number and size of colonies of NMU-induced rat mammary tumors grown in soft agar

	Colony no. (% of control)	Colony size (sq diameter)
Control	100 (16)[a]	135.9 ± 1.3[b] (678)[c]
Tamoxifen	46.2 ± 5.1[d] (12)	131.6 ± 17 (322)
DFMO	53.0 ± 4.4[d] (16)	133.3 ± 1.7 (359)
DFMO + putrescine	81.1 ± 2.6 (16)	134.9 ± 1.4 (590)
Tamoxifen + DFMO	40.6 ± 5.0[d] (12)	132.7 ± 1.9 (283)

[a] Numbers in parentheses, number of tumors.
[b] Mean ± S.E.
[c] Numbers in parentheses, number of colonies measured.
[d] $p < 0.01$ *versus* DFMO + putrescine.

DISCUSSION

Our data clearly show that the soft-agar culture system allows growth of at least 2 subpopulations of NMU-induced rat mammary tumor stem cells. On the basis of the response to the antiestrogen tamoxifen, it appears that approximately one-half are hormone responsive, whereas the remaining one-half are hormone resistant. We considered that the cytostatic effect of tamoxifen may be mediated through the polyamine pathway. Various hormones, including estrogens, have been found to stimulate ODC activity and, consequently, polyamine formation in normal tissues (6, 10). Thus, it is possible that estrogens may also stimulate ODC activity in hormone-responsive neoplastic tissue such as the NMU-induced mammary tumor. Furthermore, the stimulation of polyamine biosynthesis may mediate the mitogenic effect of estrogens on tumor growth. Our preliminary data, demonstrating that the effects of tamoxifen and DFMO are not additive, support this hypothesis. Clearly, these hypotheses need to be tested more extensively in future experiments. Specifically, it will be important to demonstrate that the inhibition of colony formation induced by tamoxifen will be reversed by the same concentrations of putrescine that reversed the effect of DFMO. Nonetheless, our data clearly indicate a role for polyamines in the growth of the NMU-induced rat mammary tumor. Inhibition of ODC activity with DFMO has been found to have a cytostatic effect *in vitro* and/or *in vivo* in a variety of tumor models including the rat hepatoma (5), L1210

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mouse leukemia (5, 7), 9L rat gliosarcoma (11), and murine mammary sarcoma (8). Since this effect is reversed by exogenous administration of polyamines, depletion of tissue polyamines secondary to the decrease in ODC activity appears to be responsible for the observed antitumor action.

Colony size, in contrast to colony number, was not affected by any of the treatments used. This finding suggests that "sensitive" stem cells were completely inhibited by tamoxifen and DFMO. In contrast, "resistant" stem cells fully retained their replicative potential. However, caution should be used in drawing this conclusion, since the "all-or-none" effect on colony size contrasts somewhat with the dose-dependent effect of DFMO and putrescine on colony number. We plan to investigate this issue in future experiments by using more quantitative methods to assess the mitogenic activity of colonies such as thymidine incorporation, which has been successfully introduced in this culture system (12). In conclusion, we have shown that the soft-agar culture technique, until now primarily used to study chemosensitivity, can be used successfully to investigate the endocrine mechanisms affecting the growth of individual experimental mammary cancers. It may also be used potentially to study tumor heterogeneity which is a prominent feature of experimental and human breast cancer.

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