Polyamines and Autocrine Control of N-Nitrosomethylurea-induced Rat Mammary Tumor Growth in Vitro by Progesterone

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ABSTRACT

These experiments were designed to test whether autocrine/paracrine mechanisms are involved in the growth-promoting action of progesterone (Pg) in the N-nitrosomethylurea-induced rat mammary tumor cultured in vitro in soft agar, cosexogenetic assay. In support of our hypothesis, we observed that conditioned media obtained from Pg-treated tumors (Pg-CM), consistently stimulated colony formation in our system to the same degree as Pg itself (approximately 140% of control). Treatment with heat, trypsin, and concanavalin A abolished the colony-stimulating effect of Pg-CM, thus suggesting the possible glycoprotein nature of the Pg-inducible growth factor(s). The growth-promoting action of Pg-CM was rather specific since CMs obtained from tumors exposed to a variety of other steroid hormones rarely stimulated colony formation and usually only to a modest degree. Administration of the polyamine biosynthetic inhibitor, α-difluoromethylornithine, abolished the colony-stimulating effect of Pg-CM. The inhibitory effect of α-difluoromethylornithine was reversed in a dose-dependent fashion by exogenous administration of spermidine, which entirely restored the growth-promoting action of Pg-CM. Addition of increasing amounts of spermidine, however, did not potentiate Pg-CM action under our experimental conditions. Our results indicate that autocrine/paracrine mechanisms may mediate, at least in part, the colony-stimulating effect of Pg in our system. The polyamine pathway plays an essential role in the expression of such control of tumor growth by Pg.

INTRODUCTION

The role of progesterone in the growth of hormone-responsive breast cancer remains poorly elucidated. Using the NMU3-rat mammary tumor cultured in soft agar, we have recently shown that progestins, tested over a wide range of concentrations, exert a significant colony-stimulating effect (1). The aim of this study was to evaluate whether autocrine/paracrine mechanisms are involved in the growth-promoting action of progestins in this experimental system as we have already shown for estradiol (2) and prolactin (3). To test this hypothesis, we evaluated the colony-stimulating effects in soft agar of conditioned media obtained from progesterone-treated tumors. To assess the specificity of our observations, we tested the effects on tumor colony formation of several additional steroids as well as conditioned media generated from tumors exposed to these compounds in vitro. Finally, we evaluated the interaction between the polyamine pathway and the autocrine control of tumor growth by progesterone.

MATERIALS AND METHODS

Materials. NMU, progesterone, 17α-hydroxyprogesterone, testosterone, dihydrotestosterone, dexamethasone, hydrocortisone, spermidine,

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2 To whom requests for reprints should be addressed, at Division of Endocrinology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033.

The abbreviations used are: NMU, N-nitrosomethylurea; DFMO, α-difluoromethylornithine; Con A, concanavalin A; Pg-CM, conditioned media obtained from progesterone-treated tumors; C-CM, conditioned media obtained from control tumors.

and trypsin were all obtained from Sigma Chemical Company, St. Louis, MO. DFMO was supplied by Merrell Research Center, Merrell Dow Pharmaceutical Inc., Cincinnati, OH. Con A-Sepharose was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Spectra/Por membranes were obtained from Fisher Scientific Company, King of Prussia, PA.

Tumor Induction and Culture Technique. The method of NMU mammary tumor induction (4) as well as the details of the culture technique in soft agar have been previously published by us (5, 6). All of our experiments were conducted under serum-free media conditions to obviate the confounding effects of the hormones and growth factors present in the serum. The number of colonies (aggregates of 50 or more cells) formed on day 6 after plating was the parameter used to assess treatment effects on tumor growth. The validity of this parameter as an indicator of growth in soft agar has been supported by its correlation with incorporation of [3H]thymidine into DNA (7).

Preparation of Conditioned Media. For each experiment, different sets of hormone- and control-conditioned media were used. To prepare the conditioned media, single cell suspensions of NMU mammary tumors were plated in soft agar in the presence or absence of either progesterone or a different steroid as indicated in the tables and figures. The concentration of these compounds was always 10−8 M. Conditioned media from hormone-treated and control dishes were obtained as previously described in detail by us (2, 3, 8). Following concentration of the conditioned media, single cell suspensions of NMU mammary tumor colony formation of several additional steroids as well as conditioned media generated from tumors exposed to these compounds in vitro. Finally, we evaluated the interaction between the polyamine pathway and the autocrine control of tumor growth by progesterone.
methasone, and hydrocortisone simultaneously added to single cell suspensions of NMU mammary tumors plated in soft agar in the absence of serum. In the second set of six experiments, we simultaneously tested under identical conditions the colony-stimulating effects of the conditioned media obtained from NMU mammary tumors exposed to the above-mentioned steroids.

Interaction between Pg-CM and Polyamines in Supporting Tumor Growth. Initially, we tested whether DFMO, an irreversible and specific inhibitor of polyamine biosynthesis, was able to block the effect of Pg-CM on colony formation. Simultaneously, the ability of exogenous spermidine administration to reverse the inhibitory effect of DFMO was also investigated in a dose-response fashion. In these experiments, NMU mammary tumors were plated under control conditions and in the presence of the following treatments: (a) Pg-CM; (b) Pg-CM + DFMO (1 µM); (c) Pg-CM + DFMO + spermidine (1 µM); (d) Pg-CM + DFMO + spermidine (10 µM); (e) Pg-CM + DFMO + spermidine (100 µM). For control purposes, the same tumors were simultaneously incubated in parallel replicate dishes with C-CM instead of Pg-CM under otherwise identical experimental conditions.

Next, we tested whether exogenous spermidine administration was able to potentiate the colony-stimulating effects of Pg-CM in our system. NMU mammary tumors were plated in soft agar in the presence of Pg-CM with and without increasing concentrations of spermidine (1, 10, and 100 µM). Simultaneous identical dose-response studies using C-CM were also conducted. DFMO and spermidine were initially dissolved in Hanks' balanced salt solution and then added to both layers of the media in the final desired concentrations.

Statistical Analysis of the Data. Since each experiment was internally controlled, statistical analysis of the data was performed individually for each experiment using analysis of variance according to the Newman-Keuls test (11). In addition, whenever repetitive experiments were analyzed (Tables 1–3), a global analysis of the data was also performed. Using analysis of variance with treatment as a factor and experiment as a block effect, we performed the Dunnett’s t-test of treatment mean colony numbers against control mean colony numbers.

Table 1 Effects of C-CM and Pg-CM on NMU-mammary tumor colony formation in soft agar

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Colony no.</th>
<th>C-CM</th>
<th>Pg-CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>105 ± 2.4</td>
<td>104 ± 3.2</td>
<td>158 ± 1.6</td>
</tr>
<tr>
<td>1</td>
<td>150 ± 3.0</td>
<td>152 ± 2.6</td>
<td>164 ± 3.2</td>
</tr>
<tr>
<td>2</td>
<td>183 ± 2.2</td>
<td>101 ± 1.0</td>
<td>119 ± 2.7</td>
</tr>
<tr>
<td>3</td>
<td>59 ± 2.1</td>
<td>59 ± 2.5</td>
<td>114 ± 3.3</td>
</tr>
<tr>
<td>4</td>
<td>108 ± 2.2</td>
<td>109 ± 2.6</td>
<td>133 ± 1.1</td>
</tr>
<tr>
<td>5</td>
<td>102 ± 3.3</td>
<td>100 ± 2.2</td>
<td>122 ± 1.5</td>
</tr>
</tbody>
</table>

* Data represent mean ± SEM colony numbers of 5 replicate dishes for each experimental condition. The colony-stimulating effect of Pg-CM was statistically significant (P < 0.01, Newman-Keuls test) in all experiments, while C-CM significantly stimulated colony formation only in Experiment 3. Global analysis of the data also revealed that the colony-stimulating effect of Pg-CM was statistically significant (P = 0.01, Dunnett’s 2-sided t-test).

RESULTS

Colony-stimulating Effect of Pg-CM. As can be seen in Table 1, Pg-CM administration significantly stimulated NMU mammary tumor colony formation in all six experiments. This treatment increased colony number to a mean of 140% ± 11.2 (SEM) of control. In contrast, C-CM administration did not influence colony formation except in Experiment 3 where a modest growth-promoting action was observed. This effect, however, was inferior to that of Pg-CM simultaneously tested.

Effect of Con A, Heat, and Trypsin Treatment on the Colony-stimulating Effect of Pg-CM. As can be seen in Fig. 1, pretreatment of Pg-CM with heat, Con A, and trypsin totally abolished the colony-stimulating effect of Pg-CM. In contrast, the addition of C-CM, either untreated or similarly pretreated, did not alter NMU mammary tumor colony formation in soft agar. Similar results were obtained in three additional experiments identical to that depicted in Fig. 1 (data not shown).

Table 2 Effects of different steroids on NMU mammary tumor colony formation in soft agar

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Colony no.</th>
<th>Pg</th>
<th>17α-OH-Pg</th>
<th>T</th>
<th>DHT</th>
<th>Dex</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>81 ± 1.5</td>
<td>123 ± 1.6&quot;</td>
<td>123 ± 1.5&quot;</td>
<td>84 ± 1.2</td>
<td>95 ± 1.2&quot;</td>
<td>96 ± 1.6&quot;</td>
<td>81 ± 1.0</td>
</tr>
<tr>
<td>1</td>
<td>103 ± 0.7</td>
<td>114 ± 0.5&quot;</td>
<td>113 ± 0.7&quot;</td>
<td>103 ± 0.5</td>
<td>103 ± 0.7</td>
<td>105 ± 0.5</td>
<td>108 ± 0.5&quot;</td>
</tr>
<tr>
<td>2</td>
<td>55 ± 0.9</td>
<td>79 ± 0.9&quot;</td>
<td>57 ± 0.5</td>
<td>59 ± 1.4</td>
<td>59 ± 1.0</td>
<td>53 ± 0.5</td>
<td>54 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>40 ± 1.2</td>
<td>55 ± 1.2&quot;</td>
<td>44 ± 1.7&quot;</td>
<td>35 ± 1.8</td>
<td>35 ± 1.8</td>
<td>35 ± 0.6</td>
<td>37 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>114 ± 1.5</td>
<td>149 ± 1.2&quot;</td>
<td>118 ± 1.0</td>
<td>133 ± 2.2&quot;</td>
<td>120 ± 1.7&quot;</td>
<td>128 ± 1.0&quot;</td>
<td>121 ± 4.5</td>
</tr>
<tr>
<td>5</td>
<td>66 ± 2.6</td>
<td>102 ± 1.7</td>
<td>71 ± 1.4&quot;</td>
<td>66 ± 2.0</td>
<td>66 ± 1.9</td>
<td>63 ± 2.1</td>
<td>65 ± 0.7</td>
</tr>
</tbody>
</table>

* Data are expressed as in Table 1.

In the global analysis, the colony-stimulating effect of 17α-OH-Pg was statistically significant (P = 0.05) in the one-sided but not in the two-sided Dunnett’s t test. None of the other treatments significantly affected colony formation in the global analysis.

Interaction between Pg-CM and Polyamines in Supporting Tumor Growth. In initial experiments, we tested the role of progesterone (Table 2). Subsequently, we evaluated the action of the conditioned media obtained from tumors exposed to these compounds (Table 3). As expected, on the basis of our previous findings (1), progesterone administration significantly stimulated NMU mammary tumor colony formation in all six experiments (Table 2). The average increase in colony formation induced by progesterone was 138% ± 6.0 (SEM) of control, an effect virtually identical to that observed with the addition of Pg-CM (Tables 1 and 3). In Experiments 1 and 2, 17α-hydroxyprogesterone had a similar growth-promoting action. With the exception of these two instances, however, a colony-stimulating effect was only sporadically observed with the other steroids tested and, when present, it was found to be significantly (P < 0.05) inferior to that produced by the administration of progesterone (Table 2). Table 3 illustrates the effects on NMU mammary tumor colony formation of the various steroid-conditioned media. In agreement with our previous results shown in Table 1, Pg-CM significantly stimulated colony formation in each experiment. The magnitude of the effect of the Pg-CM was virtually identical to that reported in Table 1, with an average increase in colony formation of 141% ± 5.4 (SEM) above control. In contrast, the remaining steroid-conditioned media only rarely had a significant colony-stimulating effect, which, in every case, was inferior (P < 0.05) to that observed with the addition of Pg-CM (Table 3).
polyamines as mediators of the action of Pg-CM in our system. As can be seen in Fig. 2, the addition of the polyamine biosynthesis inhibitor DFMO totally abolished the stimulation of colony formation induced by Pg-CM. The specificity of the DFMO effect through the polyamine pathway was indicated by the ability of spermidine to completely reverse the inhibitory effect of DFMO in a dose-dependent fashion (Fig. 2). In contrast, colony formation was not affected by DFMO with and without increasing concentrations of spermidine when C-CM was added instead of Pg-CM (Fig. 2). Similar results were obtained in three additional experiments identical to that depicted in Fig. 2 (data not shown).

Next, we tested whether exogenous spermidine administration potentiates the colony-stimulating effect of Pg-CM. As can be seen in Fig. 3, no potentiation was observed under these experimental conditions. A similar lack of additive or synergistic effects between Pg-CM and spermidine was observed in three additional experiments identical to that reported in Fig. 3 (data not shown).

**DISCUSSION**

The role of progesterone in breast cancer growth remains controversial with both stimulatory (12, 13) and inhibitory (14) effects being reported. We have recently shown that in our *in vitro* experimental mammary tumor model, progesterone has a definite growth-promoting effect (1). The results presented here suggest that autocrine/paracrine mechanisms may mediate progesterone action in our system. Our contention is supported by the observation that conditioned media obtained from progesterone-treated tumors consistently stimulated colony formation to an average of approximately 140% of control. The magnitude of the Pg-CM effect was virtually identical to that of progesterone itself.

We are fully aware that our data are indirect and that more conclusive evidence supporting an autocrine control of tumor growth by progesterone awaits biochemical identification and characterization of the progesterone-inducible growth factor(s). Due, however, to the limited number of cells that grow in soft agar (in the thousand range) compared to standard liquid culture conditions (in the million range), repeated attempts to biochemically characterize the nature of the growth factor(s) present in our media have been so far unrewarding because of...
sensitivity problems. Our data, nevertheless, for the first time, provide some evidence that progesterone may support the growth of hormone-responsive breast cancer through the stimulation of secretory products which act locally in an autocrine/paracrine fashion. The ability of heat, trypsin, and concanavalin A to abolish the colony-stimulating effect of the Pg-CM suggests that the progesterone-induced growth factor(s) may be a glycoprotein. In support of our contention, recent data in the literature indicate that progesterins are able to stimulate the synthesis of cellular (15) and secretory (16) proteins in human breast cancer cell lines as well as human endometrium (17) in culture. The relationship, however, of these proteins to the control of cell proliferation by progesterins was not investigated in those studies.

In our experimental system, progesterone seems to act similarly to estradiol (2) and prolactin (3), the two primary hormones involved in the growth of the NMU mammary tumor in vivo (5). Thus, we deemed it important to determine the specificity of our observations by testing the colony-stimulating effects of several additional steroids as well as conditioned media obtained from exposure of NMU mammary tumors to them. In contrast to Pg and Pg-CM, which exerted a reproducible colony-stimulating effect, the remaining steroids and conditioned media rarely affected colony formation. Even when present, their stimulatory effects were modest and usually inferior to those induced by Pg and Pg-CM (Tables 2 and 3). Thus, our results suggesting an autocrine/paracrine control of tumor growth by progesterone (present data) as well as by estradiol (2) and prolactin (3) are likely to reflect biologically important events and not artifacts of our culture conditions.

Our data indicate that the polyamine pathway closely interacts with the hormonal regulation of NMU-mammary tumor growth in soft agar. In previous reports, we have provided evidence supporting a critical role of polyamines in the synthesis (10, 18) as well as action (2, 8) of estradiol- and prolactin-regulated growth factors. The data presented here indicate that polyamines play an important role also in the action of progestosterone-regulated growth factors. Even though polyamines were unable to potentiate the effect of Pg-CM, inhibition of polyamine biosynthesis with DFMO totally abolished the colony-stimulating effect of Pg-CM. In support of the specificity of the DFMO effect through the polyamine pathway, exogenous spermidine administration reversed the DFMO effect and completely restored colony formation. In these experiments, we did not test the potential involvement of polyamines in the synthesis of progestosterone-regulated growth factors.

A similar important role for polyamines has recently been proposed in the endocrine control of proliferation of hormone-responsive human breast cancer cell lines grown in liquid culture (19–21). These results seem to contrast with those reported in normal endocrine target tissues such as prostate (22) and uterus (23) where hormonal stimulation of polyamine biosynthesis has been found to be dissociated from hormonal effects on cell proliferation.

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REFERENCES

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