Anchorage-independent Growth-conferred Factor Production by Rat Mammary Tumor Cells

James A. Zwiebel, Margot R. Davis, Elise Kohn, David S. Salomon, and William R. Kidwell

ABSTRACT

Conditioned medium from cultures of 7,12-dimethylbenz-(a)anthracene-induced rat mammary tumor cells contain factors that resemble sarcoma growth factor and other transforming growth factors in biological activity but differ in their physical properties. The mammary tumor factors (MTF) are acid stable and heat and protease sensitive. They inhibit the binding of epidermal growth factor, but not insulin, to mouse embryonal carcinoma cells. MTF confers upon normal rat kidney and BALB/c-3T3 cells the ability to grow in soft agar. This effect is enhanced synergistically by high concentrations of fetal calf serum but not by epidermal growth factor. Anchorage-independent growth promotion, however, is not seen with normal mammary epithelial cells, although MTF is mitogenic for these cells as well as normal rat kidney cells, BALB/c-3T3 cells, and chick embryo fibroblasts in monolayer culture. MTF is not mitogenic for primary cultures of the tumor cells from which the factors are derived. Two major molecular weight species of MTF, eluting at M, 6,000 and 65,000 to 70,000 on Bio-Gel P-100 columns, are present in acid-ethanol extracts of 7,12-dimethylbenz(a)anthracene- and nitrosomethylurea-induced rat mammary tumors. Transplantable tumors derived from primary 7,12-dimethylbenz(a)anthracene- or nitrosomethylurea-induced tumors have little or no MTF activity. These results demonstrate that different chemically induced rat mammary tumors contain transforming growth factor-like activities. Furthermore, it is possible that MTF is unnecessary for the maintenance of tumorigenicity, since some tumors contain no detectable MTF.

INTRODUCTION

Cells transformed by certain RNA tumor viruses produce growth-promoting peptides which confer several properties ascribed to the transformed phenotype upon normal epithelial and mesenchymal cell lines (4, 6, 7, 22, 37, 39). Specifically, Moloney murine sarcoma virus-transformed cells produce and secrete into the culture medium SGF (4, 6, 7). SGF is a heat-stable, acid-soluble polypeptide which has been shown to secrete into the culture medium SGF (4, 6, 7). SGF is a family of peptides with a molecular weight range of 7,000 to 10,000, TGFs isolated from human sarcoma and carcinoma cell lines have molecular weights in the 7,000 and 20,000 to 23,000 size classes (40, 41). Other TGFs have also been isolated from solid human and animal tumors (21, 29), chemically transformed cells (19), and even a variety of nonneoplastic tissues (26, 28), including human placenta, mouse embryos (25, 43), and Pedersen-prepared bovine fetuin (1). In contrast to SGF, many of these other TGFs do not compete with EGF for receptor binding (19, 27, 28).

Here we describe TGF-like activities which have been recovered from DMBA- and NMU-induced rat mammary tumors. These activities resemble TGFs in their biological properties. The present report details the partial fractionation and characterization of the MTFs.

MATERIALS AND METHODS

Cell Cultures. NRK cells were obtained from Dr. Joseph DeLarco, National Cancer Institute, and were grown as described previously (5). Mouse OTT-6050 EC cells were grown in monolayer culture as reported earlier (30). CEF cells were obtained from the American Type Tissue Culture Collection, Rockville, Md. CEF cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum (v/v) supplemented with 4 mM glutamine, 20 mM HEPES (pH 7.4) (Grand Island Biological Co., Grand Island, N. Y.), streptomycin (100 μg/ml), and penicillin (100 units/ml) (NIH Media Unit), in a humidified atmosphere of 95% air-5% CO2, at 37°. BALB/c-3T3 cells, obtained from Dr. Janice Chou, NIH, were propagated in DMEM containing 10% fetal calf serum, glutamine, and antibiotics as described for the CEF cells. Primary cultures of normal rat mammary epithelial cells were initiated from isolated ductal and alveolar structures according to previously described procedures (16, 32, 44). These cells and those derived from DMBA-induced rat mammary tumors (44) were cultured in serum-free growth medium as described previously (30, 32). This medium [improved Eagle's minimal essential medium, supplemented with 4 mM glutamine, 20 mM HEPES, streptomycin (100 μg/ml), and penicillin (100 units/ml)] contained the following hormonal supple-

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ments: insulin (5 µg/ml, bovine; Sigma Chemical Co., St. Louis, Mo.); bovine fetuin (1 mg/ml; type III, Sigma); human transferrin (5 µg/ml; Sigma); and hydrocortisone (50 µg/ml). EGF was omitted.

Preparation and Fractionation of MTF from Conditioned Medium. Freshly isolated cells derived from primary DMBA-induced tumors were plated in the serum-free and EGF-free medium as described at a seeding density of about 2 × 10^5 cells/10 ml medium. After the cells were allowed to attach for 48 hr, the medium was replaced with serum-free medium in which the fetuin and transferrin were omitted. Two days later, the medium was harvested from the dishes and analyzed for growth-promoting activity, EGF receptor-competing activity, or soft agar growth-conferring activities. Second- and third-harvests of TCM were also obtained without any appreciable cell degeneration, but there was little tumor cell growth in the absence of fetuin and transferrin. In studies in which insulin receptor-competing activities were to be tested, tumor cells were plated and maintained in the serum-free medium from which insulin was also omitted. The conditioned medium (typically 200–300 ml) was first filtered through a 0.45-µm filter and then dialyzed against 50 volumes of cold 1% acetic acid utilizing a 3500 molecular-weight-cutoff dialysis tubing (Spectrapor, Fisher-Scientific). The retained material was then lyophilized to dryness and resuspended in 50 ml of 0.1% acetic acid. The sample was then fractionated by ultrafiltration utilizing Diaflo membranes (Amicon Corp., Lexington, Mass.) with exclusion limits ranging from M, 3000 to 5000. The ultrafiltrates were then lyophilized to dryness and resuspended in various buffers or radioreceptor assay-binding buffer for analysis. As a control, an equivalent amount of growth medium not exposed to cells was processed in an identical manner.

Isolation of MTF from Tumors. Ten to 70 g of pooled primary DMBA- or NMU-induced tumors or the transplantable tumors derived from primary tumors were homogenized and extracted with acid-ethanol following the procedure which Roberts et al. (29) have previously described for the isolation of TGFs from murine tumors and virally transformed murine cells. The tumors utilized here ranged up to 10 g each; necrotic tumors were discarded. The acid-ethanol-soluble material was precipitated with anhydrous ethanol and ether. The fraction recovered by extraction of the filtered precipitate with 1 M acetic acid was dialyzed against 1% (0.17 M) acetic acid. Material retained in the Spectrapor dialysis tubing (M, 3500 cutoff) was lyophilized to dryness and stored desiccated at 4°C. Upon resuspension of the solid extract [3 mg of extract per ml PBS (HEM Research, Inc., Rockville, Md.) or 3 mg per ml binding buffer at room temperature], only about one-third of the lyophilized preparation became soluble. The insoluble residue was then removed by centrifugation. The final preparation obtained from 0.3 to 0.6 mg protein per ml as shown by protein analysis following the procedure of Lowry et al. (17).

Colony Formation in Soft Agar. Anchorage-independent growth-promoting activity of the conditioned medium or tumor extracts was assessed as follows. A cell suspension layer, containing 2 to 3 × 10^3 NRK or BALB/c-3T3 cells per ml in 1.0, 0.5, or 0.3 ml of 0.3% Difco agar (supplemented with DMEM plus 10% fetal calf serum), was layered over the same volume of 0.6% agar-medium base layer in each well of a 6-, 12-, or 24-well cluster dish (Costar, Cambridge, Mass.), respectively. The dishes were incubated at 37°C for 10 to 14 days, and the colonies were stained with nitro blue tetrazolium (Sigma) (29). An Artrek Model 880 colony counter was used to count colonies and to determine their size distribution. Colonies greater than 30 to 40 µm in diameter were scored and counted.

Assay for Mitogenic Activity of the Fractions in Monolayer Cultures. Growth-promoting activity was determined by measuring the change in cell number of the various cell types as a function of time after the addition of test samples. For normal mammary epithelial cells, serum-free medium containing fetuin, transferrin, insulin, and hydrocortisone in the amounts indicated earlier was utilized. EGF was omitted and the primary epithelial cells were plated at 1 × 10^5 in 1.5 ml of medium. Cells were counted at 0, 1, and 3 days after plating and dishes containing MTF were compared to cultures supplemented with an equal volume of control buffer, boiled extract, or, in the case of conditioned medium, a similarly prepared extract of growth medium never exposed to cells. NRK cells were seeded at 5 × 10^3/ml in serum-free medium without EGF onto polylysine-coated culture dishes (30) or, alternatively, onto uncoated culture dishes with DMEM containing 5% fetal calf serum. Cell growth was comparable in either system. BALB/c-3T3 and CEF cells were plated in DMEM containing 10% fetal calf serum and then switched to the same medium with only 1% serum in order to obtain serum-starved G1-arrested cells (23). Twenty-four hr after the medium change, tumor extract or control buffer was added. Cell numbers were determined 72 hr after the addition of tumor extract for NRK, 3T3, and CEF cells.

EGF and Insulin Radioreceptor Assays. EGF and [125I]-EGF (specific activity, 80 to 125 µCi/µg) were obtained from KOR Biochemicals, Cambridge, Mass.; Collaborative Research, Cambridge, Mass.; or Mellon Laboratories, Springfield, Va. Binding assays were performed with mouse EC cells either in suspension or in monolayer (30). In the monolayer assay, approximately 2 × 10^5 cells were seeded onto 35-mm Costar cluster dishes and assayed 48 hr after plating. Binding assays were performed in 1 ml of binding buffer (DMEM containing bovine serum albumin (1 mg/ml), streptomycin (100 µg/ml), penicillin (100 units/ml), 20 µM Hepes (pH 7.4), and 4 mm glutamine) containing 10% FBS (New England Nuclear, Boston, Mass.) and the EC cells in monolayer as described above (30). Quantitation of the number and affinities of EGF receptors on normal and tumor mammary epithelial cells was performed in monolayer cultures after culturing the cells for 3 days in serum-free medium in the absence of EGF (32). All determinations were done in duplicate.

Protein Assays. The protein content of samples was determined by the dye-binding procedure of Bradford (2) utilizing commercially supplied reagents (Bio-Rad Laboratories, Rockville Centre, N.Y.) or, alternatively, by the procedure of Lowry et al. (17) using bovine serum albumin standards.

Purification of MTF from Tumor Extracts. Approximately 20 mg of soluble protein recovered from DMBA-induced tumors were applied to a 1.5-× 90-cm column of Bio-Gel P-100 equilibrated with PBS. The column was eluted with the same buffer at a flow rate of 12 ml/hr into tubes containing 0.1 ml of 0.1% bovine serum albumin or 0.1% bovine fetuin. If the albumin was omitted, little EGF-competing activity was detected in the eluate, especially in the low-molecular-weight-range fractions. Likewise, omission of fetuin resulted in the failure to detect any soft-agar colony-forming activity. Column fractions were sterilized by filtration through 0.22-µm filters and assayed for cell- or colony growth-stimulating activities or tested directly for competition with [125I]-EGF for receptor binding in the radioreceptor assay.

RESULTS

Production of MTF by Primary Cultures of Mammary Tumor Cells. As was found for Moloney murine sarcoma- and Kirsten sarcoma virus-transformed cells (4, 6, 7, 23, 39, 40), transformation of normal rat mammary epithelium by DMBA results in a decrease in the number of assayable EGF receptors on the transformed cells as compared to their normal counterparts (Chart 1). Both the normal and the tumor mammary cells possess high-affinity EGF-binding sites with equivalent dissociation constants (Kd 0.4 versus 0.5 nM, respectively). Scatchard analysis of the mammary cells in primary culture (Chart 1, inset) indicates that the tumor cells possess only one-third as many EGF receptors as do the normal epithelial cells (2.5 × 10^4 sites/cell versus 8 × 10^4 sites/cell, respectively). This
flaf Mammary Tumor-derived Growth Factors

The difference in EGF receptor number on the mammary tumor and normal cells suggested that the tumor cells might produce factors which interact with their EGF receptors, as was found for the murine sarcoma virus-transformed cells (4, 6, 7). Conditioned medium harvested from primary cultures of DMBA-induced mammary tumor cells was tested and found to contain factors which inhibited the binding of I25I-EGF to EGF receptors on EC cells (Chart 2A). Ultrafiltration studies of TCM seemed to indicate that the factor(s) inhibiting binding resided in fractions with molecular weights greater than 50,000. As shown below, a low-molecular-weight factor was also recovered when tumor extracts were fractionated by gel filtration. This component could not be recovered from the ultrafiltration apparatus, apparently due to absorption. Based upon standard radioreceptor binding curves generated by the displacement of I25I-EGF by unlabelled EGF, the tumor cells release into the growth medium activity the equivalent of 1 µg of EGF per 10^6 cells from the 48th to the 96th hr after plating. Production then declines coincident with the drop in cell division. The EGF receptor-competing activity appears to be selective based on the inability of the high-molecular-weight ultrafiltration fraction to compete with I25I-insulin for receptor binding on the EC cells (Chart 2B). From Scatchard analysis, it was also clear that the inhibition of binding by the high-molecular-weight fraction retained by XM-50 filters reduced the number of EGF receptors rather than altered their affinity for EGF (data not shown). Inhibition of binding was dependent on the temperature at which the radioreceptor assay was performed. For example, at 37° an aliquot of the ultrafiltrate inhibited EGF binding by 70%, while at 4° the binding was reduced by only 20%.

In addition to the EGF receptor-competing activity, the TCM ultrafiltrate also contained potent growth-stimulating activity (Chart 3). In serum-free, hormonally defined medium, both normal rat mammary epithelial and NRK cell growths in monolayers were stimulated in a dose-dependent manner. With the

difference in receptor number was not due to variations in the amount of stromal versus epithelial cells present in the primary cultures, since indirect immunofluorescence indicated that both normal and tumor cell cultures were free of cells synthesizing type I collagen, a stromal cell marker (16, 32).
them to grow as colonies. Essentially, no colony growth was seen if the TCM-derived fraction was omitted. This is demonstrated in Fig. 1. The NRK cell colonies grew as tight, symmetrical clusters of cells ranging from 30 to more than 180 μm in diameter, depending on the amount of factor added.

The effects of the proteolytic enzyme, pepsin, on the EGF-competing and soft-agar growth-promoting activities of TCM are shown in Table 1. A 2-hr digestion of the material retained by the XM-50 filter in a solvent of 1% acetic acid (100 μg pepsin/200 μl TCM) resulted in a marked reduction but not an elimination of the activities responsible for soft-agar growth promotion and EGF-binding inhibition.

Isolation of EGF Binding-Inhibitory and Soft-Agar Growth-Promoting Activities from Tumors. Acid-ethanol extracts prepared according to the method of Roberts et al. (29) yielded about 8 to 20 mg of lyophilized protein per g of starting tumor, with one-third to one-fifth of this material being soluble in either 1 M acetic acid or PBS. As shown in Chart 4, considerable EGF-competing activity was present in this extract, although the EGF-competing activity was not present in all mammary tumors examined. Recoverable activity was highest in primary tumors induced with NMU, variably intermediate to high in primary tumors induced by DMBA, but virtually absent in extracts prepared from transplantable DMBA and NMU tumors. These results were reproduced in 4 separate experiments and therefore suggest that the EGF-competing activity is not necessary for the growth of all mammary tumors. This is also apparently the case for the soft-agar growth-promoting activity in the tumor extracts (Chart 5). For example, a 100-μl/ml addition of primary tumor extract produced 50 NRK cell colonies/sq cm in soft agar, while an equivalent amount of extract from the transplantable DMBA tumor gave no NRK cell colony growth in soft agar. Similar results were obtained with 3T3 cells in soft agar. The DMBA-induced tumor extract stimulated the growth of BALB/c-3T3, CEF, and NRK cells in serum-deprived monolayer culture (Table 2). Data from heat inactivation studies (see below) suggested that the mitogenic activity was unrelated to the other 2 activities. The potency of the

### Table 1

<table>
<thead>
<tr>
<th>TCM-induced soft-agar colony formation</th>
<th>Colonies/dish</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated TCM</td>
<td>920 ± 170a,b</td>
<td>100</td>
</tr>
<tr>
<td>Pepsin digest</td>
<td>580 ± 28</td>
<td>63</td>
</tr>
</tbody>
</table>

**Effect of pepsin digestion upon the activities derived from TCM**

For the soft-agar assay, 2 x 10⁵ NRK cells were plated in 2 ml of 0.3% agar-medium overlying 2 ml of 0.6% agar-medium in 60-mm plastic culture dishes. TCM was added to give a concentration of 100 μl/ml, and the dishes were incubated for 10 days at 37°C. Dishes were stained with nitro blue tetrazolium and counted. For the EGF-binding assay, EGF radioreceptor assay was carried out in monolayer as described in "Materials and Methods." TCM was added to give a concentration of 100 μl/ml.

<table>
<thead>
<tr>
<th>TCM inhibition of EGF binding</th>
<th>% of inhibition</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated TCM</td>
<td>82 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>Pepsin digest</td>
<td>33 ± 7</td>
<td>40</td>
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</table>

<table>
<thead>
<tr>
<th>MTFCONTROL</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MTF</td>
<td>1.208,000</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

**Effect of MTF upon growth of NRK, BALB/c-3T3, and CEF cells in serum-deprived monolayer culture**

NRK cells (2 x 10⁵) and CEF or 3T3 cells (1 x 10⁶) were plated in duplicate 35-mm Costar cluster wells containing DMEM plus 10% fetal calf serum. After 24 hr incubation at 37°C, the medium was replaced with DMEM plus 1% fetal calf serum, and the MTF was added. MTF was prepared by dissolving 3 mg of lyophilized DMBA-induced primary tumor extract per ml of PBS, centrifuging, and then filtering the supernatant through a Millipore GS-0.22-μm filter. Aliquots of MTF were added to give a concentration of 100 μl/ml medium. The cells were incubated for 72 hr at 37°C, trypsinized, and counted with a Bio/Physics Model 6300A cytograph.

### Table 2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell count</th>
<th>MTF:control</th>
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</thead>
<tbody>
<tr>
<td>NRK</td>
<td>416,000</td>
<td>2.9</td>
</tr>
<tr>
<td>MTF</td>
<td>1,208,000</td>
<td>2.9</td>
</tr>
<tr>
<td>CEF</td>
<td>200,000</td>
<td>2.9</td>
</tr>
<tr>
<td>MTB</td>
<td>575,000</td>
<td>2.9</td>
</tr>
<tr>
<td>BALB/c-3T3</td>
<td>205,000</td>
<td>2.8</td>
</tr>
<tr>
<td>Control</td>
<td>572,000</td>
<td>2.8</td>
</tr>
</tbody>
</table>
DMBA-induced primary mammary tumor extracts, in terms of both EGF-competing and soft-agar growth-promoting activities, varied significantly between different tumor preparations. The NMU-induced tumors, however, have not exhibited this problem of batch variability. Consequently, we have primarily used extracts of these tumors as the source of MTF for further purification.

EGF was unable to induce soft-agar colony growth, even in the presence of a high concentration of serum (Table 3). In contrast, DMBA primary tumor extract and serum in high concentrations acted synergistically to promote NRK colony formation in agar. Heat inactivation studies indicated that the soft-agar growth-promoting and EGF-competing activities were similar in their heat sensitivities (Table 4). Growth stimulation in monolayer, although reduced, was still present in the unfractionated primary DMBA-induced tumor extract after it was heated for 5 min at 100°, a treatment that completely inactivated the EGF-competing activity. The observations that heating the primary tumor extract inactivated both the EGF-competing activity and the soft-agar growth-promoting activity suggested that these 2 activities might also reside in the same molecule. Data from gel filtration studies, which are described below, however, do not entirely support this conclusion. In addition, the observation that the mitogenic activity was heat stable suggests that this activity is unrelated to the other 2 activities.

To assess the number and apparent molecular weight of the proteins in the Bio-Gel P-100 fractions, gel filtration was performed on the ethanol extract of primary DMBA-induced rat mammary tumor. Twenty mg of acid-ethanol extract was redissolved in 5 ml of PBS (pH 7.4), centrifuged, and the supernatant was diluted with PBS to a total volume of 10 ml per fraction. One-ml fractions were collected in 0.1% bovine serum albumin (BSA), and 200-pl aliquots from each fraction were tested in the EGF radioreceptor assay and soft-agar colony assay. Arrows, elution positions of ferritin (M, 500,000), void volume (Vo); bovine serum albumin (M, 66,000), insulin (M, 6,000) and bacitracin (M, 125,000). "", absorbance at 280 nm; ○, percentage of inhibition of EGF binding in EGF radioreceptor assay. The data from a Bio-Gel P-100 filtration experiment, including EGF-binding competition and soft-agar colony forming activities in the primary tumor extracts, the material which was soluble in PBS was fractionated by gel filtration on Bio-Gel P-100 columns. Initial analyses of the DMBA-induced primary tumor extract indicated the presence of EGF-competing activity with a size equivalent to that of a bovine serum albumin molecular weight marker of approximately 68,000. However, when the column fractions were collected into tubes in which small amounts of bovine serum albumin carrier protein (0.1%, w/v) were present, a major peak of competing activity was also detected in fractions corresponding to the insulin marker (M, 6,000) (Chart 6). Small amounts of competing activity were seen at other elution positions, but the 2 peaks at M, 6,000 and 68,000 represented about 80% of the total EGF-competing activity. NMu primary tumor extracts showed an identical elution profile. The data from a Bio-Gel P-100 filtration experiment, including EGF-binding competition and soft-agar colony forming activities.
The EGF-competing and anchorage-independent growth-conferring activities nearly cofractionate by gel filtration, but at this stage of purification it is uncertain whether the 2 activities reside in the same molecules. The activities are both temperature and protease sensitive, and when one activity is absent in a tumor (for example, the NMU- and DMBA-induced transplantable tumors) the other activity is also missing. Additional data concerning the relationship between the 2 activities come from the observation that a third biological activity has been recovered from these tumor extracts (13). Collagen synthesis in fibroblasts and in normal rat mammary epithelial cells is differentially stimulated 4- to 10-fold by acid-ethanol extracts prepared for the primary rat mammary adenocarcinomas. The collagen synthesis-stimulating activity nearly coelutes with MTF as a high- and a low-molecular-weight species after gel filtration. However, the colony-inducing and collagen synthesis-stimulating activities can be resolved from each other following preparative isoelectric focusing, suggesting that these activities are associated with different molecules. Concerning the relationship between the high- and low-molecular-weight forms of MTF, there is no evidence to indicate whether they are different forms of the same molecule, whether the high-molecular-weight species is a precursor or binding protein for the smaller species, or whether they are 2 unrelated molecules.

Pedersen-prepared fetuin was required in the soft-agar assay in order to detect the colony-forming activities which were recovered following gel filtration. Alone, at a concentration of 1 mg/ml, the crude fetuin preparation did not induce colony formation. It is not known whether the fetuin is merely stabilizing the partially purified MTF or behaving in a synergistic manner to promote soft-agar colony growth. In this respect, Bjornson and Moses (1) have reported the isolation of a M, 10,000 TGF from Pedersen-prepared fetuin. In addition, Roberts et al. (27, 28) have observed a synergistic soft-agar colony growth response when EGF was added to TGFs recovered from both neoplastic and nonneoplastic murine, bovine, and human tissues. In the present study, a synergistic effect was clearly observed when the tumor extract was combined with serum at high concentrations in the soft-agar assay. Whether this is due to the action of MTF in concert with other TGFs or with no other factor remains to be investigated. No additive or synergistic effects were observed for EGF in combination with MTF.

The finding that transplantable rat mammary tumors possess little or no MTF activity also suggests that such an activity may not be essential for mammary tumor growth in vivo. For example, under certain circumstances, malignant cells might lose a hormonal regulatory step which ordinarily would be under the control of TGFs; these factors would no longer be needed in such a case. Subsequently, after serial transplantation or extended tumor growth, the population of cells producing TGFs might be lost as well. Alternatively, certain tumors may be nonproducers but still dependent upon TGFs supplied by other tissues. In the present study, a synergistic effect was clearly observed when the tumor extract was combined with serum at high concentrations in the soft-agar assay. Whether this is due to the action of MTF in concert with other TGFs or with no other factor remains to be investigated. No additive or synergistic effects were observed for EGF in combination with MTF.

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The observation that there appears to be a correlation between the degree of hormone dependence and cellular differentiation of the rat mammary tumors and the amount of TGF...
which can be recovered supports some of these possible explanations. Primary rat mammary tumors which have been induced by either NMU or DMBA appear histologically as well-differentiated adenocarcinomas which are estrogen dependent (10, 45). Our experience with these tumors for the past several years is that 70 to 90% of them are hormonally dependent, with the NMU-induced tumors at the upper end of the scale. Their transplantable counterparts are generally poorly differentiated carcinomas which are hormone independent (11). We have been unable to recover significant amounts of MTF from the transplantable tumors. The association of MTF with the well-differentiated tumors under hormonal control suggests a possible means of host regulation of MTF production as well as a possible role for MTF in the normal hormone-dependent proliferative cycle of the rat mammary gland. In support of this concept has been the finding that TGF activity can be recovered in acid-ethanol extracts obtained from estrogen- and progesterone-stimulated bovine mammary glands. 5

The term TGF for this class of growth factors may be a misnomer. There are 2 observations which suggest that this may be a misclassification for this family of growth factors. (a) So-called TGFs have been recovered from many nonneoplastic sources (26–28, 43). In fact, EGF alone has been shown to have weak TGF-like activity (27, 28). (b) The ability of cells to grow in soft agar may not be an accurate index of tumorigenicity. For example, Peehl and Stanbridge (24) have found that the phenotype of anchorage-independent growth can be conferred upon normal human fibroblasts when these cells were grown in the presence of high levels of serum and hydrocortisone. In addition, Freedman and Shin (9) have demonstrated that human diploid fibroblasts, following single-step chemical mutagenesis, formed colonies in methylcellulose but were not tumorigenic in nude mice. Thus, the in vitro phenomena collectively termed transformation may not actually represent the neoplastic state, but one of several phases leading to this state. Therefore, TGFs may be inducing in vitro and in vivo a normal process of proliferation which may be one of several stages in the transition to a neoplastic state (35).

In summary, NMU- and DMBA-induced primary rat mammary adenocarcinomas contain factors which resemble TGFs in many but not all of their biological and physicochemical properties. MTF inhibits EGF binding to receptors on EC cells; it stimulates NRK and BALB/c-3T3 cells to grow as colonies in soft agar; and in monolayer culture it acts as a mitogen for both epithelial and mesenchymal cells, while reducing their serum requirement and causing them to lose their contact inhibition of growth. MTF may not be necessary for the growth of all rat mammary tumors. It could play a role in the maintenance of the normal proliferating state of the mammary gland as has been proposed for EGF (18, 36, 42, 46).

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We would like to thank Kenneth E. Burdette and Flora Grantham for excellent technical assistance.

REFERENCES

from virally and chemically transformed cells by acid/ethanol extraction.


Fig. 1. Growth of NRK cells in soft agar in the presence and absence of TCM. Cells were grown for 10 days with or without factor and then stained with nitro blue tetrazolium and photographed. A, factor present; B, factor absent. X 100.
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