ABSTRACT

Conditioned medium (CM) from a human mammary carcinoma cell line, MCF-7, and ten individual clones derived from these cells was examined for the presence of transforming growth factors (TGFs). Concentrated CM from all of the MCF-7 cell lines was found to stimulate the anchorage-independent growth of normal rat kidney cells in soft agar and to inhibit the binding of epidermal growth factor (EGF) to mouse NIH/3T3 fibroblasts and to A431 human epidermoid carcinoma cell membranes. The soft agar stimulating activity was heat stable but sensitive to treatment with dithiothreitol. EGF receptors were measured on the MCF-7 cell lines to determine whether the amount of TGFs associated with the CM from the various cell lines was correlated with the level of EGF receptors being expressed on these cells. Moreover, the intrinsic cloning efficiency of these lines in soft agar was measured to ascertain if any correlation might exist between the level of TGFs associated with these cells and the ability of these cell lines to form colonies in soft agar. Although all the MCF-7 cell lines had approximately the same number of EGF receptors per cell, ranging from 3 to 6 x 10^3 sites/cell, CM from these lines varied in potency with respect to inducing the growth of normal rat kidney cells as colonies in soft agar and in inhibiting the binding of EGF to NIH/3T3 cells. Likewise, the level of TGFs associated with the CM from the various clones showed no correlation with the ability of these individual lines to grow as colonies in soft agar. TGF activity was also detected in acid-ethanol extracts prepared from MCF-7 cells propagated in nude mice as tumors and in the extracts from two transplantable human mammary adenocarcinomas, Clouser I and II. In addition, approximately 50% of the normal rat kidney colonies formed in response to the Clouser II tumor extracts exhibited a branching morphology in contrast to spherical colonies produced by Clouser I or MCF-7 extracts. These results demonstrate that human mammary carcinoma cells from both established cell lines and cells maintained in nude mice as tumors contain TGF-like activities. Furthermore, the variation in TGFs associated with the CM from the MCF-7 clones suggests that the parent MCF-7 cell line contains a heterogeneous population of cells.

INTRODUCTION

Cells transformed by several retroviruses such as Moloney, Kirsten, feline, and Rous have associated with their culture medium a growth factor, SGF, which can reversibly confer upon nontransformed chicken, murine, rat, and human fibroblasts several biological properties ascribed to transformed cells (1, 8, 9, 24, 32, 35, 37, 42, 43). These properties include the promotion of anchorage-independent growth of cells in soft agar as colonies, a decreased serum and growth factor requirement for the growth of cells in monolayer culture, and a loss of contact inhibition of growth. Recently, SGF-like factors have also been demonstrated in the CM from rat fibroblasts transformed by certain DNA tumor viruses including SV40, polyoma, and adeno-virus (11, 18, 19). It is now generally accepted that SGF is actually a prototype for a new class of growth factors collectively termed TGFs (21, 43). TGFs have been partially purified from several human tumor cell lines (15, 25, 42, 43); from solid mouse, rat, and human tumors (1, 15, 28, 43, 45); from the CM of chemically transformed rat and mouse cells (21, 27); and from a variety of nonneoplastic human and rodent tissues including human placenta, kidney, lung, mouse embryos, human urine, serum, and platelets (3, 6, 27, 36, 37, 41, 43, 44). Some, but not all, of these TGFs such as SGF and the TGFαs resemble EGF in that they are able to compete with EGF for binding to EGF receptors (5) and share partial amino acid sequence homology with both mouse and human EGF (25) but fail to cross-react with antibodies generated against mouse EGF (2, 3, 5, 43). However, others, including the TGFβ subclass, fail to compete with EGF for receptor binding but in combination with EGF or TGFβs are potentiated in their ability to promote the growth of nontransformed fibroblasts in soft agar (1-3, 5, 15, 28, 35, 36). More recently, the presence of other growth factors not related to EGF but related to PDGF and multiplication-stimulating activity has been demonstrated in the CM from several tumor cell lines (14, 23, 42).

SGF and TGFs (α and β) belong to a family of heat- and acid-stable peptides that require intrachain disulfide bonds for activity (1-3, 25, 36, 43). Generally, TGFs have molecular weights ranging from 6,000 to 30,000, depending upon their source and the tester cell line utilized to screen for colony formation in soft agar (3, 20, 27, 28, 37, 41, 43, 45). In fact, several human tumors and tumor cell lines produce multiple species of TGFs, each of which is capable of stimulating the anchorage-independent growth of different fibroblast tester cells in soft agar, and some of which are potentiated by EGF (15, 27, 28). In addition, it has been demonstrated recently that a variety of normal growth factors can induce the growth in agar of cells that are on the verge of expressing the anchorage-independent phenotype, suggesting that they may function as TGFs under certain circumstances (20, 26, 34).

We have shown previously that the CM from cultures of primary chemically induced rat mammary adenocarcinomas or the acid-ethanol extracts prepared from such tumors contain a factor(s) which we have termed MTF that resembles a TGF (45). MTF induces the anchorage-independent growth of NRK and BALB/c-3T3 cells in soft agar as colonies, stimulates the monolayer growth of both normal mesenchymal and epithelial cells,
and inhibits the binding of EGF to mouse embryo carcinoma cells. Two major species of MTF were detected following gel filtration chromatography, M, 6,000 and 68,000 species. The present study was undertaken to ascertain whether comparable TGF-like activities could be detected in the concentrated CM from a human mammary carcinoma cell line, MCF-7, in several clones derived from this line and in the acid-ethanol extracts prepared from MCF-7 cells propagated in nude mice as tumors and in 2 transplantable human mammary adenocarcinomas, Clouser I and II. The CM from all the MCF-7 cell lines and the acid-ethanol extracts prepared from the human mammary tumors stimulated the growth of NRK cells in soft agar as colonies and inhibited the binding of 125I-EGF to mouse NIH/3T3 cells and A431 human epidermoid carcinoma cell membranes. In addition, although the CM from all of the MCF-7 clones contained demonstrable TGF activity, the amount of TGF found in the CM varied between the different clones and did not correlate with the number of EGF receptors on these cells or the intrinsic ability of these clones to grow in soft agar as colonies.

MATERIALS AND METHODS

Cell Cultures. NRK cells (clone 49F) were obtained from Dr. Joseph DeLarco, National Cancer Institute, and were grown as described previously (9, 45). NIH/3T3 cells were generously supplied by Dr. Robert Bassin, National Cancer Institute, and were propagated in DMEM containing 10% FCS (v/v) supplemented with 4 mM glutamine, 20 mM HEPES (pH 7.4), streptomycin (100 µg/ml), and penicillin (100 units/ml) in a humidified 95% air-5% CO2 atmosphere at 37°. The MCF-7 cell line was obtained from the Breast Cancer Task Force, National Cancer Institute. The MCF-7 clones were derived and generously supplied by Dr. Patricia Horan Hand, National Cancer Institute (16). The MCF-7 parent line and clones were grown in DMEM containing 10% FCS (v/v), unless otherwise indicated, and supplemented with bovine pancreatic insulin (10 µg/ml; Sigma Chemical Co., St. Louis, MO) and glutamine, HEPES, and antibiotics as described for the 3T3 cells.

Preparation of CM. MCF-7 cells (passage 125) were propagated in 15-cm tissue culture flasks (Costar, Cambridge, MA) until confluent. The cultures were washed 3 times with PBS and cultured for an additional 3 days in serum-free, improved Eagle’s minimal essential medium as described previously (39, 45). CM (180 ml) was harvested, centrifuged to remove any cells and cellular debris, filtered through a 0.45-µm filter, and subsequently dialyzed for 48 hr at 0-4° against 20 volumes of 1% triton X (Altex) equilibrated previously with PBS at 23°. The column was eluted at 37° with 3T3 cells or at 23° with A431 membranes. All assays were conducted in duplicate.

Gel Filtration Chromatography. CM or tumor extracts were chromatographed on a 60-cm TSK-3000 SW HPLC gel filtration column (Altex) equilibrated previously with PBS at 23°. The column was eluted with the same buffer at a flow rate of 2 ml/min using a Beckman HPLC chromatography pump equipped with a UV detector. Fractions of 4 ml were collected, dialyzed against distilled water for 24 hr at 0-4°, lyophilized to dryness, and assayed for either NRK colony-stimulating activity or for competition with 125I-EGF in the EGF RRA using A431 membranes.

RESULTS

Presence of EGF-Inhibiting and Colony-stimulating Activities in Human Mammary Tumor Cells. As was demonstrated previously for primary cultures of rat mammary adenocarcinoma cells (45), concentrated CM prepared from MCF-7 cells, an established human breast cancer cell line (4, 30), is able to stimulate the growth of NRK cells in soft agar as colonies (Table 1). Moreover, aliquots of comparably prepared CM concentrates were also tested for their ability to inhibit the binding of 125I-EGF to mouse NIH/3T3 cells. EGF-inhibitory activity was found to be associated with these same samples in an EGF RRA (Table 1). The amount of EGF-inhibitory activity in the MCF-7 CM concentrates was proportional to the volume of CM tested in the EGF RRA (Chart 1) and exhibited a similar, but less pronounced, displacement of 125I-EGF as native, unlabelled EGF. The failure of the crude CM concentrates to completely inhibit the binding
of EGF-competing activity (25). A431 cells also possess specific receptors for insulin. For example, a 50-μl aliquot (100 μg protein) obtained from 2 liters of concentrated CM from the MCF-7 parent cells produced a 90% inhibition of 125I-EGF binding in the EGF RRA using A431 membranes. However, an equivalent aliquot from the same pool of CM was ineffective in competing with 125I-EGF but not 125I-insulin to isolated membranes obtained from A431 human epidermoid carcinoma cells. These cells possess an unusually high number of EGF receptors and have been used by other investigators to screen for the presence of EGF-competing activity (25). A431 cells also possess specific receptors for insulin. For example, a 50-μl aliquot (100 μg protein) obtained from 2 liters of concentrated CM from the MCF-7 parent cells produced a 90% inhibition of 125I-EGF binding in the EGF RRA using A431 membranes. However, an equivalent aliquot from the same pool of CM was ineffective in competing with 125I-insulin for binding to insulin receptors on these membranes. In this case, when A431 membranes were incubated with 10 ng of 125I-insulin, 9 × 10^4 cpm of 125I-insulin were bound to the membranes. Inclusion of 10 μg of unlabeled insulin in the receptor assay produced a 60% displacement of the bound 125I-insulin, whereas 50 μl of CM showed no effect in competing with 125I-insulin for binding.

Although neither the EGF-inhibitory activity nor the NRK colony-stimulating activity has been extensively purified, the NRK colony-stimulating activity in the MCF-7 CM was inactivated by prior treatment of the samples with dithiothreitol (Table 2). The activity was stable to dialysis (4° for 48 hr) and to heat treatment (100° for 30 min). This latter property is in contrast to the activity (MTF) isolated from rat adenocarcinomas which is heat labile (45). However, like the MTF isolated from the CM of rat mammary adenocarcinoma cells, the EGF-competing activity and the NRK colony-stimulating activity were stable to acid extraction. Preliminary experiments (data not shown) indicate that the NRK colony-stimulating activity in the CM from MCF-7 cells exhibits a molecular weight of approximately 6000 following gel filtration under low-salt conditions at neutral pH on a TSK-3000 SW HPLC gel

---

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>125I-EGF specifically bound (pg/10^5 cells)*</th>
<th>Colony-stimulating activity (NRK colonies/dish)*</th>
<th>MCF-7 colony-stimulating activity (NRK colonies/dish)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent MCF-7</td>
<td>31.6 (4.7 × 10^4)</td>
<td>63</td>
<td>209 ± 38*</td>
</tr>
<tr>
<td>2A8</td>
<td>45.9 (6.3 × 10^4)</td>
<td>66</td>
<td>336 ± 105</td>
</tr>
<tr>
<td>2B3</td>
<td>18.5 (2.8 × 10^4)</td>
<td>56</td>
<td>201 ± 73</td>
</tr>
<tr>
<td>2E5</td>
<td>34.8 (5.2 × 10^4)</td>
<td>63</td>
<td>230 ± 48</td>
</tr>
<tr>
<td>3E5</td>
<td>38.2 (5.7 × 10^4)</td>
<td>62</td>
<td>141 ± 33</td>
</tr>
<tr>
<td>4C10</td>
<td>25.1 (3.8 × 10^4)</td>
<td>47</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>5A9</td>
<td>32.4 (4.9 × 10^4)</td>
<td>75</td>
<td>56 ± 10</td>
</tr>
<tr>
<td>5H7</td>
<td>30.3 (4.6 × 10^4)</td>
<td>47</td>
<td>89 ± 30</td>
</tr>
<tr>
<td>6F1</td>
<td>31.0 (4.7 × 10^4)</td>
<td>73</td>
<td>183 ± 95</td>
</tr>
<tr>
<td>8B6</td>
<td>45.6 (6.9 × 10^4)</td>
<td>52</td>
<td>92 ± 33</td>
</tr>
<tr>
<td>10B8</td>
<td>60.8 (9.1 × 10^4)</td>
<td>83</td>
<td>243 ± 128</td>
</tr>
</tbody>
</table>

* MCF-7 cells were incubated with various concentrations of 125I-EGF, and the amount of EGF specifically bound at saturation was determined as described in Chart 3.

** Aliquots of CM (100 μl/ml) prepared from the MCF-7 cell lines were assayed in the EGF RRA using 3T3 cells to determine the amount of EGF-inhibiting activity expressed as percent inhibition or on NRK cells in soft agar to determine the potency for colony-stimulating activity.

---

### Table 2

<table>
<thead>
<tr>
<th>Conditioned Medium (μl)</th>
<th>EGF (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>Dithiothreitol (0.065 M in PBS, 2 hr, 20°)</td>
<td>0</td>
</tr>
<tr>
<td>Heat (100°, 30 min)</td>
<td>43 ± 33</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
filtration column and possesses a pH of approximately 4.0 following isoelectric focusing on a 110-ml LKB Model 8100 Ampholine column using Ampholines over the pH range of 3.0 to 10.0.

Several clones have been isolated previously from the parent MCF-7 cell line obtained from the Breast Cancer Task Force at passage 157 by end-point dilution (16). Ten of these clones were also assayed for the presence of EGF-inhibitory and NRK colony-stimulating activities in CM concentrates to determine if there was any heterogeneity within the original cell population of the MCF-7 cell line with respect to the amount of these activities associated with their CM (Table 1). The CM from all 10 clones possessed amounts of EGF-inhibitory activity which were comparable to the levels measured in the parental MCF-7 cell line. However, the ability of CM concentrates to stimulate the growth of NRK cells as colonies in soft agar varied among the clones. For example, the CM from clones 2A8, 2B3, 2E5, 6F1, and 10B5 had an equivalent amount of NRK colony-stimulating activity as did the parental MCF-7 cell line. In contrast, the CMs from clones 4C10, 5A9, 5H7, and 8B6 were 50 to 75% less active in promoting NRK colony growth than was the CM obtained from the parent cell line.

The presence of these 2 activities (EGF-competing and NRK colony-stimulating activities) was not restricted to CM obtained from a human mammary tumor cell line. Crude acid-ethanol extracts prepared from MCF-7 cells which were propagated in nude mice as tumors and from 2 transplantable human mammary adenocarcinomas, Clouser I and II, also possessed both activities (Table 3). The level of both activities recovered from the MCF-7 tumors was relatively comparable to the amounts of these 2 activities recovered in the CM samples from the parent MCF-7 cell line. Likewise, there was no significant difference in the amount of these 2 activities which could be recovered from the 2 human, transplantable Clouser adenocarcinomas. Moreover, the EGF-competing activity recovered in the tumor extracts, like the activity in the MCF-7 CM, appears to be selective since aliquots of the tumor extracts were unable to compete with 125I-insulin for binding to human A431 cell membranes (data not shown). It should be noted that the morphology of the NRK colonies produced in response to the crude acid-ethanol extracts prepared from the MCF-7 tumors and from the Clouser II tumors was strikingly different (Fig. 1, A and B). In the presence of MCF-7 or Clouser I (not shown) tumor extracts, the NRK colonies grew as spherical aggregates of cells (Fig. 1A). In contrast, the colonies formed in response to an equivalent amount of the Clouser II extracts showed branching structures (Fig. 1B). Approximately 50% of the colonies formed in the presence of the Clouser II extract exhibited this branching morphology, and 50% of the colonies were spherical. A partial characterization of the EGF-competing activity was achieved by gel filtration chromatography of the Clouser I tumor extract on a TSK-3000 HPLC column under isotonic and neutral conditions. A broad peak at approximately M, 60,000 to 70,000 was observed which contained 90% of the EGF-competing activity (Chart 2). The remainder, approximately 10%, of the EGF-competing activity eluted near the void volume of the column probably as a result of aggregation, because the gel filtration was performed in low salt. Although the material present in the tumor extracts appeared to selectively compete with EGF for receptor binding, 2 other possible explanations were considered. The "competition" might be due to: (a) EGF binding proteins present in the extracts that would reduce the concentration of 125I-EGF accessible for receptor binding or (b) the presence in the extracts of proteases which would selectively degrade EGF. Both of these possibilities were eliminated by the following experiments. Three hundred μg of the Clouser I extract, an amount equivalent to that utilized to test for the presence of EGF-competing activity in the RRA, were incubated with 125I-EGF (2.5 ng) for 1 hr at 23° in the RRA binding buffer. The mixture was then chromatographed on a TSK-3000 gel filtration column under conditions identical to that described in Chart 2 for the detection of the EGF-competing activity in the crude tumor extracts (data not shown). Ninety-nine % of the radioactivity eluted at a position from the column which was identical to that where free 125I-EGF was recovered when EGF was incubated in binding buffer and chromatographed. Likewise, no EGF binding protein activity could be detected in the M, 60,000 to 70,000 region of the Clouser I extract. When this region of the column was pooled, concentrated, incubated with 125I-EGF, and rechromatographed on the HPLC sizing column, greater than 99% of the radioactivity eluted at a position identical to that of EGF. Moreover, aliquots of the pooled and concentrated M, 60,000 to 70,000 region were incubated with 125I-EGF, subjected to sodium dodecyl sulfate-gel
electrophoresis in 15% acrylamide gels, and fluorographed. Other than a band which comigrated with $^{125}$I-EGF, no other bands could be detected in the gel with molecular weights less than EGF. These results indicate the apparent absence of proteases in this region of the column (M, 60,000 to 70,000) which could degrade the $^{125}$I-EGF and therefore give an apparent competition in the RRA.

Binding of $^{125}$I-labeled EGF to MCF-7 Cell Lines. To determine whether a correlation might exist between the amount of EGF-inhibitory activity present in the CM obtained from the MCF-7 cell lines and the number of EGF receptors on these clones, the binding of $^{125}$I-EGF was measured on subconfluent cultures. Chart 3 illustrates the specific binding of $^{125}$I-EGF as a function of increasing concentrations of the labeled growth factor to the parent MCF-7 cell line and clone 10B5. At 37°, maximum binding of $^{125}$I-EGF was reached after 60 to 90 min (data not shown). Nonspecific binding accounted for less than 10% of the total amount of $^{125}$I-EGF bound at the various concentrations which were tested. At saturation, which occurred between 10 and 15 ng of $^{125}$I-EGF per ml, approximately $8 \times 10^3$ cpm of $^{125}$I-EGF (60 pg) were specifically bound to $10^6$ cells from the parent MCF-7 line. The amount of growth factor bound at saturation varied by a factor of 2 among the various MCF-7 clones as determined by Scatchard analysis (Table 1). With the possible exception of clones 4C10 and 5H7, all of the remaining 8 clones and the parent MCF-7 cell line possessed a similar number of EGF receptors per cell as determined by Scatchard analysis of binding curves comparable to those depicted in Chart 3. No correlation was found between the amount of EGF-inhibitory activity present in the CM and the number of measurable EGF receptors on the various MCF-7 cell lines. However, the number of EGF receptors may fluctuate, depending upon the saturation density of the cultures (38) and the availability of free unoccupied receptor sites. Therefore, the validity of these calculations is merely the approximations from several assays of the number of EGF receptors using semiconfluent cultures.

Clonogenicity of MCF-7 Cell Lines in Soft Agar. Varieties of human tumor cells from cell lines and from biopsies are able to grow in soft agar as colonies (33). Moreover, EGF has been demonstrated to produce a 2-fold increase in the cloning efficiency in semisolid medium of tumor cells obtained from a number of tumor cell lines and from biopsies (33). To determine whether there was a direct correlation with the amount of NRK colony-stimulating activity present in the CM obtained from the MCF-7 cell lines and the intrinsic ability of these cells to grow as colonies in soft agar, cells were seeded in soft agar in medium containing 15% FCS to optimally sensitize the cells for anchorage-independent growth (see Refs. 20, 34, and 45). As illustrated in Table 1, no correlation could be unequivocally demonstrated between these 2 phenotypic properties. For example, although the CM from clones 2A8, 3E5, and 6F1 had a considerable amount of colony-stimulating activity for NRK cells, these same cells had a relatively low cloning efficiency in soft agar.

**DISCUSSION**

It has been demonstrated previously that the CM from a variety of tumor cell lines and from the cells transformed by several different retroviruses or DNA tumor viruses contains TGFs as assessed by the ability of concentrated CM aliquots to induce the growth of NRK cells in soft agar as colonies and/or to inhibit the binding of EGF (1, 2, 8, 21, 25, 32, 35, 37, 42, 43, 45). The former property is common to all TGFs that have been described and some normal growth factors (20, 21, 26), while the latter property is generally restricted to the TGFα subclass (1-3, 5, 43). It has been inferred from these studies that TGFs are being produced and secreted by these cells since they are associated with, and in some cases have been partially purified from, the CM obtained from these cells. The present study demonstrates that a human breast tumor cell line and several clones derived from this line also have associated with their CM TGF-like activities. Moreover, comparable TGF-like activities possessing similar biological activities can also be recovered in the acidethanol extracts prepared from MCF-7 tumors and in 2 transplantable, human mammary adenocarcinomas (Clouser I and Clouser II). It is not clear whether the activities found in association with the CM from the MCF-7 cells are physicochemically identical to the activities extracted from the tumors. In rat mammary adenocarcinomas, the EGF-competing and NRK colony-stimulating activities copurify (45). In contrast, the NRK colony-stimulating activity associated with the CM has a molecular weight of approximately 6,000, while the EGF-competing activity in the Clouser I tumor extract is approximately 60,000 to 70,000. It can also not be definitively stated from the present data as
to whether the EGF-competing and NRK colony-stimulating activities are actually being synthesized by the human breast cancer epithelial cells. To fully address this question, these activities have to be purified to a point where antibodies can be generated against them for use in biosynthetic labeling studies with isotopic precursors in vitro. Furthermore, since neither of these activities has been extensively purified from this cell line or from the human mammary tumor extracts, it is not clear whether the EGF-competing activity is due to the presence of a TGF-α which is directly inhibiting the binding of 125I-EGF to EGF receptors on mouse NIH/3T3 fibroblasts (2, 5). It is possible that this activity may be indirectly modulating the binding of 125I-EGF to live cells analogous to the inhibition of EGF binding produced by phorbol esters (38). This, however, seems unlikely, since this activity can compete with EGF for binding to EGF receptors on isolated membranes from A431 cells. Under these assay conditions, phorbol esters do not affect EGF binding (38). Moreover, the EGF-competing activity which is present in the crude Clouser extracts cannot be accounted for by: (a) a general nonspecific perturbation of the membranes since the binding of 125I-insulin to isolated A431 membranes is not affected; (b) the sequestration of 125I-EGF due to the presence of an EGF-binding protein in the tumor extracts; or (c) a degradation of 125I-EGF due to the presence of protease activity in the semipurified tumor extracts. It is also possible that the TGF-like activities detected in the CM samples could arise from serum carryover during the conditioning interval. It is known that serum does contain TGFs (6) and other growth factors (20) and that a majority of this TGF activity in serum is apparently derived from platelets (3). However, the observation that the different clones of MCF-7 cells have variable amounts of TGFs associated with their CM would tend to mitigate against such a possibility. Moreover, the presence of comparable biological activities in the acid-ethanol extracts obtained from rat mammary adenocarcinomas (45) and in the acid-ethanol extracts prepared from biopsies of human mammary tumors would also tend to preclude this possibility. Considering these qualifications, we can only assume from the presence of these TGF activities in the CM of mammary carcinoma cells and from their detection in mammary tumor extracts from several sources that these cells are synthesizing and secreting these factors.

It is interesting to note that, for the NRK colony-stimulating activities isolated from the extracts of MCF-7 and the Clouser II tumors, the morphologies of the NRK colonies were different. Some of the NRK colonies produced in response to the Clouser II tumor extracts were branching and may be ductal in nature, reminiscent of structures formed by capillary and vascular endothelial cells in vitro after the addition of CM from tumor cells or a variety of angiogenesis factors (12). However, a more detailed histological analysis of these branching, irregularly shaped colonies will be necessary to substantiate whether these structures actually possess a lumen and are therefore true ducts. These data do suggest, however, that 2 activities might be associated with the Clouser II extracts, one capable of stimulating cell growth in soft agar as colonies and a second, possible morphogenic factor(s). This latter factor(s) may induce the formation of structures which resemble glomerular tubules, since NRK cells are derived from embryonic kidney tissue. Moreover, the apparent absence of this second activity from the Clouser I extracts suggests that this factor(s) is different from any previously described TGFs and that TGFs might be one of several factors needed for morphogenesis during embryonic development (43). Further purification of these 2 activities from the Clouser II tumor extracts will obviously be necessary to determine whether these biological activities reside in the same or different protein(s).

The presence of multiple TGF-like activities which are capable of stimulating different types of fibroblast cells to grow in soft agar has been found in various normal tissues and tumor cell lines (15, 27, 36). Some of these factors are potentiated by either EGF, TGF-α, or high concentrations of serum, suggesting that they probably represent TGF-βs (1–3, 15, 27, 28, 36). Nickell et al. (28) have described recently the partial purification of distinct activities which can stimulate the anchorage-independent growth of NRK and mouse AKR-2B cells from the acid-ethanol extracts prepared from specimens of several human neoplasms including adenocarcinoma of the breast. These activities appear to reside in separate proteins for either NRK or AKR-2B fibroblast cells of which some are also potentiated in their activity by EGF (1, 28, 35, 36). In addition, several normal host-derived growth factors such as PDGF and FGF have likewise been demonstrated to promote the anchorage-independent growth of partially transformed cells or transformed cells which produce low levels of TGFs which are insufficient by themselves to induce the anchorage-independent growth of these same cells (20, 26). In this respect, the viral oncogene product, p28k, of the simian sarcoma virus has been demonstrated to be structurally related to PDGF, indicating that a viral transforming protein may be a normal growth factor that is inappropriately or constitutively expressed in a neoplastic cell (10). The variation in production of NRK colony-stimulating activity and in the cloning efficiency in soft agar of the various MCF-7 clones suggests that multiple TGF-like activities (α and β) might also be produced by these different clones of MCF-7 mammary tumor cells. The biological potency of some of these TGF-like activities (i.e., NRK colony-stimulating activity) could possibly be accentuated by the presence of other growth factors present in serum such as EGF, PDGF, or FGF (20, 26, 34, 45). It has been demonstrated recently that SGF derived from the CM of Moloney sarcoma virus-transformed cells and further purified by reverse-phase HPLC actually consists of a mixture of α and β TGFs (2). TGF-α in the SGF preparation was demonstrated to be the activity which was responsible for competing with EGF for binding to EGF receptors (5), while the soft-agar colony-stimulating activity was due to the combined, synergistic action of both TGF-α and TGF-β (2). The MCF-7 clones may actually represent populations of cells which are either low or high producers of some or all of these TGF-α and TGF-β activities. Therefore, the parent MCF-7 cell line may consist of a mixed population of cells containing a spectrum of various cells producing different amounts of these activities, exemplifying further the concept of tumor cell heterogeneity which has been observed for several phenotypic properties (31). Initial attempts to detect TGF-βs in the CM of the parent MCF-7 cells have been unsuccessful. Crude CM samples were assayed on NRK cells in soft agar in the absence or presence of EGF at 5 ng/ml. The number of colonies formed was comparable under either condition. However, considering that none of these activities have

---

**CANCER RESEARCH VOL. 44**

---

been extensively purified from the CM of this cell line, that TGFβ activity cannot usually be detected in crude CM samples (2), and that other clones derived from the parent cell line may be producing relatively more of this activity, it is not unexpected that these results fail to conclusively demonstrate that TGFs are not being produced by mammary tumor cells.

Although EGF has been demonstrated to be a mitogen for MCF-7 cells propagated in the presence or absence of serum which is mediated by the presence of EGF receptors on these cells (4, 29, 30), the results of the present study have shown using various MCF-7 clones that the number of EGF receptors is not correlated with the level of EGF competing activity or with the NRK colony-stimulating activity associated with the CM from these clones. In this respect, Imai et al. (17) have demonstrated that there was no association between the level of EGF binding and its mitogenic effect on the growth of several human breast cancer cell lines in monolayer cultures. Likewise, Pathak et al. (33) were also unable to show any clear correlation between the number of EGF receptors and the ability of EGF to enhance the growth of a variety of human carcinoma cells in soft agar as colonies. It is therefore possible that one phenotype in a cell population may fluctuate independently of the expression of another phenotype (16, 17, 31, 33). It should also be stressed that the assay routinely used to monitor for the presence of all known TGFs is the ability of these factors to promote the anchorage-independent growth of a variety of fibroblast cell lines, particularly NRK cells, in soft agar as colonies (1–3, 8, 25, 35–37, 43). More importantly, with the possible exception of crude preparations of SGF (22), none of these TGFs has been assayed on nontransformed epithelial cells with respect to promoting their growth in soft agar. Moreover, primary cultures of fibroblasts fail to respond to these activities with respect to the induction of anchorage-independent growth (8, 20, 21), suggesting that established cell lines have undergone at least one of the steps in the transformation process, namely, cell immortalization, which may sensitize these cells to exhibit anchorage-independent growth in response to exogenous TGFs (20). Although we were unable to demonstrate a correlation between the amount of TGFs associated with the CM from the MCF-7 clones and the intrinsic ability of these cell lines to grow in soft agar, it is therefore possible that the use of NRK fibroblasts as an indicator cell line may be inappropriate for detecting TGF-like factors that could be produced in these cells which would stimulate the anchorage-independent growth of epithelial cells, in the particular primary cultures of normal mammary epithelial cells. We have observed that normal mouse or rat mammary epithelial cells do respond mitogenically to TGFs isolated from the crude CM of MCF-7 cells. However, these same cells fail to grow in soft agar in the presence of MCF-7-derived TGFs. However, a crude mammary-derived TGF is able to enhance the growth of an MCF-7 clone in soft agar which intrinsically exhibits a low capacity to form colonies in soft agar (clone 2A8). In the absence of TGFs, 2A8 cells form 16 ± 4 (S.E.) colonies/dish in soft agar. The inclusion of mammary-derived TGF to these cells increases the number of colonies by approximately 3-fold to 42 colonies/dish. Under comparable assay conditions, this TGF also enhances the ability of NRK cells to form colonies in soft agar by approximately 20-fold (2 colonies/dish versus 47 ± 5 colonies/dish). These results confirm previous findings demonstrating that NRK cells are generally more sensitive to the anchorage-independent growth-confering effects of TGFs obtained from various sources (1–3, 8, 25, 35–37, 43). More importantly, the results demonstrate that breast carcinoma cells in contrast to normal mammary epithelial cells are capable of responding to TGFs in soft agar, although to a lesser extent than NRK cells. Moreover, these same cells (2A8) are capable of producing TGFs which are active in inducing the anchorage-independent growth of NRK cells (cf. Table 1). These results essentially agree with those obtained by Nickell et al. (28), demonstrating that the TGFs produced by one cell type may be able to stimulate the anchorage-independent growth of a particular indicator cell type (i.e., NRK cells) yet may be insufficient by themselves to facilitate the soft agar growth of these same cells or another tester cell.

Although the exact biological function of these TGF-like activities is unknown, they may possibly induce the expression of other biological properties which could be associated with the transformed phenotype in mammary epithelial cells. These would include a reduction in the growth factor requirement for the proliferation of these cells in monolayer cultures on different collagen substrates (39), a change in cell morphology, a loss of contact inhibition of growth, and/or an induction in the expression of tumor-associated cell surface antigens (16). Moreover, their presence in human breast tumors and their association with the CM from a human mammary tumor cell line suggest that they may be synthesized and secreted by a human mammary tumor cell line and in varying amounts by several clones derived from this line. These cells may therefore provide an in vitro system in which to study TGF synthesis and/or secretion in response to various hormones and other growth factors. Moreover, the availability of clones which may differ in their ability to produce TGFs should prove useful in determining whether any relationship exists between TGF production and the expression of other phenotypes, such as tumor-associated cell surface antigens (16), tumorigenicity (7, 40), metastatic potential, growth rate (17, 33), or sensitivity to other growth factors or hormones (4, 17, 20, 21, 26, 30, 33, 39, 45).

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Kenneth E. Burdette and Earl Smith for their excellent technical assistance.

REFERENCES


Fig. 1. Growth of NRK cells in soft agar in the presence of acid-ethanol extracts from human breast tumors. Cells were grown for 14 days with aliquots (200 µl/ml, 2 mg/ml) of MCF-7 (A) or Clouser II (B) tumor extracts, stained, and photographed. Nitro blue tetrazolium, x 100.
Presence of Transforming Growth Factors in Human Breast Cancer Cells

David S. Salomon, James A. Zwiebel, Mozeena Bano, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/9/4069

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.