Stimulation of Thymidine Uptake and Cell Proliferation in Mouse Embryo Fibroblasts by Conditioned Medium from Mammary Cells in Culture

Eugene F. Howard, David F. Scott, and Carol E. Bennett

Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30902

SUMMARY

Undialyzed conditioned medium from several cell culture sources did not stimulate thymidine incorporation or cell overgrowth in quiescent, density-inhibited mouse embryo fibroblast cells. However, dialyzed conditioned medium (DCM) from clonal mouse mammary cell lines MCG-V14, MCG-T14, MCG-T10; HeLa cells; primary mouse adenocarcinoma cells; and BALB/c normal mouse mammary epithelial cells promoted growth in quiescent fibroblasts. The amount of growth-promoting activity produced per cell varied from 24% (HeLa) to 213% (MCG-V14) of the activity produced by primary tumor cells. The production of growth-promoting activity was not unique to tumor-derived cells or cells of high tumorigenicity. The amount of growth-promoting activity produced per cell in the active cultures was not correlated with any of the following: tumorigenicity, growth rate, cell density achieved at saturation, cell type, or species of cell origin. It is concluded that transformed and nontransformed cells of diverse origin, cell type, and tumorigenicity can produce growth factors in culture.

The growth-promoting potential of the active media from primary tumor cultures accumulated with time of contact with cells and was too great to be accounted for entirely by the removal of low-molecular-weight inhibitors by dialysis. The results are consistent with the hypothesis that conditioned medium from the active cultures contained a dialyzable, growth-promoting activity. Different cell lines exhibited differential sensitivity to tumor cell DCM and fetal bovine serum. Furthermore, quiescent fibroblasts were stimulated by primary tumor cell DCM in the presence of saturating concentrations of fetal bovine serum. These observations support the notion that the active growth-promoting principle in primary tumor cell DCM may not be a serum factor(s).

INTRODUCTION

There have been reports that cells in culture produce growth factors that can be recovered in the medium [reviewed by Pardee (11)]. In most cases, the producer cells have been transformed in culture or derived from tumors (1, 5, 6, 8, 10, 13, 15). Primary normal fibroblasts of murine or human origin do not produce detectable growth factors in culture (6, 15). This could mean that the production of growth factors in vitro is a unique property of malignant transformed cells. Secretion of similar factors in vivo might promote the growth of stromal cell elements, which are responsible for tumor vascularization and connective tissue support (5). Nair and DeOme (10) reported that CM2 from primary cell cultures of spontaneous mouse mammary adenocarcinomas stimulated the growth of density-inhibited secondary cultures of MEF. The experiments described in this report were designed to determine whether CM with growth-promoting activity could be obtained from established mammary cell cultures of normal and tumor origin as well as from primary cultures of normal mammary epithelial cells. In addition, CM’s from primary mammary tumor cells, HeLa cells, and secondary MEF were tested for growth-promoting activity. We show that the production of growth-promoting activity was not a unique feature of tumor-derived cells. Furthermore, the capacity of cells to produce growth-promoting activity was not correlated with the tumorigenicity, growth rate, growth potential, cell type, or species origin of the producer cells. We conclude that transformed and nontransformed cells of diverse origin, cell type, and growth properties may produce growth factors in culture. Evidence is also presented that the active principle in primary tumor cell DCM is a factor produced by the tumor cells that is different from serum growth factors and is manifested only after the removal of low-molecular-weight inhibitory substances by dialysis.

MATERIALS AND METHODS

Fetal bovine serum, McCoy’s Medium 5A (modified), Fungizone, penicillin, and streptomycin were obtained from the Grand Island Biological Company, Grand Island, N. Y. Collagenase (type I) was purchased from Sigma Chemical Company, Saint Louis, Mo. Plastic T-flasks and Petri dishes for cell culture were obtained from Corning Glass Works, Science Products Division, Corning, N. Y. Tritiated thymidine (65 Ci/m mole, Lot ZR-1551) was supplied by Schwarz/Mann, Orangeburg, N. Y.

1 This work was supported by Contract CB 33909 and Program Project Research Grant CA-17059 from the National Cancer Institute.

Received April 2, 1976; accepted August 31, 1976.

DECEMBER 1976

4543
Preparation of CM from Primary Tumor Cultures

Spontaneous mammary adenocarcinomas were excised from C3H/BiKi mice. Necrotic tissue was removed, and the tumors were minced with scalp blades. Aliquots of tissue mince equivalent to 2 tumors (1-cm diameter) were digested separately in 10 ml serum-free McCoy's Medium 5A that contained 1 mg collagenase, 5 μg Fungizone, 400 units penicillin, and 400 μg streptomycin per ml. Each 10 ml digestion mixture was placed in a tightly stoppered, 50-ml Erlenmeyer flask and agitated gently in a 37° water bath for 2 hr. The enzyme-digested tissue was then pipetted gently to disperse the cells. The cell suspensions were pooled, concentrated by centrifugation at 90 x g for 3 min, and then resuspended in McCoy's Medium 5A that was supplemented with 10% fetal bovine serum.

Primary monolayer cultures of tumor epithelial cells were established by seeding the cell suspensions into plastic, screw-capped culture flasks (75-sq cm growing surface). Each culture flask received all of the cells obtained from 2 tumors. Accurate cell counts could not be made due to a preponderance of cell aggregates in these suspensions. The primary tumor cultures became confluent in 4 to 6 days. The spent medium was removed from these cultures and replaced with antibiotic-free medium, which contained 0.25% fetal bovine serum to prevent cell detachment. After 6 days, this medium was removed, centrifuged at 10,000 x g for 30 min, filtered through a 0.22-μm Millipore filter, and frozen at −80° until used as CM. For some experiments, CM was dialyzed for 24 hr against 100 volumes of serum-free McCoy's Medium 5A (2 changes) before filtration.

MEF Cultures

MEF were obtained in the following manner. Embryos were removed from 17-day-pregnant BALB/cKi mice, minced, and digested with collagenase according to the protocol described above for primary tumors. The cells obtained from the enzyme digestion grew as fibroblast monolayers in culture flasks. Confluent monolayers were dispersed with 0.05% trypsin in Puck's saline. The cells were then transferred to plastic Petri dishes (9 sq cm). The growth curve of these secondary MEF cultures is shown in Chart 1. Density-dependent inhibition of growth occurred after 5 days in culture. After this time, there was no further increase in cell number, and the incorporation of triitated thymidine into acid-insoluble material had virtually ceased. Cell viability as measured by trypan blue dye exclusion remained greater than 90% during the entire 8-day culture period. Chart 2 shows that thymidine incorporation could be stimulated in quiescent MEF culture by the addition of 10% fetal bovine serum. Three different lots of fetal bovine serum were tested; all stimulated thymidine uptake in MEF to the same extent. Addition of fresh, serum-free McCoy's Medium 5A in this experiment did not cause a detectable stimulation of thymidine incorporation. Therefore, the observed stimulation was probably due to serum factors. The increase in thymidine incorporation was followed by cell growth. Forty-eight hr after addition of serum, the number of cells in the culture dish had doubled. No increase in cell number was seen in unfed cultures.

Bioassay for Growth-promoting Activity of Tumor Cell CM

Secondary MEF cultures were used to assay growth-promoting activity in tumor cell CM. On the 7th day of culture (Chart 1), the spent medium was removed from the quies-
cent MEF, and was replaced with the solution to be assayed. Twenty-four hr later, thymidine incorporation was measured in treated and control cultures. A significant stimulation of thymidine uptake at 24 hr was interpreted as an indication of growth promotion. In some experiments, this assumption was verified by measuring the increase in cell number 48 hr after addition of test solutions. Exposure of the MEF to serum-free McCoy's Medium 5A for 24 hr prior to the addition of test solutions did not alter the magnitude or the timing of the stimulation of thymidine incorporation or the increase in cell number.

Thymidine Incorporation into TCA-insoluble Material

Twenty-four hr after addition of the test solution to quiescent MEF, 1 μCi of tritiated thymidine (65 Ci/m mole; final thymidine concentration, 7.5 nM) was added to each culture dish. One hr later, the medium was removed from the cultures and replaced with 2 ml of cold 5% TCA. After 30 min at 4°, the TCA was removed, and the acid-insoluble residue in each culture dish was dissolved in 1 ml of 0.5 N NaOH (15 min, 37°). This solution was washed quantitatively from the culture dish into a centrifuge tube. Ten ml of cold 5% TCA were added. After 20 min at 4°, the precipitate was collected by centrifugation at 10,000 x g. The pellet was washed twice with cold 5% TCA and once with cold 95% ethanol, and then it was dried in an oven. The pellet was then hydrolyzed in 2.5 ml of 5% TCA at 90° for 15 min. The acid-insoluble residue was removed by centrifugation, and an aliquot of the supernatant was assayed for radioactivity in a liquid scintillation counter. Another aliquot was assayed for DNA content with the diphenylamine method of Burton (3). Data were expressed as tritium dpm per μg DNA per hr. In some experiments, cells were removed from the culture dish with trypsin and counted; their viability was measured by trypan blue dye exclusion before precipitation with 5% TCA. Data were expressed as tritium dpm per 10^6 viable cells per hr.

Primary Normal Mammary Epithelial Cultures

The 4th and 5th inguinal mammary glands were dissected from virgin BALB/cKi mice and carefully cleaned of connective tissue, blood vessels, lymph nodes, and muscle under a binocular dissecting microscope. During this operation, the glands were placed in serum-free McCoy's Medium 5A that contained excess antibiotics (400 units penicillin and 400 μg streptomycin per ml). The cleaned glands were then minced and digested with collagenase as described for primary tumor cells. The glands from 2 mice were digested separately, and the cells obtained were seeded into 1 plastic Petri dish (9 sq cm). A total of 12 cultures (12 Petri dishes) were prepared for each experiment.

Established Mammary Cell Lines

All of the mammary cell lines used in this study were established in this laboratory. A detailed description of their properties is in preparation.

MCG-V14. This cell line was derived from normal mammary tissue that was obtained from a 16-month-old virgin A/Ki mouse. The primary cells, which were obtained from collagenase-digested mammary tissue, were epithelioid.

The cell line that was derived from the primary cells was cloned after 1 year and 50 passages. Clone 7 was used in this study. These cells are fibroepithelioid and have a modal chromosome number of 125. They grow with a doubling time of 33 hr in log phase and achieve a saturation density of 0.75 x 10^6 cells/sq cm. When 5 x 10^6 cells were injected s.c. into 12 A/Ki mice, a spindle cell tumor appeared in 1 mouse after 6 months. No tumors have appeared in any of the other mice after 12 months. MCG-V14-7 is considered to be a low-tumorigenic cell clone.

MCG-T14. The source of this cell line was a mammary adenocarcinoma that had appeared spontaneously in a BALB/cKi mouse and had subsequently been propagated as a s.c. implant. The primary cells from the tumor were subcultured 76 times during a period of 14 months. At this point, the line was cloned. Clone 3 was used in this study. The cells are epithelioid and have a modal chromosome number of 50. The doubling time of MCG-T14-3 cells is 14 hr in log phase. The saturation cell density is 2.10 x 10^6 cells/sq cm. When 5 x 10^6 cells were injected s.c. into BALB/cKi mice, palpable adenocarcinomas were formed in 10 to 14 days. MCG-T14-3 is considered to be a high-tumorigenic cell clone.

MCG-T10. The source of this cell line was a spontaneous mammary adenocarcinoma from a CBA/StKi mouse. The cells derived from this tumor were cloned after 45 passages and 1 year in culture. The clone used in this study, clone 10, is fibroblastic and has a modal chromosome number of 40. MCG-T10-10 cells grow with a doubling time of 10 hr in log phase and achieve a saturation density of 2.62 x 10^6 cells/sq cm. MCG-T10-10 cells produced spindle cell carcinomas in CBA/StKi mice within 2 weeks and are considered to be highly tumorigenic.

MCG-T19. This tumor cell line was derived from a spontaneous mammary adenocarcinoma from an A/Ki mouse. After 1 year and 30 passages in culture, the line was cloned. Clone 9 was used in this study. The chromosome number in clone 9 cells is bimodal with modes at 43 and 80 chromosomes. The doubling time of this clone is 10 hr in log phase. The saturation density is 1.89 x 10^6 cells/sq cm. When 5 x 10^6 cells were injected s.c. into A/Ki mice, palpable adenocarcinomas were formed in 1 to 2 weeks after injection s.c. into A/Ki mice.

The clonal mammary cell lines used in this study will be referred to without the clone number; e.g., MCG-V14-7 will be written as MCG-V14.

Culture of Established Cell Lines

All of the clonal cell lines used in this study were maintained as monolayer cultures in McCoy's Medium 5A supplemented with 5% fetal bovine serum. Cultures were grown at 37° in a 5% CO_2-95% air atmosphere. Subculture was accomplished with standard trypsinization techniques. Cells in all cultures used in this study were counted on a hemocytometer after trypsinization. Cells were renewed from frozen stocks at 3-month intervals. Thus, all of the clonal cell lines used in this study were subcultured no more than 12 times after cloning. All cells were free of contaminating bacteria, fungi, and Mycoplasma.
RESULTS

Growth-promoting Activity of CM from Primary Tumor Cells. The ability of tumor cell CM and those of other additives to stimulate thymidine incorporation into acid-insoluble material by quiescent MEF were compared (Chart 3). The acid-insoluble radioactivity, recovered from cells that had been exposed to fetal bovine serum [final concentration, 10% (v/v)] for 24 hr and then labeled with tritiated thymidine for 1 hr, was given a normalized value of 1.0. The absolute value of the acid-insoluble radioactivity in these serum-fed cultures averaged 4521 ± 783 dpm/μg DNA (n = 8). Thymidine incorporation in cultures in which the spent medium was not renewed or supplemented (unfed cultures) was 8% of that seen in serum-fed cultures (Chart 3). When quiescent MEF were exposed to serum-free McCoy's Medium 5A, thymidine incorporation 24 hr later was about 18% of the response induced by 10% serum. This indicated that renewal of medium constituents caused only a small stimulation of thymidine incorporation. The activity of McCoy's Medium 5A supplemented with 0.25% fetal bovine serum was identical to that of McCoy's Medium 5A alone. Furthermore, undialyzed tumor cell CM that contained 0.25% serum (see "Materials and Methods") had the same activity as serum-free medium. Therefore, 0.25% serum did not stimulate thymidine incorporation in quiescent MEF above the level exhibited by serum-free medium. In contrast to the previous additives, DCM had a stimulatory activity comparable to that of 10% fetal bovine serum.

In addition to an apparent effect on DNA synthesis, DCM stimulated cellular overgrowth in density-inhibited MEF (Chart 4). The magnitude of cellular overgrowth (approximately 2-fold) was equivalent to that caused by 10% fetal bovine serum.

Growth-promoting Activity of CM from Established Cell Lines, Normal Mammary Epithelium, and Normal MEF. CM and DCM from several cell lines were assayed for their ability to stimulate thymidine uptake into acid-insoluble material by density-inhibited MEF. The mouse mammary cell lines were MCG-V14, MCG-T14, MCG-T10, and MCG-T19, described in "Materials and Methods." HeLa cells were also used as a source of DCM. In addition, medium from normal mammary epithelium and from secondary cultures of MEF was tested. For every cell culture, the protocol for obtaining CM was the same as that described for primary tumor cells (see "Materials and Methods"). Table 1 shows the results of duplicate experiments. It can be seen that the stimulatory activity of CM (relative to 10% serum) from the various cell cultures ranged from 0.02 (secondary MEF) to 0.28 (MCG-V14). With the exception of cell line MCG-T19, dialysis of CM from all of the other cell cultures caused an increase in stimulatory activity from 1.8-fold (HeLa) to 14-fold (MCG-T10).

In these experiments, CM was obtained from cells in the stationary phase of growth. The plateau or saturation density achieved by each cell line varied from 0.40 × 10⁶ cells/sq cm (primary mammary epithelial cells) to 2.62 × 10⁶ cells/sq cm (MCG-T10). Consequently, CM was obtained from different numbers of cells in each cell line. In order to compare the potential of each cell line to produce growth-promoting activity, the relative activity of the various DCM's was normalized for cell density (Table 2). The saturation density of each cell line (Table 2, Column A) is expressed as a multiple of the cell density achieved by primary tumor cells [relative cell density (Table 2, Column B)]. The relative growth-promoting activity of the DCM obtained from each
Growth Stimulation by Mammary Cell CM

Table 1
Stimulation of thymidine uptake in stationary MEF by CM from various cell cultures

<table>
<thead>
<tr>
<th>Source of medium</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>McCoy's Medium 5A + 10% fetal bovine serum</td>
<td>1.00</td>
</tr>
<tr>
<td>MCG-T14 CM</td>
<td>0.79 ± 0.10*</td>
</tr>
<tr>
<td>MCG-T19 CM</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>MCG-T10 CM</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>MCG-V14 CM</td>
<td>0.10 ± 0.14</td>
</tr>
<tr>
<td>HeLa CM</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>Primary normal mammary epithelium</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>Secondary MEF CM</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>CM</td>
<td>0.02 ± 0.001</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

cell line is listed in Table 2, Column C. These values have been corrected for the activity exhibited by fresh serum-free medium (Chart 1). This is done because DCM is obtained by dialysis of CM against serum-free medium and presumably some of the activity of DCM (relative activity, 0.2 compared to fetal bovine serum) is due to renewal and removal of low-molecular-weight medium components. The corrected relative growth-promoting activity of the DCM obtained from a cell line was divided by the relative cell density to estimate the relative activity produced per cell (Table 2, Column D). It can be seen that cell line MCG-V14 produced more than twice as much growth-promoting activity per cell as did primary tumor cells. The relative activity per cell in the other cell cultures ranged from zero (MEF and MCG-T19) to 0.56 (MCG-T14). Primary mammary epithelial cells produced 32% as much activity per cell as did the primary adenocarcinoma cells.

Further Observations on Primary Tumor Cell DCM. If primary tumor cells constantly release a nondialyzable growth factor, one might expect that the growth-promoting activity of CM would increase as a function of the time the medium was in contact with cells. Chart 5 shows that CM that was harvested after 24 hr and then dialyzed (Chart 5, DCM 24 hr) had one-half the activity of DCM that had been in contact with primary tumor cells for 6 days. Therefore, the stimulatory activity of DCM does appear to increase with time. The primary tumor cells from which 24-hr CM had been harvested were refed with fresh medium and 0.25% serum. Six days later, this medium was harvested and dialyzed (Chart 5, DCM 6 days-rinsed). The DCM from the rinsed cultures had the same activity as did 6-day DCM from unrisned cultures (i.e., from confluent, primary tumor cultures where the spent growth medium was replaced with McCoy's Medium 5A and low serum without an intervening

Table 2
Relative activity of DCM from various cell cultures normalized for cell density

<table>
<thead>
<tr>
<th>DCM source</th>
<th>A. Saturation cell density ( \times 10^8 \text{ cells/sq cm} )</th>
<th>B. Relative cell density</th>
<th>C. Corrected relative activity (C/B)</th>
<th>D. Relative activity/cell (C/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor</td>
<td>1.58</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>MCG-V14</td>
<td>0.75</td>
<td>0.47</td>
<td>1.00</td>
<td>2.13</td>
</tr>
<tr>
<td>MCG-T14</td>
<td>2.10</td>
<td>1.33</td>
<td>0.74</td>
<td>0.56</td>
</tr>
<tr>
<td>MCG-T10</td>
<td>2.62</td>
<td>1.66</td>
<td>0.64</td>
<td>0.39</td>
</tr>
<tr>
<td>Primary mammary epithelium</td>
<td>1.85</td>
<td>1.20</td>
<td>0.50</td>
<td>0.32</td>
</tr>
<tr>
<td>HeLa</td>
<td>2.54</td>
<td>1.61</td>
<td>0.93</td>
<td>0.56</td>
</tr>
<tr>
<td>MCG-T19</td>
<td>1.69</td>
<td>1.20</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td>MEF</td>
<td>1.70</td>
<td>1.07</td>
<td>0.20</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Chart 5. Accumulation of stimulatory activity in primary tumor cell DCM. The ability of DCM, which was obtained from primary mammary tumor cells after 24 hr or 6 days, to stimulate thymidine uptake into acid-insoluble material by density-inhibited MEF was compared. In addition, confluent monolayers of primary tumor cells were rinsed for 24 hr with fresh medium that contained 0.25% (v/v) fetal bovine serum. This medium was replaced by fresh medium that contained 0.25% serum. Six days later, this medium was dialyzed and assayed as "DCM-6 days-rinsed." The stimulatory activity of the various DCM was expressed as a multiple of the activity (1.0) of fresh medium that contained 10% (v/v) fetal bovine serum.
24-hr "rinse"). This result indicates that the active principle in DCM is probably not derived from residual, spent growth medium in the primary tumor cultures.

One possible explanation for the growth-promoting activity in tumor cell DCM is that the tumor cells sequester serum factors from the growth medium and then release them into the GM. If this occurs, then the active principle in tumor cell DCM might be a serum factor(s). This possibility is very difficult to rule out. However, the following experiments provide indirect evidence that the growth-promoting activity in tumor cell DCM is different from the growth factor(s) in fetal bovine serum.

The ability of 10% fetal bovine serum and tumor cell DCM to stimulate thymidine uptake was compared in the cell lines utilized previously (Chart 6). All cell cultures were allowed to reach a quiescent, stationary phase during which cell growth and DNA synthesis had ceased before they were fed with serum or with DCM. The absolute magnitude of the basal and stimulated levels of thymidine incorporation varied greatly between these cell lines; therefore, normalized data were not presented in this case. This experiment showed that the response to serum and DCM is very different in some cell lines. For example, MCG-V14 is stimulated by 10% serum but is apparently refractory to DCM. In contrast to MCG-V14, MCG-T10 was 3.6 times more sensitive to DCM than to 10% serum. MEF, HeLa cells (not shown), and MCG-T14 mammary tumor cells had a nearly equal response to serum and DCM. Finally, quiescent MCG-T19 cells were not stimulated by serum. However, DCM caused a 3-fold stimulation of thymidine uptake compared to unfed controls. If the active principle in DCM is a serum factor(s), then one would expect that a given cell type would respond similarly to DCM or to serum. This does not occur in MCG-V14, MCG-T10, or MCG-T19 cells.

The maximum serum stimulation of quiescent MEF occurred with 10% serum. Addition of more serum did not cause any further increase in thymidine incorporation (results not shown). An experiment was performed to determine whether DCM could stimulate MEF in the presence of saturating concentrations of serum (Chart 7). When MEF were fed with DCM and 10% fetal bovine serum, the stimulation of thymidine incorporation was greater than when cells were fed with serum or with DCM alone. The magnitude of the response in cells fed with DCM and 10% serum (5985 dpm/μg DNA) was approximately 7% less than the sum (6419 dpm/μg DNA) of the response in cells that were fed with serum alone or DCM alone. This implies that the effect of serum and DCM on thymidine incorporation is additive at saturating concentrations of serum. These data are consistent with the hypothesis that the active principle in DCM is different from the active factor(s) in serum.

DISCUSSION

These experiments show that DCM but not CM from 3 mouse mammary clonal cell lines, HeLa cells, and primary mouse mammary epithelial cells stimulate thymidine uptake into acid-insoluble material by quiescent MEF. Cells from 1 malignant cell line (MCG-T19) and MEF did not produce any detectable growth-promoting activity. In addition, we have confirmed the observation of Nair and DeOme (10) that DCM from primary monolayer cultures of mouse mammary adenocarcinoma cells stimulated thymidine uptake and cell overgrowth in MEF cultures.

Our observations show that among the cell cultures tested production of growth-promoting activity was not a unique property of tumor-derived cells. MCG-V14, which is a cell line derived from normal mammary tissue, produced more than twice as much growth-promoting activity per cell as the primary tumor cells. MCG-V14 exhibited density-
dependent inhibition of growth at low cell density and in this respect resembled a nontransformed cell line. However, MCG-V14 is an established cell line which has produced a tumor in 6 months when injected s.c. into homologous mice (see "Materials and Methods"). Therefore, MCG-V14 is likely to be a transformed cell line with low tumorigenicity. The production of an extracellular TAF by established cell lines (WI-38 and BALB/c-3T3), which were derived from normal tissue and exhibited "contact inhibition" of growth in culture, has been reported by Klagsbrun et al. (8). In addition, a variety of tumor-derived and viral-transformed cell lines produced TAF. However, primary cultures of MEF and low-passage cultures of human fibroblasts produced no TAF. These observations could mean that production of TAF is a general property of transformed cells in culture but is not a property of nontransformed cells. This possibility is supported by Suddith et al. (15), who reported that CM obtained from a variety of clonal cell lines of tumor origin stimulated the proliferation of cultured endothelial cells, while CM from cultures of nontransformed, primary fibroblasts, nonmalignant peripheral lymphocytes, and cells derived from amniogenesis had no detectable activity. Although it is difficult to compare growth-promoting activities detected in different assay systems, our data have a bearing on the proposition that the production of growth factors is a unique property of transformed cells in culture. As in the cited reports, primary MEF produced no growth-promoting activity. However, primary cultures of mouse mammary epithelial cells produced approximately one-third as much growth-promoting activity per cell as did primary tumor cells. Thus, in this system growth-promoting activity was produced by nontransformed cells in culture. To determine whether this is an isolated phenomenon or a general property of primary epithelial cells will require the testing of a broad spectrum of primary cell cultures from a variety of epithelial tissues.

The amount of growth-promoting activity produced per cell by primary epithelial cells was considerably less than that produced by primary tumor cells. This could mean that the production of growth factors is amplified by the process of transformation. If this were the case, one might expect to find a correlation between the amount of growth-promoting activity produced per cell and those properties of cells in culture that are associated with the transformed state, such as high tumorigenicity, increased growth rate during log phase, and the capacity to achieve high-saturation cell densities. In our experiments none of these parameters were correlated with the capacity of cells to produce growth-promoting activity. Thus, MCG-V14 exhibited low tumorigenicity when measured by the criteria of time of tumor production in homologous mice (6 to 8 months after the s.c. injection of 5 × 10⁶ cells), low growth rate (doubling time, 33 hr in log phase), and a high saturation density (0.75 × 10⁶ cells/sq cm). MCG-T19 exhibited high tumorigenicity (10 to 14 days after injection of 5 × 10⁶ cells), high growth rate (doubling time, 10 hr in log phase), and a high saturation density (1.89 × 10⁶ cells/sq cm). By these criteria, MCG-T19 appeared to be a cell line in which the degree of transformation was greater than that of MCG-V14. However, MCG-V14 produced the greatest amount of growth-promoting activity per cell of all cell lines tested. MCG-T19 did not produce detectable growth-promoting activity. Cell lines MCG-T14 and MCG-T10 were similar to MCG-T19 with respect to tumorigenicity, growth rate, and saturation density achieved in culture. These cells produced growth-promoting activity but not at the level exhibited by MCG-V14 cells. These observations suggest that, among the cultures tested, transformation, when measured by the criteria of tumorigenicity, growth rate, and growth potential, was not correlated with the capacity of cells to produce growth-promoting activity. Similarly, the extent of production of growth-promoting activity was not correlated with cell type or species origin. Active fibroblasts (MCG-T10) and inactive MEF, active epithelial cells (MCG-T14, HeLa, primary mammary epithelium), and inactive epithelial cells (MCG-T19) were identified. In addition to the active murine cell cultures, cells of human origin (HeLa) produced an active principle. These observations suggest that transformed and nontransformed cells of diverse origin, cell type, and tumorigenicity may produce growth factors in culture. However, the proposition that the growth-promoting activity in DCM, obtained from active cultures, actually contained a growth factor(s) of cellular origin requires further scrutiny.

Calderone and Unanue (4) have shown that macrophage-rich peritoneal exudate cells from the mouse release a dialyzable inhibitor and a nondialyzable growth factor to the medium when they are placed in in vitro culture. A similar phenomenon that would explain the relative inactivity of CM compared to DCM could have occurred in our active cultures. However, there are alternative explanations of our data to be considered. It is conceivable that CM because of its contact with nongrowing cell monolayers acquires low-molecular-weight inhibitory substances only. These could be derived from dead or dying cells and/or could be released as normal export products from healthy cells. CM that contained the inhibitory substances would not promote growth. However, dialysis of the CM would remove inhibitors, and the DCM would then exhibit stimulatory activity. This explanation implies that there is no growth factor; the activity of DCM could be ascribed wholly to the removal of a dialyzable inhibitor. If these were the case, dialysis of CM from any source should produce a DCM with growth-promoting activity. Furthermore, since CM is dialyzed against serum-free medium, the growth-promoting activity of DCM would not be expected to exceed that of serum-free medium or of medium that is supplemented with an amount of fetal bovine serum (0.25%) equivalent to the level that is present during the conditioning period. These predictions are not borne out by our results. Dialysis does not always produce a DCM in which relative growth-promoting activity is different from untreated CM (cell line MCG-T19). In most cases, the relative activity of DCM is 50 to 100% that of fetal bovine serum. This is significantly greater than the relative activity (20%) of serum-free medium or medium supplemented with 0.25% serum. Thus, it would appear that the growth-promoting activity of DCM is too great to be accounted for entirely by the removal of a dialyzable inhibitor.

If the premise is accepted that active DCM contains a growth factor(s), then it is of interest to speculate on the nature and source of this material. One possibility is that the
active principle in DCM is a serum growth factor(s) that is derived from the original spent-growth medium or from the 0.25% serum present during the conditioning period. We have already discussed the observation that the relative potency of active DCM from primary tumors exceeds that of 0.25% serum. If the growth-promoting activity of primary tumor cell DCM is due to residual serum from spent medium, then the activity of DCM should not increase with prolonged exposure to the cell monolayer. However, we have shown (Chart 5) that the activity of primary tumor cell DCM does increase with time of exposure to cells. The increase in activity occurs with similar magnitude when the cell monolayer was washed to remove spent medium before fresh CM is applied. Thus, it appears that the active principle in primary tumor cell DCM accumulates in the culture and that it is probably not derived from spent medium.

The active principle in DCM also could be a serum growth factor(s) that is sequestered by the cells during growth in high concentrations of serum and then is slowly released to the CM. If this occurred, the relative activity of DCM would still be expected to increase with time of contact with the cell monolayer. Two of our observations give indirect evidence that the active principle in primary tumor cell DCM is different from the growth factors in serum. First, some cell lines react differentially to serum and primary tumor cell DCM (Chart 6). For example, thymidine incorporation into density-inhibited MCG-V14 cells is stimulated by serum but not by primary tumor cell DCM. MCG-T10 is much more sensitive to DCM than to serum. Quiescent MCG-T19 cells are refractory to serum, but thymidine incorporation is stimulated by primary tumor cell DCM. If the active principle in primary tumor cell DCM is the same as the active principle in fetal bovine serum, then one would expect that a given cell line would react similarly to each additive. However, Leffert (7) has shown that both relative and absolute levels of serum factors influence the proliferation of cultured fetal rat hepatocytes. Furthermore, some serum growth factors resemble peptide hormones (12), and some cell lines differ in their hormone requirements for growth in vitro (2). It is possible, therefore, that the differential response of some cell lines to DCM and to whole serum is due to differences in the relative and absolute levels of peptide hormone-like growth factors in these additives.

The 2nd observation, which suggests that primary tumor cell DCM contains growth factors that are different from serum growth factors, was the response of quiescent MEF cells to the addition of both DCM and 10% serum. The increase of thymidine uptake into acid-insoluble material, following the simultaneous addition of both DCM and serum, was equal to the sum of the increase in cultures given serum plus the increase in cultures given DCM (Chart 7). The response of MEF to serum plateaued at the 10% level. Further addition of serum to 25% did not cause a further increase in thymidine uptake, nor did it result in cell toxicity (E. Howard, unpublished observation). Therefore, 10% serum was a saturating dose with respect to the stimulation of thymidine uptake by quiescent MEF. If serum and DCM contain the same growth factors, these factors should interact with the same cellular receptors that are responsible for the regulation of thymidine uptake, and the simultaneous addition of DCM and a saturating concentration of serum should not result in a stimulation of thymidine uptake above that seen with 10% serum. Thus, the observation that the response to both DCM and 10% serum is additive strongly suggests that the active principle in DCM interacts with cellular receptors that are different from those involved in the response to serum growth factors and that the active principle of DCM is different from the serum growth factors. However, alternative interpretations of these data, which involve an increased response of a cellular receptor to a unique combination of several serum growth factors in DCM and 10% serum, cannot be ruled out at this time.

On the basis of the experiments with primary tumor cell DCM, we propose that our active cell cultures also produced a growth factor(s) that could stimulate cell proliferation in quiescent MEF cells. Our data do not reveal whether the various active cell lines produced qualitatively different growth factors, or whether the growth-promoting activity in primary tumor cell DCM resembled the tumor-produced stromal growth factors that may function in vivo to stimulate the proliferation of stromal tissues required for sustained tumor growth (5). The fact that primary tumor cell DCM stimulated DNA synthesis and cell division in malignant cell lines of epithelial origin (HeLa, MCG-T14, MCG-T19) as well as it did in MEF, suggests that primary tumor cell DCM contained a general growth-promoting activity not specific for fibroblasts. The growth of all of the cell lines tested, including the tumor cells, was completely arrested when they were exposed to DCM. Thus, the growth-promoting activity of DCM in these experiments represented a reversal of growth arrest at high cell density. It will be interesting to see whether DCM can also promote or support the attachment of freshly plated target cells or the growth of these cells at low density.

A definitive answer to whether or not active DCM contains dialyzable inhibitors or macromolecular growth factors awaits the isolation, purification, and biochemical characterization of these substances. Although the literature abounds with reports of factors that will stimulate or inhibit cell growth [reviewed by Pardee (11)], few of these factors have been isolated or characterized. Instances in which cultured cells produce readily identifiable growth factors are rare. Rat liver cell clones exist that apparently produce a multiplication-stimulating factor that resembles growth factors in whole serum (14). Nerve growth factor, which is a natural product of the mouse submaxillary gland, is produced by mouse fibroblasts (16) and by mouse neuroblastoma cells in culture (9). Nair and DeOme (10) reported that the active principle in primary mammary tumor cell DCM could be precipitated with ammonium sulfate. We have confirmed this observation in preliminary experiments. Thus, we are encouraged that further efforts to isolate and characterize the putative growth factors in DCM obtained from active cell cultures will be successful.

ACKNOWLEDGMENTS

The authors acknowledge the capable technical assistance of Judy Greene.
REFERENCES

Stimulation of Thymidine Uptake and Cell Proliferation in Mouse Embryo Fibroblasts by Conditioned Medium from Mammary Cells in Culture

Eugene F. Howard, David F. Scott and Carol E. Bennett


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/12/4543

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, use this link http://cancerres.aacrjournals.org/content/36/12/4543. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.