A Growth-stimulating Factor from Solid Mouse Mammary Tumors

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SUMMARY

A soluble fraction (SF) prepared from solid spontaneous primary mouse mammary tumors stimulated the growth of density-inhibited mouse embryo cells in monolayer culture. Dialysis of the SF increased its growth-stimulating activity. At higher concentrations, both nondialyzed and dialyzed SF's were inhibitory to cell growth. A similar preparation from normal mouse mammary gland showed some growth-stimulating activity. SF from the kidney of tumor-bearing or multiparous old mice showed high activity, while that from tumor-free virgin young mice did not show significant activity. Hyperplastic alveolar nodules of the mammary gland showed activity as high as that of tumors. The growth-stimulating factor in the SF was precipitated with 60% saturated ammonium sulfate, and the ammonium sulfate precipitate was further separated by Sephadex G-100 column chromatography.

INTRODUCTION

From histological studies, earlier workers have reported that tumors have a growth-stimulating effect on adjoining normal tissue (2, 3, 13, 20). Conjoint organ cultures of normal and tumor tissues provided additional support for the histological observations (12, 23). Medium harvested from the cultures of Rous sarcoma cells and human cancer cells contain growth-stimulating substances (24, 26). In another report from this laboratory (16) we have demonstrated the occurrence of a GSF in the culture medium of mouse mammary tumor cell cultures. The existence of a GSF in solid spontaneous primary mouse mammary tumors and its assay in monolayer culture are reported in this paper.

MATERIALS AND METHODS

Preparation and Processing of the Soluble Fraction

Female BALB/cfC3H/CRGL mice (obtained from Cancer Research Laboratory, University of California, Berkeley, Calif.) bearing spontaneous primary mammary tumors were killed and tumors were dissected out under sterile conditions. Tumor tissue was weighed and minced with a sterile razor blade. Distilled water was added to bring the homogenate to 20% w/v. Sometimes Tris- or phosphate-buffered saline was used instead of distilled water (Tris-buffered saline: 0.8% NaCl, 0.038% KCl, 0.01% Na₂HPO₄, 0.1% glucose, 0.38% Trizma base, 5 x 10⁴ units penicillin, and 50 mg streptomycin per liter, pH adjusted to 7.2 with HCl; phosphate-buffered saline: 0.14 M NaCl and 0.01 M phosphate). The tissue was homogenized in the solvent in the cold in a Waring blender (Scientific Products, Evanston, Ill.) for 3 min (all preparative steps were done at 4°C). After 3 min the homogenate was poured into 50-ml centrifuge tubes and centrifuged for 15 min at 17,000 x g. The supernatant was decanted and the sediment was again homogenized in the solvent and centrifuged. The 2 supernatants were combined. The homogenate had a final concentration of 10% w/v. The supernatant was then centrifuged at 100,000 x g for 2.5 hr to remove mammary tumor virus and subcellular particles. The clear supernatant called SF was saved and the pellet was discarded. The nucleoproteins were removed before assay as described in Ref. 5.

An aliquot of SF was dialyzed (with stirring) in the cold against buffer for 48 hr, with frequent buffer changes. Protein determinations were made from absorbancy measurements at 280 nm. Colorimetric determinations by the method of Lowry et al. (11) showed 1 absorbance material was layered on the column. The same buffer was used instead of distilled water. A280 material was layered on the column. The same buffer was used instead of distilled water.
the material from the column. Six-ml fractions were collected every 30 min. The A280 of the fractions was read and series of fractions with the same A280 were pooled. A total of 5 pools were made (Chart 3). Since the sample was diluted about 10-fold in the column, the pooled fractions total of 5 pools were made (Chart 3). Since the sample was diluted about 10-fold in the column, the pooled fractions were concentrated in a Diaflo pressure filtration apparatus (Amicon Corp., Lexington, Mass.), with a PM-10 membrane (Amicon Corp.) that eliminated molecules up to 10,000 M.W. The A280 of the concentrated fraction was determined.

Assay for GSF

A detailed account of the assay was given elsewhere (16), and the sections relevant to the present work are described below. Basically, the SF's to be assayed for GSF activity were added to density-inhibited cultures of mouse embryo cells and the growth stimulation was determined from the rate of DNA synthesis and cell division. The rate of DNA synthesis was studied by labeling the mouse embryo cell cultures with 3H-labeled thymidine. Cell counts were made to determine the increase in cell number due to stimulation of growth.

Rate of Uptake of 3H-labeled Thymidine. After the addition of SF's the cultures were labeled with 3H-labeled thymidine (specific activity, 13.7 Ci/mmole, Schwarz Bioresearch, Orangeburg, N. Y.) to a final concentration of 2 \( \mu \)Ci/ml medium for 1 hr. After 1 hr, the labeled medium was removed and the cells were washed 4 times with cold Tris-buffered saline. The acid-soluble fraction was extracted with 5% trichloroacetic acid for 10 min in the cold. The cells were rinsed twice with cold 5% trichloroacetic acid, and the acid-precipitable fraction was hydrolyzed at 70° for 2 hr in a water bath. One ml hydrolysate was counted in a liquid scintillation counter in 12 ml cocktail containing Omnifluor (10 g; New England Nuclear, Boston, Mass.), Triton X-100 (1 liter, scintillation grade; Beckman Instruments, Inc., Palo Alto, Calif.) and toluene (2 liters).

Cell Number. Culture medium was removed from treated dishes with 1 ml STV (22). Cell counts were made in the same STV.

RESULTS

Nondialyzed and dialyzed SF's were tested for their capabilities to induce growth in density-inhibited cultures of mouse embryo cells. Fifteen-day-old BALB/cfC3H mouse embryos were dispersed to single cells with STV and cultured in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal serum; this medium is called SMEC. Mouse embryo cells (106) from primary cultures were plated in 35-mm tissue culture dishes containing 2.5 ml SMEC. Subsequently, the SMEC was replaced with modified SMEC, which contained 2% rat serum and 1% fetal calf serum, for reasons described earlier (16). Sixty hr after the addition of modified SMEC, 0.1 ml dialyzed or nondialyzed SF was added to those cultures. Dialyzed SF (0.1 ml) contained 0.25 A280, and 0.1 ml nondialyzed SF contained 0.52 A280 protein. Control cultures received 0.1 ml buffer. The addition of SF's caused an increase in the uptake of 3H-labeled thymidine and cell number (Table 1).

The dialyzed SF was almost twice as active as the nondialyzed SF on a A280 basis. At higher concentrations, both nondialyzed and dialyzed fractions showed an inhibitory effect on the incorporation of 3H-labeled thymidine by embryo cells (Chart 1). SF's from other tissues were prepared by the same procedure that was used for tumor tissue. Since they were different in their protein concentrations, the activity was tested on a unit basis. For this purpose, the more concentrated SF's were diluted with Tris-buffered saline, and each test culture dish received the same amount of protein, namely, 0.8 A280 protein in 0.2 ml volume. Control cultures received 0.2 ml of Tris-buffered saline. Results of the assays of SF's from tissues other than tumor are included in Table 2. The SF from normal pregnant or lactating mouse mammary gland was moderately active in stimulating DNA synthesis of density-inhibited mouse embryo cells. Since mammary glands are epithelial organs, other epithelial organs were also examined for growth-stimulating activity. Liver did not show any stimulatory effect; on the contrary, it showed an inhibitory effect on DNA synthesis and caused cell death at higher doses. Kidney from tumor-bearing, multiparous old mice showed high activity, whereas kidney from young, virgin tumor-free mice showed only slight activity. The high activity of the kidney was seen in mice carrying mammary tumor virus (BALB/cfC3H) and in the kidney of mice that were free of virus (BALB/c). The addition of a nonspecific protein, bovine serum albumin, had no significant effect. The hyperplastic alveolar nodule, which is preneoplastic in mouse mammary tumorigenesis (7), showed activity as high as

<table>
<thead>
<tr>
<th>Test material</th>
<th>cpm/dish/hr at 0 hr</th>
<th>2 hr</th>
<th>4 hr</th>
<th>6 hr</th>
<th>12 hr</th>
<th>18 hr</th>
<th>Cell no. (10^6) at 30 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed SF</td>
<td>869 ± 68*</td>
<td>789 ± 62</td>
<td>921 ± 93</td>
<td>1826 ± 89</td>
<td>2190 ± 193</td>
<td>2.06 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Nondialyzed SF</td>
<td>621 ± 41</td>
<td>691 ± 71</td>
<td>826 ± 109</td>
<td>1281 ± 102</td>
<td>1864 ± 92</td>
<td>1.20 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Tris-buffered saline</td>
<td>948 ± 61</td>
<td>723 ± 64</td>
<td>646 ± 66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 6 values.
as that of tumor. Dose response studies were done for the SF from each tissue and they all had the maximum activity at a concentration of about 1 A₂₈₀ and an inhibitory effect at higher concentrations. This also explains why 0.8 A₂₈₀ was chosen for comparing the activity of different SF's (B. K. Nair, unpublished data).

Active material from the SF was precipitated with 60% saturated ammonium sulfate and assayed (Chart 2). Addition of 0.1 ml solution containing 0.4 A₂₈₀ protein caused a more than 2-fold increase in the uptake of radioactive thymidine by density-inhibited cultures of mouse embryo cells.

The separation of ammonium sulfate precipitate on a Sephadex G-100 column and the results of the assay of the fractions are shown in Chart 3. The entire effluent from the column was divided into 5 pools. Materials above 100,000 M.W. appeared in the void volume (Pools I and II). Slight activity was detected in Pools III and IV. Most of the activity remained in Pool V.

DISCUSSION

The growth-stimulating effect of different preparations obtained from mouse mammary tumors supports the histological observations of earlier workers (2, 3, 12, 20). The

![Chart 1. Growth-stimulating activity of nondialyzed and dialyzed SF from mouse mammary tumors. Nondialyzed and dialyzed SF's had different concentrations of protein. Therefore they were brought to the same concentration by dilution with Tris-buffered saline. Since the optimum dose for maximum stimulation was not known, the assay was done using different concentrations. At very high concentration, both inhibited uptake of ³H-labeled thymidine. Activity is expressed as percentage increase over control cultures that received Tris-buffered saline. This seemed appropriate to express the results obtained from several experiments whose control values varied.](chart1)

![Chart 2. Growth-stimulating activity of ammonium sulfate precipitate obtained from the SF of mammary tumors; 0.4 A₂₈₀ protein in 0.1 ml solution was added to 60-hr-old cultures of mouse embryo cells. Control cultures received 0.1 ml Tris-buffered saline (Tris-saline). After the addition of test materials, the cultures were labeled with ³H-labeled thymidine at different time intervals.](chart2)

**Table 2**

**Assay of growth-stimulating activity in the SF of tumor and nontumor tissues**

Since the SF's from different sources were different in protein concentration they were brought to the same concentration by dilution with Tris-buffered saline, which is used as control. At approximately 1.0 A₂₈₀, liver was toxic. The volume of SF added in each dish was constant (0.2 ml). Bovine serum albumin was included as a nonspecific protein. The 1 A₂₈₀ was approximately 1 mg protein, as determined by colorimetric methods. SF's were added to 50-hr-old density-inhibited cultures of mouse embryo cells and, 20 hr later, the cultures were labeled with ³H-thymidine. Rate of uptake of the isotope was determined by liquid scintillation counting.

<table>
<thead>
<tr>
<th>Source of SF</th>
<th>Concentration</th>
<th>cpm/dish/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>0.8</td>
<td>4259 ± 381*</td>
</tr>
<tr>
<td>Hyperplastic alveolar tissue</td>
<td>0.8</td>
<td>4084 ± 448</td>
</tr>
<tr>
<td>Kidney from multiparous old tumor-bearing mice</td>
<td>0.8</td>
<td>3252 ± 264</td>
</tr>
<tr>
<td>Kidney from multiparous retired breeders (MTV +)</td>
<td>0.8</td>
<td>3271 ± 502</td>
</tr>
<tr>
<td>Kidney from multiparous retired breeders (MTV -)</td>
<td>0.8</td>
<td>2962 ± 317</td>
</tr>
<tr>
<td>Liver from tumor-bearing mice</td>
<td>0.1</td>
<td>1876 ± 161</td>
</tr>
<tr>
<td>Liver from tumor-bearing mice</td>
<td>0.2</td>
<td>1732 ± 174</td>
</tr>
<tr>
<td>Kidney from 1-month-old virgin</td>
<td>1.0</td>
<td>2090 ± 208</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.8</td>
<td>2013 ± 442</td>
</tr>
<tr>
<td>Tris-buffered saline</td>
<td></td>
<td>1936 ± 127</td>
</tr>
</tbody>
</table>

*Mean ± S.E. of 6 values.

* MTV +, Mammary tumor virus carrying; MTV −, Mammary tumor virus free.
higher activity after dialysis might be due to the elimination of a toxic dialyzable molecule. The occurrence of toxic molecules in cancer was reported by earlier workers (6, 10, 24, 25). The inhibitory effect of soluble fractions at higher concentrations is similar to that reported by Rounds (24) for the culture medium from human cancer cell cultures. The growth stimulatory activity was not detected in some tumors that were necrotic (B. K. Nair, unpublished observations). This might be due to lack of GSF or predominance of the toxin. Ludford and Barlow (12) as well as Randive and Bhide (23) observed different grades of stimulatory effect in different types of tumors, including lack of any stimulatory effect in some cases.

The stimulatory effect of the SF from the mammary tumors does not seem to come from mammary tumor virus borne by the donors, because no difference was observed between the tissues obtained from virus-carrying (BALB/cfC3H) and virus-free (BALB/c) mice. Moreover, centrifugation at 100,000 × g for 2.5 hr and precipitation of nucleoproteins with streptomycin would have eliminated the mammary tumor virus in the extract.

The increase in GSF activity in the SF from tumor-bearing and old multiparous mice could be an effect of age or parity. Nandi (18) described a parity factor in multiparous mice that caused an increase in the sensitivity of mammary gland tissue to certain hormones.

The kinetics of DNA synthesis induced by the GSF is similar to that caused by the addition of serum to density-inhibited cultures of mouse embryo cells (16). There was a lag period of about 12 hr before increase in the incorporation of radioactive thymidine. This might be due to the arrest of density-inhibited cells at G1 phase in the cell cycle and other preparations before cells enter the S phase (19, 30). The effect of partial depletion of nutrients in the medium of assay cultures is reflected in the low degree of stimulation by GSF. When the GSF was assayed in cultures whose medium was 30 hr old, the degree of stimulation (as shown from the high cpm and the presence of more labeled nuclei in autoradiographs) was much higher than the stimulation elicited in cultures whose medium was 60 hr old (B. K. Nair, unpublished data). A similar situation was described for the growth stimulation induced by serum in density-inhibited cultures of mouse embryo cells (14, 17).

The precipitability of GSF with 60% saturated ammonium sulfate is similar to the preparation of growth factor from the culture medium of Rous sarcoma cells (26) and mouse mammary tumor cells (16). During column chromatography, the bulk of the total protein appeared in the void volume, decreasing considerably the amount of active GSF. However, this small amount of protein was about 20-fold as active as the protein in the rest of the column. Slight activity in pools III and IV could be due either to aggregation of active molecules to form heavier complexes or to the association of active molecules to larger ones that appeared in the leading front.

The chemical nature and function of the GSF are unknown. The nondialyzable nature, precipitability with ammonium sulfate, UV absorption maximum at 280 nm, fractionation on Sephadex G-100 column, and stainability with stains for proteins during isoelectric focusing (15) indicate that GSF is a protein or a mixture of proteins. Increased activity of certain enzymes was described in some carcinomas (8, 9, 21, 28, 32). An increase in peptidase and protease activity has been shown in the periphery of growing tumors (9, 31, 32). Experimentally, proteases induce growth in density-inhibited cultures by altering the surface properties of cells (4, 27). One possible source of such proteases is lysosomal enzymes (1, 33). Further studies are required in order to determine whether the GSF is a surface-active lysosomal protease.

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