Purification and Properties of a Mammary-Uterine-Pituitary Tumor Cell Growth Factor from Pregnant Sheep Uterus*

(Received for publication, April 26, 1983)

Tatsuhiko Ikeda and David A. Sirbasku§

From the Faculty of Nutrition, Kobe-Gakuen University, Iga-awadani-Choo Ariae, Nishi-ku, Kobe, Japan 673 and the Department of Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, Texas 77225

A mammary-uterine-pituitary tumor cell growth factor has been purified from lyophilized powders of pregnant sheep uteri by a five-step procedure. Uterine-derived growth factor (UDGF) was extracted from the powders with 0.1 M acetic acid, heated at 95 °C, and further purified by sulfopropyl-Sephadex C-25, Sephadex G-50, and carboxymethyl-Sephadex C-25 chromatography. From 500 g of uterine powder, 40 to 50 mg of UDGF can be isolated at an overall yield of 33%. The degree of homogeneity of the final preparations was estimated by 8 M urea, 0.1% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), and by PAGE under nondissociating conditions at either pH 8.5 or 4.5. In all PAGE experiments, the purified UDGF preparation showed a single Coomassie blue-stained band that directly corresponded to the only area of elution of UDGF activity from duplicate unstained gels. Molecular sieve high performance liquid chromatography HPLC, reverse phase HPLC on an octylsilyl (C8) column, and hydrophobic chromatography on octyl-Sepharose CL-4B all confirm a similar degree (i.e. >90%) of homogeneity. The Mr of UDGF estimated by urea/sodium dodecyl sulfate-PAGE was 4200 ± 500 and, by molecular sieve HPLC, 6200 ± 1000. The isoelectric point of UDGF was estimated as pI = 7.3. The UDGF isolated should be a homogenous factor that has useful cell-type specificity for established cell lines that were derived from estrogen-responsive tumors; purified sheep UDGF was mitogenic for MTW9/PL rat mammary tumor cells (at 10^-10 to 10^-9 M concentrations) while showing no mitogenic activity toward normal rat diploid fibroblasts. UDGF also promoted growth of uterine-derived tumor cells and the GH3/C14 rat pituitary line. Measuring growth as an increase in cell number, UDGF supported the logarithmic growth of the MTW9/PL rat mammary tumor cells over 6 days; other known hormones and growth factors were not able to substitute for the UDGF mitogenic action on MTW9/PL cells. It is concluded that a rapid, high-yield method of purification of a new uterine-derived growth factor activity has been developed.

Polypeptide growth factors have been isolated from organs

*Supported by American Cancer Society Grant BC-255 and by National Cancer Institute Grant RO1-CA-25617. A preliminary report of this work was presented at the Annual American Society of Biological Chemists Meeting, San Francisco, CA, June, 1983 (71). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§Recipient of American Cancer Society Faculty Research Award, FRA-212. To whom reprint requests should be addressed.

1 The abbreviations used are: EGF, epidermal growth factor; MSA, multiplication stimulatory activity; IGF I and IGF II, insulin-like growth factors I and II, respectively; SmC, somatomedins C; PDGF, platelet-derived growth factor; TGF, transforming growth factor; SGF, sarcoma growth factor; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; estradiol, extr-1,3,5(10)-triene-3,17-diido; UDGF, uterine-derived growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TEMED, N,N,N',N''-tetramethylethylendiamine; CM, carboxymethyl; SP, sulfopropyl.
mary cells cultured in collagen gels (36). Nevertheless, the role of EGF in mammary growth in vivo is not clear. A question remains whether plasma levels of EGF correlate with mammary growth in vivo, since EGF activity is androgen related (37) in submaxillary glands of mice, and androgen treatment has no measurable effect on mouse plasma EGF levels while estrogen treatment causes a depression of plasma levels. However, another investigator (38) has reported that plasma EGF is elevated during pregnancy, a condition accompanied by high plasma levels of estrogens and progesterins and rapid mammary growth. Since data from in vivo experiments were contradictory, the effect of EGF on mouse mammary gland development in vitro has been studied (39); results demonstrate a potential role of EGF in mammary gland development. Also, EGF has been reported to be the main mitogenic activity found in human milk, again implying a role of this growth factor in pregnancy-related development and/or fetal development (40). In another study, an acid-stable mammary-stimulating factor different from EGF was partially purified from porcine serum (41), but the physiological significance of the activity remains to be defined.

Since we are interested in identifying polypeptide growth factors that may be specifically involved in estrogen responsive mammary, pituitary, and uterine tumor cell growth in vivo, and since we have reported evidence suggesting that newly identified estrogen-inducible growth factors from uterine (42,43), kidney (42), and pituitary (44) may be involved in mammary tumor growth in vivo, we describe here the continuation of our studies aimed at purifying to homogeneity a mammary-uterine-pituitary tumor growth factor from lyophilized powders of early pregnant sheep uteri. The purification of this new activity was assayed by measuring the tissue extract effects on growth of the MTW9/PL rat mammary tumor cell line in serum-free culture medium (45).

The methods applied here to the purification of this new activity arose from our earlier attempts (46) to isolate a UDGF from a larger scale source than rat tissue. By extraction and chromatography methods conducted at approximately neutral pH using acetone powders of pregnant sheep uteri, UDGF could be purified partially (i.e. 1,700-fold) by a 6-step procedure to yield a high specific activity (25,000-Da) product that was still only an estimated 3% pure; the overall yield from this partial purification was less than 1% which was not sufficient to allow continued purification to homogeneity or to provide sufficient material for extensive biochemical studies. At this point, either a source of growth factor richer than pregnant sheep uteri was necessary for the large scale purification, or a substantially modified procedure was required to increase yields. Using new acetic acid-extraction and heat-treatment methods followed by three chromatography steps, milligram amounts of activity have now been isolated from pregnant sheep uteri. An apparent $M_c = 4,200$ and $2,600$ was estimated by SDS-PAGE and HPLC methods, respectively; by several criteria the UDGF isolated appears to be a highly homogeneous preparation that demonstrates significant growth responses in the $10^{-10}$ to $10^{-9}$ M concentration range. A description of the methods of purification and the properties of sheep UDGF are presented next.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—The MTW9/PL rat mammary tumor cell line used in this study was established in culture from the estrogen- and prolactin-responsive MTWA tumor (47) by methods described previously (48). MTW9/PL cells are estrogen (49) and pituitary factor (44) responsive, but not estrogen responsive (50). The MTW9/PL cells were assayed periodically for the ability to form estrogen-responsive tumors in vivo as described previously (48); after more than 6 years in culture, the cells still demonstrate the same hormone-responsive tumor formation properties described in the original report (48).

Stock cultures of MTW9/PL cells were maintained in the formulation of DME medium prepared at the higher (4.5 g/liter) concentration of glucose and supplemented with 2 mm glutamine, 240 μg/ml of potassium penicillin G, 540 μg/ml of streptomycin sulfate, 50 μg/ml of amphotericin B, and 2% (v/v) in DME of 5% fetal calf serum. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air and passed every 3-4 days at a density of 6.0 × 10⁶ cells/78-cm² plastic tissue culture dish (Corning). The DME was purchased from Grand Island Biological and serum from K. C. Biologicals. All sera were used without heat inactivation.

**Growth Factor Assay Method**—The growth factor specific activity in either 0.1 M acetic acid extracts of fresh uteri or during purification from lyophilized powders of pregnant sheep uteri was determined by a bioassay procedure which measures the growth response of MTW9/PL cells in serum-free DME. During purification, Method 1 was used either with and followed by incorporation of tritium-labeled thymidine into DNA in response to varying concentrations of protein. Stock cultures used to initiate the assays were always used on day 3 after passage (counting day 0 as time of subculture); this procedure was essential since log phase cells were necessary for the assays of mitogenic activity. Stock cultures (40 ml) were grown for 24 h in serum-free medium in the wells, no UDGF growth factor activity is identified even with the most active preparations.

After 24 h in serum-containing medium the DME was removed from the cultures by gentle aspiration and replaced immediately (one well at a time) with 0.9 ml of serum-free DME. The plates were then incubated in a CO2 incubator at 37 °C for exactly 24 h, after which 0.1 ml of UDGF preparation was added. Growth factor dilutions were always made in serum-free DME. Incorporation of tritium-labeled thymidine was conducted at 2-3 h pulse-labeling duration (22-24 h) after addition of UDGF. The labeled precursor (methyl-³H) thymidine, specific activity, 70 Ci/mmol, purchased from Schwarz/Mann was added in 0.050-ml portions containing 1.0 μCi. Labeling was conducted at 37 °C in the humid CO2 incubator. Thymidine incorporation was terminated by addition of 1.0 ml of Carnoy's fixative composed of three parts methanol and one part glacial acetic acid. Addition of this solution fixed the cells to the bottom of the culture wells and, by allowing the plates to stand for 3-5 h, the soluble (non-DNA) pools of thymidine precursors were washed out. This fixation solution was then removed by aspiration, and each well washed with 2.0-ml portions of 80% methanol (250 ml) to remove all residual labeled thymidine. The methanol rinse was carried out with 24 wells per wash procedure. Then 0.3 ml of trypsin (1-300 hog pancreas preparation purchased from Nutritional Biochemicals) prepared at a concentration of 20 mg/ml in 50 mM HEPES (pH 7.5) was added to each well. After at least 2 h at room temperature, 0.7 ml of 1% SDS prepared in distilled water was added to each well, and the total 1.0 ml from each well transferred to scintillation vials. The radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer after addition of 10 ml/vial of Liquescent (Na-
RESULTS

Identification of Pregnant Sheep Uteri as a Large Scale Source of UDGF

Before initiating a large scale growth factor purification from species other than rat, the new source was evaluated from hormone responsive ewe uteri. A large scale purification of UDGF activity was performed in Fig. 1. 0.1 M acetic acid extracts of fresh uteri from pregnant sheep showed a significantly elevated specific activity compared to those extracted from fresh uteri of ovarioctomized ewes, or from fresh uteri from intact ewes that were used as described above. The final pH gradient of the experiments was estimated by slicing an unstained gel into 1-mm discs, eluting each for 12 h into 0.5 ml of degassed distilled water, and determining the pH of the resulting solution. The isoelectric point of the UDGF activity was estimated from unstained gels, analyzed by slicing each for 12 h into 0.5 ml of DME, and 0.5 ml of UDGF or other preparation diluted with serum-free DME. Either on designated days, or on day 6 only, replicate plates were harvested by trypsin treatment and total cell numbers determined with a Coulter Counter (Model ZB). The purity of the UDGF activity applied could be recovered by these methods.

The HPLC method was performed with a Waters apparatus equipped with a dual wavelength detector which allowed monitoring from 280 and 210 nm. Analysis by molecular weight sieving was accomplished by eluting the activity from unstained gels. The frozen gels were performed at 95 nm as above. The final pH gradient of the experiments was estimated by slicing an unstained gel into 1-mm discs, eluting each for 12 h into 0.5 ml of degassed distilled water, and determining the pH of the resulting solution. The isoelectric point of the UDGF activity was estimated from unstained gels, analyzed by slicing each for 12 h into 0.5 ml of DME, and 0.5 ml of UDGF or other preparation diluted with serum-free DME. Either on designated days, or on day 6 only, replicate plates were harvested by trypsin treatment and total cell numbers determined with a Coulter Counter (Model ZB). The purity of the UDGF activity applied could be recovered by these methods.
yield values of \( G_{oo} = 100 \mu g/ml \) or more.

To better define whether stage of pregnancy has a marked effect upon UDGF activity, uteri pooled from 10 or more animals at either early pregnancy (i.e. <49 days), mid-pregnancy (i.e. 50 to 110 days) or late pregnancy (i.e. >110 days) were lyophilized and extracted with 0.1 M acetic acid. These extracts showed \( G_{oo} \) values ranging from 0.75 \( \mu g/ml \) for early pregnant uteri to 1.3 \( \mu g/ml \) for mid-pregnant uteri (Fig. 2). While these differences may be significant, it was clear that the UDGF activity did not vary greatly during pregnancy and that the process of lyophilization caused the extracted activities to vary less than found with fresh tissue. Further, the extraction of lyophilized powders with 0.1 M acetic acid yielded a 2-4-fold higher specific activity than extraction from fresh uteri by the same methods, suggesting that purification started from lyophilized powders may be more effective than from fresh tissue. Finally, the total amount of extractable growth factor activity was assayed from these same three groups of lyophilized uteri (Fig. 3). Clearly, the amount of total protein extracted, and hence, the total activity extracted was 2-fold greater from the uteri pooled from early pregnant sheep than from uterus of animals at later stages of pregnancy. For this reason, purifications were planned using only lyophilized powders of early pregnant sheep uteri.

**Purification of UDGF from Early Pregnant Sheep Uteri**

Unless otherwise noted, the purification of UDGF was carried out at 4°C. The basic five-step procedure is summarized as follows.

**Step 1**—A total of 500 g of lyophilized sheep uterine powder...
was stirred for 24 h with 5.0 liters of 0.1 M acetic acid. The suspension was then clarified by centrifugation at 25,000 × g for 60 min and the supernatant filtered through glass wool to remove lipids that floated to the surface during centrifugation. This crude supernatant was stored at −20 °C. After thawing, a large precipitate was removed by an additional centrifugation at 25,000 × g for 30 min, and the clear brown supernatant adjusted to pH 4.5 by dropwise addition of glacial acetic acid to a total volume of 4.210 ml.

Step 2—Aliquot (200 ml) of extract obtained in Step 1 were heated (in a boiling water bath) to 94 °C for 10 min, and then rapidly cooled to 0 °C in a propanol/dry ice bath. The large inactive precipitate was removed by centrifugation at 13,000 × g for 30 min, and the active supernatant frozen at −20 °C. After thawing, the supernatant was acidified to pH 3.5 with glacial acetic acid and the inactive insoluble material removed by centrifugation as described above.

Step 3—A total of 55 g of dry sulfopropyl-Sephadex C-25 (Pharmacia) was suspended in 2 liters of 0.1 M acetic acid and sufficient glacial acetic acid added to lower the pH to 3.1. This gel was then washed by decantation five times with 2 liters of 0.1 M acetic acid each time. The settled beads (260 ml) were then added to the acidified supernatant from Step 2, and the slurry was stirred slowly overnight. The sulfopropyl Sephadex beads were then allowed to settle for 2-5 h and the inactive supernatant poured off and discarded. The gel was then transferred to a 5.2-cm diameter glass column and washed successively, first with 1.2 liters of 0.1 M acetic acid, then with 500 ml of 0.001 M acetic acid. These washings contained little or no UDGF activity and were discarded. The growth factor activity eluted from the sulfopropyl-Sephadex with 0.3 M ammonium acetate, pH 7.2, at a flow rate of 17 ml/h. The active fractions (21 ml/tube) were collected, pooled and lyophilized; the dried residue was then dissolved in 50 ml of 0.1 M acetic acid and any precipitate removed by centrifugation.

Step 4—The 50 ml of active UDGF from sulfopropyl-Sephadex was then applied to a Sephadex G-50 column (5.2 × 135 cm) equilibrated in 0.1 M acetic acid. The column was eluted with the same acetic acid. Fractions (21 ml/tube) were collected at a flow rate of 126 ml/h. As can be seen in Fig. 4, the UDGF eluted as a single active peak which was pooled and lyophilized. The dried residue of UDGF from the Sephadex G-50 column was then dissolved in 20 ml of 10 mM sodium phosphate, pH 6.0, and any precipitate removed by centrifugation.

Step 5—The concentrated fractions from Step 4 were desalted by gel filtration on a Sephadex G-25 fine grade column (2.5 × 120 cm), equilibrated, and eluted with 10 mM sodium phosphate, pH 6.0. The active desalted fractions were then applied to a CM-Sephadex C-25 column (2.5 × 31 cm) equilibrated in the same buffer. After washing with 160 ml of the same pH 6.0 buffer, the majority (i.e. 70%) of the growth factor activity was eluted with a linear salt concentration gradient (400-ml total volume) formed by using equal amounts of 10 mM sodium phosphate, pH 6.0, and the same buffer containing 0.3 M sodium chloride. The flow rate was 25 ml/h and 2.0-ml fractions were collected. The active fractions (see Fig. 5, fractions 86–115) were pooled and lyophilized. The dry powder was dissolved in 0.1 M acetic acid, and the solution desalted by gel filtration on a Sephadex G-25 (superfine) column (1.0 × 50 cm), equilibrated, and eluted with 0.1 M acetic acid. The purified growth factor was stored at 4 °C in 0.1 M acetic acid, and was stable for at least 3 months under these conditions.

Table I summarizes the results of the purification procedure, and shows that from 500 g of lyophilized sheep uteri the yield of the five-step purification procedure was 33%, and the final amount of UDGF isolated was 42 mg of protein which showed a specific activity of G50 = 8 ng/ml. Based on the specific activity of the UDGF in the crude acetic acid extract, the overall purification was 162-fold, although if the purification is calculated from our previously reported (46) specific activity of UDGF in crude phosphate-buffered saline extracts of fresh sheep uteri (G50 = 450 μg/ml), the overall purification achieved here is greater than 56,000-fold. Assays of aliquots...
Growth Factors for Estrogen-responsive Tumor Cells

TABLE I

| Step                  | Volume/ml | Protein/ug/ml | Total protein/ng/ml | G<sub>0</sub> | Total activity/units | Purification fold | Yield % |
|-----------------------|-----------|---------------|---------------------|-------------|----------------------|------------------|---------|
| Crude extract         | 4,210     | 4.9           | 20,929              | 1,300       | 15.9 x 10<sup>6</sup> | 3.5              | 82      |
| Heat treatment        | 4,010     | 1.2           | 4,812               | 370         | 13.0 x 10<sup>6</sup> | (246)<sup>a</sup> | 100     |
| SP-Sephadex C-25      | 50        | 19.4          | 976                 | 82          | 11.8 x 10<sup>6</sup> | 16               | 74      |
| Sephadex G-50         | 20        | 10.6          | 212                 | 21          | 10.1 x 10<sup>6</sup> | 62               | 64      |
| CM-Sephadex C-25      | 12        | 3.5           | 42                  | 8           | 5.3 x 10<sup>6</sup>  | 162              | 33      |

<sup>a</sup> One unit of activity in that amount of protein yields G<sub>0</sub> by the tritium-labeled thymidine incorporation into DNA assay described under "Experimental Procedures."

<sup>b</sup> 500 g of lyophilized sheep uteri extracted with 5 liters of 0.1 M acetic acid as described in the text.

<sup>c</sup> Numbers in parentheses indicate the calculated purification based on the phosphate-buffered saline (pH 7.2) extraction of mid-pregnant sheep uteri as described in Iio and Sirbasku (46). The starting G<sub>0</sub> of these phosphate-buffered saline extracts was 450 μg/ml.

![Image of Table I](image1)

![Image of Table II](image2)

TABLE II

Effect of trypsin on UDGF activity

| Samples                          | Activity cpm/well<sup>d</sup> |
|----------------------------------|--------------------------------|
| Control (DME without UDGF)       | 1,135 ± 65                     |
| UDGF (DME plus a fixed concentration of 69 ng/ml) | 79,965 ± 3,156                 |
| UDGF plus trypsin<sup>b</sup>    | 3,216 ± 179                    |
| UDGF plus boiled trypsin<sup>c</sup> | 81,129 ± 2,714                 |

<sup>a</sup> Counts/min indicate incorporation of tritium-labeled thymidine into DNA.

<sup>b</sup> 750 μg of UDGF incubated with 37.5 μg of trypsin for 3 hr at 37°C in 1.0 ml of 20 mM sodium phosphate buffer, pH 7.0, followed by addition of 0.1 ml of 1.0 M acetic acid and heating at 100°C for 5 min to stop the action of trypsin. The sample was then diluted 10,000-fold into the assay by Method 1 (final assay concentration of UDGF was 68 ng/ml).

<sup>c</sup> The trypsin was boiled at 100°C for 5 min before addition to the digestion as described in Footnote b.

37°C by the cells in culture. Also, the assays reported here are conducted in completely serum-free medium; this method imposed certain limitations on the interpretation of G<sub>0</sub> of UDGF (see Footnote 5 for further amplification of this point).

<sup>d</sup> It must be noted that when evaluated by the methods used in this report, the effective molar concentration of UDGF required to half replace the mitogenic response of MTW9/PL cells to 10 (v/v) FCS was in the 10<sup>-6</sup> M range. These assays were conducted in the absence of serum supplementation, and, in the absence of all other known hormones, attachment factors and growth factors already described as requirements for mammary cell growth in serum-free medium (34, 35). Our reason for using an assay for UDGF based on cell response to addition of a single factor was to ensure the isolation of a true mitogen, rather than an agent which facilitates or augments the action of other known growth factors. This assay method is different in this regard from the procedures used for measuring the mitogenic responses of cells in cultures to PDGF (11-13), EGF (68), and TGF (15).

In one case (PDGF), the assays of the growth factor were conducted in the presence of 5% (v/v) platelet-poor plasma in the culture medium (12). This supplementation of plasma provided nutrients, attachment factors, and the other known growth factors such as EGF and SMC that potentiate or complete the action of this purified factor (69, 70), and allowed measurement of half-maximal responses (G<sub>50</sub>) to PDGF in the 10<sup>-11</sup> M range. Without plasma supplementation, considerably higher concentrations of PDGF were required to achieve G<sub>50</sub> responses. In the case of EGF, low level supplementation (i.e. 1% v/v calf serum) was required for maximal target cell responses (68). Under these conditions, EGF half-maximal biological activity fell into the 10<sup>-12</sup> M range. However, as was evident from the data presented in Fig. 6 (Miniprint), when target cell responses to purified EGF were measured in the absence of the serum supplementation, G<sub>50</sub> was not achieved at concentrations of >1 x 10<sup>-8</sup> M.

* D. Danielpour and D. A. Sirbasku, unpublished data.
However, it is apparent from these data that the final preparations were able to stimulate MTW9/PL rat mammary tumor cell growth well beyond the level of 10% FCS.

The final product from Step 5 was assayed for the effect of trypsin on the activity. As shown in Table I, trypsin treatment caused a 96% loss in activity of UDGF within 3 h at 37 °C. Incubation with the equivalent amount of growth factor with boiled trypsin had no effect on activity.

**Evaluation of Homogeneity of the UDGF Preparation after Various Isolation Steps**

The purification of UDGF as summarized in Table I was monitored by 8 M urea, 0.1% SDS, 12.5% PAGE. Aliquots of each fractionation step were analyzed and the results with selected fractions are shown in Fig. 7. The acetic acid extract (Step 1) showed the presence of proteins of $M_r > 10,000$, and only very minor protein bands between $M_r = 6214$ and 2512. Analysis of the supernatant from the heated acetic acid extraction showed many high $M_r$ proteins, and the first appearance of a band between $M_r = 6214$ and 2512. After each step, the intensity of the higher $M_r$ protein bands diminished, and the band corresponding to purified UDGF increased in intensity. As shown in Fig. 7, (Step 5) UDGF preparation demonstrated a single stained band when 20 μg of protein was applied to the gel. In data not presented, we have applied up to 150 μg of protein per gel and still find only a single Coomassie blue-stained band. From the data presented in Fig. 7, and from densitometer scans conducted at 595 nm (data not shown)> 95% of the Coomassie blue intensity resides in the single stained band.

An estimate of the molecular weight of the stained band by a plot of log10 of the molecular weight of the myoglobin and myoglobin fragments versus per cent relative mobility (RM %), showed an apparent $M_r = 4,200 \pm 500$ for UDGF (Fig. 8). The range of possible $M_r$ originates from the width of the band, which may be related to the properties of the urea-SDS PAGE system used to analyze this sample, since the myoglobin fragments of exact known molecular weights also exhibit relatively broad bands under these same conditions. Also, it is possible that since these proteins are of low $M_r$ they may undergo diffusion during the fixing and staining process. Evidence for this possibility is that gels left unfixed for a length of time after electrophoresis show diffuse bands after Coomassie staining (data not shown).

**Fig. 7.** 8 M urea, 0.1% SDS, 12.5% PAGE Coomassie blue-staining analysis of the UDGF preparation after selected steps of the purification. Each gel received 20 μg of protein. The far left gel received protein from the acetic acid extract (Step 1). The next (going from left to right) received heated supernatant (Step 2), SP-Sephadex eluted activity (Step 3), and the UDGF activity eluted from the 8 M Sephadex (Step 5). The far right lane shows the migration position of the molecular weight markers of myoglobin and fragments of myoglobin as described under "Experimental Procedures."

**Fig. 8.** Estimation of UDGF $M_r$ by 8 M urea, 0.1% SDS, 12.5% PAGE. From the data presented in Fig. 7, the relative mobilities of the myoglobin $M_r$ markers and the UDGF were calculated from the migration position of bromphenol blue.
the UDGF activity. Parallel urea-SDS gels were run as described under "Experimental Procedures"; one set of gels was stained with Coomassie blue to localize the proteins, while the other set was immediately frozen at −20 °C, sliced into discs and the eluant of each disc assayed for UDGF activity.

The results of this experiment are shown in Fig. 9. The migration position of the Coomassie blue-stained component exactly corresponded to the migration position of the recoverable UDGF activity. It must be noted that while the recovery of UDGF activity from urea-SDS gels was only partial, the heating at 100 °C in 8 M urea and 1% SDS caused an initial 60% inactivation before electrophoresis. Hence, the fraction of activity found after the elution from acrylamide discs represents a 20–25% recovery of the residual activity applied.

Attempts were made to increase the yields of the activity eluted by using several different methods, including removal of residual SDS bound to eluted UDGF by a −18 °C ethanol/albumin coprecipitation. None of the other methods employed proved more effective than that used to obtain the data in Fig. 9. If boiling of UDGF in 1% SDS was omitted before electrophoresis, two minor Coomassie-stained bands (each approximately 10% of the total stain intensity) appear at M, = 12,000 and 16,000, suggesting aggregation of the UDGF prior to or during electrophoresis at pH 6.8 in phosphate buffers. This aggregation of UDGF at near neutral pH in sodium phosphate buffers is thought to be responsible for the fraction of 30% of the UDGF activity that elutes through the CM-Sephadex column during Step 5 of the purification (Fig. 5). Analysis of the CM-Sephadex flow-through fractions by urea-SDS gel electrophoresis shows that, after boiling in SDS, the protein present in this fraction migrates on urea-SDS gels as a single band equivalent to that of the UDGF eluted by the sodium chloride gradient. These data suggest that the CM-Sephadex flow-through may be an aggregated form of UDGF.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.

The UDGF preparations from Step 5 were submitted to non-SDS-PAGE at both basic and acidic pH. Analysis carried out at pH 8.5 with gels containing 7.5, 10, 12, and 15% acrylamide was applied to the gels as a single band equivalent to that of the UDGF eluted by the Coomassie blue staining. Migration direction is down (cathode). BPP, bromphenol blue.
only protein band identified by Coomassic blue staining exactly corresponded to the UDGF activity eluted from companion unstained 12.5% acrylamide gels prepared and run at pH 4.5 (data not shown).

Evaluation of the State of Homogeneity of Purified UDGF by Hydrophobic Chromatography and HPLC

The homogeneity of the UDGF Step 5 preparation was assessed by hydrophobic chromatography and by HPLC methods.

Hydrophobic chromatography on octyl-Sepharose CL-4B (Pharmacia) is shown in Fig. 12. Before chromatography, the UDGF was equilibrated with the same 6.0 M ammonium acetate in 10 mM sodium phosphate buffer, pH 6.8, used to wash and equilibrate the column. After initiating chromatography, a total of 90% of the applied protein could be accounted for in the 6.0 M ammonium acetate wash with the remaining 10% found in the reverse linear gradient fractions running 6.0-0 M ammonium acetate in 10 mM phosphate buffer (pH 6.8) (fractions 16 through 32), and in the final 50% (v/v) ethylene glycol wash. The UDGF activity (85%) eluted in the major protein fraction of the ammonium acetate wash, and no other activity could be identified in any other region of the elution.

In parallel experiments, to confirm that the UDGF was not highly hydrophobic and to further confirm homogeneity, UDGF analysis was conducted using HPLC reverse phase chromatography on octylsilyl (C8) columns washed first with 1-propanol (immobile phase), and later equilibrated with a mobile phase of 0.1% trifluoroacetic acid (pH 2.2) just before applying the UDGF. The UDGF sample was equilibrated in the same concentration of trifluoroacetic acid by passage through Sephadex G-25 fine grade, equilibrated, and eluted with 0.1% trifluoroacetic acid. From the HPLC C8 column elution times found, UDGF eluted at the same time as a marker (i.e., molecular iodine) that did not interact with this hydrophobic matrix. Approximately 90% of the UDGF activity and 85% of the total protein (i.e., 210 nm absorbing material) applied appeared in coincident fractions. No activity or protein could be further eluted by a gradient of 0-20% (v/v) 1-propanol in 0.1% trifluoroacetic acid (data not shown).

In another HPLC approach to evaluating homogeneity, a sample of UDGF Step 5 was equilibrated with 0.1% trifluoroacetic acid and applied to an in-line series of two HPLC molecular sieve TSK-125 columns equilibrated in the same acid. The activity and protein elution profile are shown in Fig. 13. In each fraction with significant absorption at 210 nm parallel UDGF activity was found. When these tandem columns were calibrated with components of known Mr, an estimated UDGF Mr = 6200 ± 1800 was obtained (data not shown).

Determination of Isoelectric Point of UDGF

The isoelectric point of UDGF from Step 5 was determined in glass tube gels as described under "Experimental Procedures." A typical Coomassie blue-stained gel is shown in Fig. 14, in which the sample was applied at the pH 3.0 electrode. The stained gel shows a broad major peak focusing near the midpoint of the gel, and another minor peak focusing at an apparently slightly more basic position. Densitometry scanning (Fig. 15) shows clearly the presence of the minor component, although from these data it is not possible to calculate the exact percentage of the total stained material this band represents. Nevertheless, the data show that the sample of UDGF Step 5 is highly homogeneous. In a parallel experiment, an unfixed and unstained gel was sliced into 1-mm thick discs, and the UDGF activity eluted and assayed. As can be seen in Fig. 16, the UDGF activity elutes over the whole region of both the major and minor band. The estimated pI of the major band is 7.3.
Fig. 13. Molecular sieve HPLC of the UDGF, Step 5, preparation. A total of 12 μg of UDGF was equilibrated with 0.1% trifluoroacetic acid by passage through Sephadex G-25 superfine. This material was then applied directly to two tandem TSK-125 columns equilibrated and eluted with the same acid. Fractions (0.7 ml) were collected at a flow rate of 0.7 ml/min. The times of elution of the following \( M_r \) markers are shown: A, immunoglobulin G, \( M_r = 150,000; \) B, ovalbumin, \( M_r = 45,000; \) C, horse heart myoglobin, \( M_r = 16,949; \) D, cyanocobalamin \( M_r = 1350. \) All of the fractions collected were assayed by dilution of 0.1 ml of each with 0.9 ml of serum-free DME. A total of 100 μl of this dilution was used per cluster well in assay Method 1. This experiment was repeated with UDGF amounts applied of 120 and 360 μg with identical results.

**DISCUSSION**

Both the results presented in the main text and in the Miniprint will be discussed here.

We have reported previously (42) an estrogen-inducible growth factor activity in extracts of rat uteri. When prepared at neutral pH, this activity was a relatively specific mitogen for tissue culture cell lines which were established from estrogen-responsive or -dependent mammary, pituitary, and kidney tumors from rats and hamsters; when correlations were made between this mitogenic activity and hormone-responsive tumor growth in vivo, it was confirmed that the level of total extractable mitogenic activity in rat uteri correlated well with the rate of mammary and pituitary tumor growth in rats (42). In attempting further characterization of this mitogen as a product of the uterus, a similar growth factor was identified in estrogen-induced accumulations of rat uterine luminal fluid (55, 56); this fluid is well known to contain uterine origin components secreted under estrogen stimulation (57, 58). Attempts to further characterize the rat uterine growth activity showed that when extracted with phosphate buffers at pH 7.2, the mammary cell growth factor obtained was a heat-labile, trypsin-labile substance that did not show properties related to either steroid hormones or other lipid-like components expected in uterine extracts (43). However, from the results available with other growth factors, it was apparent that tissue-derived or plasma-borne activities initially may be identified as high \( M_r \) factors (i.e., \( >70,000 \)) in neutral pH extracts, but later shown by treatment with acid, or other dissociating agents, to be relatively low \( M_r \) (6,000 to 10,000) species. Examples of growth factors known to demonstrate these different properties dependent upon pH of extraction are EGF (59, 60) and SmC (8). With these data in mind, the rat uterine-derived growth factor was submitted to acetic acid extraction; instead of \( M_r = 70,000, \) the activity chromatographed on Sephadex G-50 with an apparent \( M_r = 2,000-6,000. \) Further, when extracted directly into 0.1 M acetic...
The isolation of a high specific activity mammary-uterine-pituitary tumor cell growth factor from lyophilized sheep uteri has been accomplished using a high yield five-step procedure, the results of which are summarized in Table I. The UDGF isolated will half replace the serum-stimulated growth of MTW9/PI cells at 8 ng/ml without addition of other steroid or polypeptide hormones, cell attachment factors, or other known growth factors. The concentration of mitogen required for this response is 1.29-1.90 x 10^{-8} M depending upon calculations based upon $M_t = 4200$ (urea-SDS-PAGE estimation) or $M_t = 6200$ (TSK-125 HPLC estimation). A significant biological response (i.e. p < 0.01 response over serum-free controls) can be measured at concentrations as low as 3 ng/ml, which is in the range of 4.8 to 7.1 x 10^{-10} M.

These data support the conclusion that a potent effector of mammary tumor cell growth in culture has been isolated. Furthermore, not only can a high specific activity UDGF be prepared by the methods described, but from each 500 g of lyophilized early pregnant sheep uteri, 40-50 mg can be obtained. This amount calculates to a tissue level of 60-75 mg of UDGF/kg of fresh (wet) uteri, which is an amount that exceeds the known abundance of other acid-stable growth factors such as PDGF (11), SmC (8), TGF (15, 16), and SGF (14). Of the acid-stable group of polypeptide mitogens isolated, only EGF is present in tissues (i.e. mouse submaxillary gland) in such abundance (1).

This preparation of UDGF has been evaluated for degree of homogeneity by several independent methods. During the course of these studies, the properties of the growth factor have been partially elucidated. These studies are summarized as follows: (i) At each of the last two steps of the purification, which are Sephadex G-50 chromatography (Fig. 4) and CM-Sephadex chromatography (Fig. 5), the growth factor activity coincides with a single, well defined protein (i.e. 286 nm absorbing) peak. (ii) By 8 M urea, 0.1% SDS, 12.5% PAGE, only one Coomassie blue-stained band was found even when as much as 150 μg of UDGF preparation was applied per gel; further, elution of individual discs of parallel unstained gels showed UDGF recovery only at the migration position of the single Coomassie-stained band. (iii) Using non-SDS, pH 8.5, PAGE to separate by both $M_t$ and net charge, again only a single Coomassie blue-stained band was identified, even at high concentrations (i.e. 150 μg of UDGF per gel), and parallel elutions of sliced discs of unstained gels showed UDGF activity corresponding only to the position of the migration of the stained band. (iv) Repeating the non-SDS-PAGE at pH 4.5 yielded the same results as those found at pH 8.5; the migration position of the UDGF activity and stained protein band were identical. However, the polarity of the electrophoresis at pH 4.5 was opposite that of the pH 8.5, indicating that the $p_I$ of UDGF was between these pH values. Also, despite the differences in the polarity of electrophoresis and in the distances migrated by UDGF at pH 4.5 and pH 8.5, no additional Coomassie-stained bands could be identified. (v) Molecular sieve HPLC (Fig. 13) of the UDGF preparation revealed only a single 210 nm absorbing peak containing >90% of the total protein and >85% of the total UDGF activity applied. However, the $M_t = 6200 ± 1000$ estimation by HPLC is significantly higher than that estimated by urea-SDS-PAGE. The reasons for these differences are not yet apparent, but the fact that UDGF heated in 8 M urea plus 1% SDS as only 40% of the initial activity suggests that the growth factor does not assume the expected rigid rod structure in SDS solutions, but instead, may retain some secondary structure. This retention of activity, and hence possible secondary structure, may cause the apparent $M_t$ to be estimated lower by SDS than by HPLC methods. This latter method separates on the basis of the Stokes radius of the undenatured UDGF and may more accurately reflect the $M_t$ of UDGF. In other work to be reported, no evidence for amino sugars has been found by chemical analysis, suggesting that UDGF is not a glycopeptide; if UDGF were a glycopeptide, this would cause discrepancies in $M_t$ estimation by the different methods used in this report. (vi) Using different properties of the UDGF than examined before, hydrophobic chromatography on octyl-Sepharose and reverse phase HPLC on octylsilane (C8) columns showed the activity to be a polar species that eluted in the void volumes of both chromatographic systems. In both cases, the protein elution profiles and the UDGF activities corresponded in the same fractions. (vii) By isoelectric focusing between pH 3.0 and 10.0 in acrylamide gels (Fig. 14), the UDGF preparation showed a major band focused at average $p_I$ = 7.3 while the minor band focused at what initially appeared to be a somewhat more basic pl (Fig. 15). However, when determinations of the pH of the focusing gradient were done, there appears a pH discontinuity at the basic side of the major pl 7.3 band (Fig. 15), and this discontinuity corresponds to the final focusing position of the minor band. Hence, the pl of the minor band is currently estimated as 7.1.

In view of the pH gradient discontinuity in the isoelectric focusing experiment, it may be possible that the minor band is artifically generated by this gradient abnormality. This possibility is supported by the fact that UDGF growth factor activity is found throughout the region of the focusing gel corresponding to both major and minor peaks (Fig. 16). Another possibility is that there are two forms of UDGF which are very closely related, but which cannot be distinguished by any method applied other than isoelectric focusing. If this is the case, it may be that one form of UDGF is a degradation product of another and that this occurs during the focusing experiment since the relative amounts of these two bands vary between different focusing experiments. It is equally

---

*T. Ikeda and D. A. Sirbasku, unpublished results.*
possible that the two forms of UDGF may be formed by partial proteolytic degradation which occurs during the time required for conversion of the 2-10-kg amounts of fresh uteri to lyophilized powders, or partial proteolytic degradation occurring during the isolation process. At present, these possibilities cannot be distinguished, but from the sum of the data available by all methods applied, we conclude that a highly (i.e. >90%) homogeneous preparation of UDGF has been achieved.

In an effort to establish whether the UDGF purified from sheep uteri possesses the same cell type specificity as the activity identified in crude extracts of rat uteri, experiments were carried out which are presented in the Miniprint. The $G_{50}$ of sheep UDGF was determined for key cell lines that form estrogen-responsive tumors in rodents. These cell lines are the GH3/C14 rat pituitary tumor cells that form estrogen-(61) and thyroid hormone-(62) responsive tumors in Wistar Furth rats, the H-301 hamster kidney tumor cells that form estrogen-dependent tumors in Syrian hamsters (63), ULMS smooth muscle tumor line developed from an estrogen- and androgen-induced leiomyosarcoma of hamster uterus (49), and the UCS endometrial tumor cells developed from an estrogen-induced hamster uterine carcinosarcoma (49). When assayed by standard methods, UDGF is a potent mitogen with the five cell lines in decreasing order of responsiveness of MTW9/PL mammary cells > uterine UCS cells > kidney tumor cells > uterine ULMS smooth muscle cells > pituitary tumor cells. The $G_{50}$ of this series of cell tests range from 8-10 ng/ml (MTW9/PL cells) to 1400 ng/ml (GH3/C14 pituitary cells). No mitogenic activity was found with normal diploid rat fibroblasts even at concentrations of UDGF > 50 µg/ml (Fig. 5, Miniprint).

One very interesting new fact brought to light by these studies is that UDGF is a relatively potent mitogen for uterine tumor cells of endometrial origin ($G_{50}$ = 36 ng/ml), and to a lesser degree, for uterine smooth muscle tumor cells ($G_{50}$ = 176 ng/ml) (see Fig. 4, Miniprint). These observations raise the possibility that the activity isolated from uterus may be an endogenous autostimulatory growth factor (autocrine type activity) that is involved in estrogen-responsive or pregnancy-related uterine growth or, alternatively, in uterine tumorigenesis. At present, the role in normal uterine growth is being further explored, and preliminary results confirm that short term monolayer cultures of normotum rat uterine cells respond to UDGF at concentrations of <50 ng/ml in serum-free medium.

In addition to the cell type specificity assays, one other control study was essential. We have purified and characterized UDGF by following its activity as a function of the ability to stimulate incorporation of labeled precursor into DNA. This measure of cell growth is used routinely to purify and otherwise measure mitogenic activities, although it is recognized widely that this measure may not correspond to cell growth as measured by logarithmic increase in cell number. Hence, the experiments (see Fig. 3, Miniprint) confirm that UDGF is a true growth factor promoting a logarithmic rate of cell growth in culture. From the data obtained, it is apparent that UDGF promotes a continuous rate of growth of MTW9/PL cells in culture and that the UDGF must be consumed or otherwise degraded in culture relatively rapidly since MTW9/PL cell growth ceases at 48 h after a single addition of growth factor; if additional factor is not provided, the cells decrease in number sharply. These data confirm that not only is UDGF a promoting growth factor, but that it is required for continued survival of the MTW9/PL cells in culture. One additional important feature about the mitogenic activity of UDGF when measured by cell number increase is that a basal (nongrowth supporting) concentration of phosphate-buffered saline (pH 7.2) extract of lyophilized sheep uteri is required for the growth response. This may indicate that either additional factors not yet recognized are present in crude uterine extracts, or that this extract provides essential nutrients and other components required for cell growth.

After isolation and determination of the cell type specificity of UDGF, the next major consideration was whether UDGF isolated from pregnant sheep uteri is a new growth factor activity not previously characterized, or instead, an isolation of a known growth factor from another source. Studies aimed at resolving this problem are presented in the Miniprint. A number of known hormones and purified growth factors were assayed for the ability to replace or supplement the mitogenic effect of UDGF with MTW9/PL mammary cells. From the data presented in Table I (Miniprint), none of the known steroid hormones, polypeptide hormones, or purified growth factors were mitogenic for MTW9/PL cells under conditions where UDGF showed a potent (i.e. $G_{50}$ = 10 ng/ml) cell growth effect. An example of how these data were obtained is shown in Fig. 11 (Miniprint).

Purified mouse EGF alone was assayed for mitogenic effect on MTW9/PL cells at concentrations of up to 20 µg/ml. No effect was found. Similar assays with other growth factors such as MSA, SmC, insulin, and bovine fibroblast growth factor showed no mitogenic effects. In another type of specificity assay, a cell line was selected that was EGF-responsive (i.e. the Balb/c 3T3 cells) and it was shown that UDGF was at least 150 times less potent a stimulator of cell growth than EGF with these cells, again confirming that the action of at least one of the well known growth factors (EGF) cannot be replaced by UDGF. The UDGF was also treated with the reducing agents DTT and 2-ME, and it was shown that the apparent $M_{r}$ of the activity did not change as estimated by the 8 M urea, 0.1% SDS, 12.5% PAGE (Fig. 9, Miniprint) and that the biological activity was retained completely (Fig. 10, Miniprint). These data further suggest that the activity isolated from sheep uter is not PDGF-like (11), EGF-like (64) or TGF-like (65, 66) since these factors lose activity after reduction.

From the data reported here, the UDGF isolated from pregnant sheep uteri appears to be a unique activity. Final confirmation of this conclusion is now being sought by determining the complete amino acid sequence using standard procedures (67). By determining the sequence, data should be obtained which will establish conclusively the molecular identity of UDGF.

Acknowledgments—We wish to thank Judy Roscoe for expert technical assistance and preparation of the photographs. We appreciate the efforts of Wilda Ward and Anne DeForest who typed and prepared the Miniprint and main manuscript section. Also, we acknowledge gratefully the assistance of Dr. Stephen LeGrue of the Division of Organ Transplant and Immunology and the Department of Biochemistry and Molecular Biology at The University of Texas Medical School at Houston who helped conduct the HPLC studies, and Dr. Darrell N. Ward of the Department of Biochemistry at the M. D. Anderson Hospital and Tumor Institute, Houston, TX, who aided in arranging the preparation of lyophilized powders of sheep tissues by colleagues in New Zealand.

REFERENCES
1. Savage, C. R., Jr., and Cohen, S. (1972) J. Biol. Chem. 247, 7699-7611
2. Gospodarowicz, D. (1975) J. Biol. Chem. 250, 2515-2520
3. Gospodarowicz, D., Lui, G.-M., Cheng, J. (1982) J. Biol. Chem. 257, 12266-12276
4. Pollack, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M., and Sporn, M. B. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5339-5343
Growth Factors for Estrogen-responsive Tumor Cells

INTRODUCTION

The use of growth factors has been described as a possible method to target and distinguish estrogen-responsive tumor cells for cancer therapy. The following sections will describe the results of previous studies, including those by the National Institutes of Health (NIH) and the University of California, Los Angeles (UCLA). This information is intended to provide a comprehensive overview of the current state of research in this area.

EXPERIMENTAL METHODS

Cell line culture methods

Several cell lines were used in this portion of the study. The cells were derived from human breast cancer (MCF-7) and from human lung cancer (A549). The MCF-7 cells were grown in culture dishes and treated with a growth factor for 48 hours before being harvested and assayed for cell proliferation.

Growth factor assays

The in vitro growth of cells was assessed using a MTT assay. The cells were treated with increasing concentrations of growth factors and the proliferation rate was measured using a colorimetric assay. The results were analyzed using a nonlinear regression model to determine the optimal concentration of growth factors for cell growth.

RESULTS

In vitro proliferation assays

The proliferation of MCF-7 cells in response to growth factors was assessed using a MTT assay. The results indicated that the growth factors had a significant effect on cell proliferation. The optimal concentration of growth factors for cell growth was determined to be 1 μM, with an IC50 of 0.1 μM.

In vivo proliferation assays

The proliferation of MCF-7 cells in response to growth factors was assessed using a MTT assay. The results indicated that the growth factors had a significant effect on cell proliferation. The optimal concentration of growth factors for cell growth was determined to be 1 μM, with an IC50 of 0.1 μM.

CONCLUSIONS

The results of this study suggest that growth factors can be used to target estrogen-responsive tumor cells for cancer therapy. Further research is needed to determine the optimal conditions for using growth factors in clinical settings.

Figures

Figure 1: Growth factor assay results of MCF-7 cells in response to different concentrations of growth factor. The results indicate that the optimal concentration of growth factor for cell proliferation is 1 μM, with an IC50 of 0.1 μM.

Figure 2: Effect of growth factor concentration on cell proliferation. The results suggest that increasing the concentration of growth factor beyond 1 μM does not result in increased cell proliferation.

Figure 3: Effect of growth factor concentration on cell viability. The results indicate that increasing the concentration of growth factor beyond 1 μM leads to decreased cell viability.

References

[1] Brown, J. et al. (2018). The role of growth factors in cancer therapy. Journal of Clinical Oncology, 36(24), 2753-2764.

[2] Chen, W. et al. (2019). The effect of growth factors on cell proliferation. Journal of Cellular Physiology, 234(7), 10791-10798.

[3] Lee, S. et al. (2020). The role of growth factors in cancer metastasis. Cancer Research, 80(11), 2802-2810.
Growth Factors for Estrogen-responsive Tumor Cells

Figure 1. Demonstration of UDF as a promoter of MMTV-Py tumor growth as measured by BrDUlabeled cell number increase. The methods used were similar to those described in Fig. 2. Cell growth was measured daily by Method 4 in one culture. Affinity-purified, high-purity estrogen receptor (apoER) was added to the cultures which had already received UDF and the neutral extract. These cultures continued to grow (solid circles). Solid circles show the cell number when additions of fresh UDF were not made on days 2 and 4. Growth in 1% FCS is shown by closed triangles. Cell number standard deviation was ± 1% of mean.

When later additions of UDF were made on days 2 and 4, the cells showed continued growth; if these later additions were not made on days 2 and 4, the growth of the MMTV-Py cells stopped and cell number per plate showed marked decreases.

Cell Type Specificity of UDF in Explant Propagation

Our previous results using crude estrogen-suppressed sera, PK 7,2, extracts of rat uteri (20,21) showed that the proteins extracted by this method promoted the growth of various cell lines in the relative order of effectiveness as ovarian tumor cells > kidney tumor cells > Pituicytary tumor cells > fibroblasts. Interpretations of these results came from the obvious promise that neutral extracts could be used to screen for other putative growth factors present in crude extracts, each of which promotes growth of a different cell type. Now, with purified sheep UDF, the cell type specificity with several of these cell lines was determined again using the assay of stimulation of tritium-labelled thymidine incorporation into DNA. As shown in Fig. 3, UDF promoted the growth of two different cell lines: the growth responses were 10.4 ± 0.9% for the 3T3 cells and 8.5 ± 0.5% for the 10T1/2 cells, respectively, suggesting that sheep UDF may be a more potent stimulus for the tumoral cell line than for the normal mouse cells of the uterus (the origin of the UDF cell line) for the smooth muscle cells of the uterus (the origin of the 3T3 cell line).

In another test of specificity, UDF in concentrations of up to 60 ng/ml did not promote significant incorporation of labelled thymidine into the DNA of normal rat fibroblasts in culture (Fig. 4). While UDF effects were tested with another presumed fibroblast line, 3T3 cells, UDF promoted significant incorporation into DNA. However, since the akin origin of the 3T3 cell line has not been established conclusively, the reasons for the anetocytic effects of purified UDF on these cells remains to be defined. Parallel tests with 3T3 cells were conducted with UDF and UDF by the assay method used in this study. UDF showed significant growth response at 0.1 to 1.0 ng/ml and maximum growth at 1.0 ng/ml, while UDF effects occurred at levels of 100-1000 fold higher concentrations. Hence, UDF and UDF were very different in response curves with mouse 3T3 cells.

In experiments with two other cell lines that form estrogen-responsive as dependent tumors, UDF promoted significant growth of the MMTV-Py rat pulmonary tumor cells at 5.0 ± 1.4 ng/ml (Fig. 5). Similar analyses showed that UDF promoted growth of the 9-114 mammary hormone tumor cells with a G(50) = 47 ng/ml (Fig. 6).

Figure 2. Effect of UDF on growth of normal rat adrenal fibroblasts. The normal rat fibroblasts were established in culture as described in Experimental Procedures (Materials). The assay was conducted by Method 3. The UDF used showed G(50) = 10 ng/ml with MMTV-Py cells.

Figure 3. Effect of UDF on growth of NIH-3T3 cells. The assay procedure used was in Method 4, with the exception that the C3H control represents the mean response ± 5% (n=5) cell culture. The UDF used showed G(50) = 10 ng/ml with MMTV-Py cells.

Figure 4. Growth of MMTV-Py mammary tumor, 3T4 sterile smooth muscle tumor and UC sterile subcutaneous tumor growth in response to UDF. The designated concentrations of UDF were added to six-well cultures of the three designated cell lines and growth responses monitored by following the incorporation of tritium-labelled thymidine into DNA as described in Method 1 in Experimental Procedures (Materials and Methods). The growth responses of the 3T4 and 3T4 cells to 24 hours of UDF (50 ng/ml) are shown. The G(50) for UDF effect on these cells is calculated from these data.

In a test of specificity, UDF at concentrations of up to 60 ng/ml did not promote significant incorporation of labelled thymidine into the DNA of normal rat fibroblasts in culture (Fig. 4). While UDF effects were tested with another presumed fibroblast line, 3T3 cells, UDF promoted significant incorporation into DNA (Fig. 6). However, since the akin origin of the 3T3 cell line has not been established conclusively, the reasons for the anetocytic effects of purified UDF on these cells remains to be defined. Parallel tests with 3T3 cells were conducted with UDF and UDF by the assay method used in this study. UDF showed significant growth response at 0.1 to 1.0 ng/ml and maximum growth at 1.0 ng/ml, while UDF effects occurred at levels of 100-1000 fold higher concentrations. Hence, UDF and UDF were very different in response curves with mouse 3T3 cells.

In experiments with two other cell lines that form estrogen-responsive as dependent tumors, UDF promoted significant growth of the MMTV-Py rat pulmonary tumor cells at 5.0 ± 1.4 ng/ml (Fig. 5). Similar analyses showed that UDF promoted growth of the 9-114 mammary hormone tumor cells with a G(50) = 47 ng/ml (Fig. 6).
Growth Factors for Estrogen-responsive Tumor Cells

Figure 9. PAGE analysis of UDGF both with and without reducing agent treatment. Duplicate samples of 0.72 mg UDGF were incubated in 1.0 ml of 1X Tris-HCl, pH 8.0, with either 1M 2-ME or 10 mM DTT for 10 to 18 min at 10°C. The samples were then compared for electrophoretic mobility of UDGF to samples incubated under identical conditions without the reducing agents. A total of 44 μg of UDGF (1 reducing agent) was applied to each gel. PAGE was conducted as described in Experimental Procedures (Main Text). The designated lanes show mobilities of the myoglobin Mr markers (see Fig. 1, Main Text).

Figure 10. Effect of reducing agents on the mitogenic activity of UDGF. Incubation of UDGF (10 μg/ml) with either 1M 2-ME or 10 mM DTT was performed in 1.0 ml of 1X Tris-HCl buffer, pH 8.0, for 10 to 18 min at room temperature. After the incubation, the samples were diluted into serum-free DME to yield the designated concentrations of UDGF. The assay was conducted by Method 1 described in Experimental Procedures (Main Text).

Figure 11. Effect of UDGF and EGF on MTW/PL cell growth. The EGF used in this study was receptor grade (Collaborative Research Corp.), although culture grade yielded the same results. The assays were performed as in Method 1 (Main Text).

When the other known growth factors such as BSA, MSA, PDGF, insulin, vasopressin, relaxin and plasminogen activator were assayed for activity, similar results were obtained. Table 1 summarizes the results of these experiments. In all experiments presented in Table I, no effect (none) indicates that C100 was not achieved even at the highest concentrations tested. In most cases a range of concentration of each agent was assayed. The results shown here, as well as those of our previous reports (44,50), confirm that UDGF mitogenic activity is unique and cannot be replaced by any of the other potential mitogens tested.

Table 1

| Hormone Tested       | Concentration Range | Assay Method | Effect on Growth |
|----------------------|---------------------|--------------|------------------|
| Estradiol            | 10^{-12} to 10^{-8} M | 1 & 2        | None             |
| Androgen             | 10^{-12} to 10^{-5} M | -            | -                |
| Insulin              | 10^{-8} to 10^{-5} M | -            | Inhibitory       |
| Thyroxine            | 4 x 10^{-8} M       | 2            | Stimulation      |
| Prealbumin           | 10 ng/ml to 10 μg/ml | 1 & 2        | None             |
| Relaxin              | 10 ng/ml to 1 μg/ml  | -            | -                |
| Growth Hormone       | 10 ng/ml to 1 μg/ml  | -            | -                |
| Epidermal Growth Factor | 10 ng/ml to 10 μg/ml | -            | -                |
| Fibroblast Growth Factor | 1 ng/ml           | -            | -                |
| Insulin              | 50 ng/ml to 5 μg/ml  | -            | -                |
| Multiplication       | -                   | -            | -                |
| Stimulating Activity | -                   | -            | -                |
| Phosphatidylcholine   | 10^{-9} to 10^{-6} M | 2            | -                |
| Vasopressin          | 10^{-8} M           | -            | -                |
| Thyrotrypsin Releasing Hormone | 10 ng/ml to 1 μg/ml | 1 & 2        | None             |
| Frontalin PCI6       | 1.6 x 10^{-9} to 10^{-5} M | 2 | Inhibitory |
| Frontalin PCI12      | 1 x 10^{-9} to 10^{-5} M | - | - |
| Frontalin PCI16      | 1 x 10^{-9} to 10^{-5} M | - | - |
| Oxytocin             | 0.1 ng/ml to 10 μg/ml| 1 & 2        | None             |
| Catechol Oxytocin    | 10^{-10} to 10^{-6} M | - | - |

*Assay Method 1 measures tritium labeled thymidine incorporation into DNA, while assay Method 2 measures growth as a function of increase in cell number.

Test of Ability of Known Growth Factors and Hormones to Substitute for the UDGFogenic Growth Properties of MTW/PL Cells

A wide range of known hormones and growth factors was assayed for mitogenic activity with MTW/PL cells in vitro, either by the Method 1 assay which measures labeled thymidine incorporation into DNA, or by Method 2 which measures increase in cell number. An example of the results obtained using Method 1 is shown in Fig. 11 in which the effect of commercially prepared mouse EGF on MTW/PL cells in comparison to UDGF. Clearly, EGF does not show mitogenic activity toward MTW/PL cells.
Purification and properties of a mammary-uterine-pituitary tumor cell growth factor from pregnant sheep uterus.

T Ikeda and D A Sirbasku

*J. Biol. Chem.* 1984, 259:4049-4064.

Access the most updated version of this article at [http://www.jbc.org/content/259/7/4049](http://www.jbc.org/content/259/7/4049)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/259/7/4049.full.html#ref-list-1) to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/259/7/4049.full.html#ref-list-1](http://www.jbc.org/content/259/7/4049.full.html#ref-list-1)