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HUMAN TERM PLACENTA CONTAINS TRANSFORMING GROWTH FACTORS

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Growth factors of apparent molecular weights of 6,000, 10,000, 20,000 and one in excess of 30,000 daltons can be isolated from acid-ethanol extracts of human term placentas. Each size class of growth factor resembles transforming growth factor (TGF) in that it stimulates anchorage independent growth of normal rat kidney cells and competes with EGF for binding to EGF membrane receptors. The 6,000, 10,000 and 20,000 molecular weight polypeptides also resemble TGF in their acid and heat stability, and their requirement for intact disulfide bonds for growth promoting activity. By homologous radioimmunoassay, neither the 6,000 nor 10,000 dalton polypeptide is related to human epidermal growth factor (hEGF). The presence of these TGFs in ample concentrations (approximately 100 ng of EGF equivalents per term placenta for the 10,000 dalton polypeptide) indicates the usefulness of this tissue source for study of human TGFs.

INTRODUCTION

Cellular transforming growth factors (TGFs), have been isolated from a variety of animal embryonic (1), normal 2), and malignant cells or tissues (3), as well as malignant human tissue (4). In addition extracellular TGFs are found in conditioned medium from human tumor cell cultures (5,6) and virus-transformed rodent cells (7,8), as well as in human urine of both malignant and embryonic origin (3). These TGFs are defined by their capacity to interact with cell surface epidermal growth factor (EGF) receptors, and to stimulate anchorage-independent growth in normal rat cells (5,7).

The human placenta has been a tissue source for isolation of a number of growth factors directed towards several target organs (9-11). Although placenta is rich in EGF receptors (12), and EGF influences hormone secretion differentially in normal and malignant placental cells in culture

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(13), no studies have examined the in vitro synthesis of EGF, or other growth factors, by human placenta. To examine the possibility that placenta, as an example of normal fetal tissue, might also contain TGF, we have extracted growth-stimulatory factors from term human placental tissue by established procedures (4). We now document the presence in this fetal tissue of three major peaks of transforming growth factor activity at apparent molecular weights of 6,000 and 10,000 and 20,000 daltons. These heat and acid stable peptides require disulfide bonds for biological activity, promote anchorage-independent growth of non-transformed indicator cells, and compete with EGF for EGF membrane receptors. Moreover, by a sensitive radioimmunoassay neither the 6,000 dalton or the 10,000 dalton class of cellular placental TGF's demonstrated immunological cross-reactivity with EGF.

MATERIALS AND METHODS

Tissue Source and Extraction Procedure

Within eight hrs of collection and storage at 4°C, normal spontaneously-delivered term human placentas were extracted using a modification of an acid-ethanol procedure described by Roberts et al. (1,4). Briefly, tissue was homogenized in 2 ml/g of a solution (4°C) containing 375 ml of 95% (vol/vol) ethanol and 7.5 ml of concentrated HCl, using 33 mg of phenylmethylsulfonylfluoride and 56 trypsin inhibitory units of aprotinin from bovine lung as protease inhibitors. The volume was adjusted to 3 ml/g with distilled water and extracted overnight at 4°C. Following centrifugation at 8,000 rpm for 2 hr, the supernatant was adjusted to pH 5.2 with concentrated ammonium hydroxide. One ml of 2 M ammonium acetate buffer, pH 5.2, was added per 100 ml of extract. The resultant precipitate was removed by centrifugation and the supernatant precipitated with four volumes of cold anhydrous ether and two volumes of cold ethanol (-20°C for 24 hr). The precipitate was collected by centrifugation, redissolved in 1 M acetic acid (2 ml/g of placental tissue) and extensively dialyzed against 0.2 M acetic acid (Spectraphor tubing, molecular weight cutoff 3,500, Spectrum Medical Industries L.A., CA.). The extract was lyophilized and stored at -20°C until used for Bio-Gel P-100 (Bio-Rad, Richmond, CA) column chromatography.

Assays

EGF-competing activity of lyophilized aliquots of individual P-100 column fractions was determined as previously described (5). Assays were performed in multi-well tissue culture plates (Linbro/Flow Labs, Hamden, Conn.) using A431 human carcinoma cells seeded at 8×10^3 cells per well and mouse ^{125}I-EGF as the probe.

Soft agar assays were carried out in Dulbecco's modified Eagle's medium containing 10% calf serum as previously described (7). A 0.5% base layer and a 0.3% agar overlay containing the test sample were used. Normal rat kidney (NRK) cells, clone SA6, were seeded at 3×10^4 cells per 60 mm plate. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere and

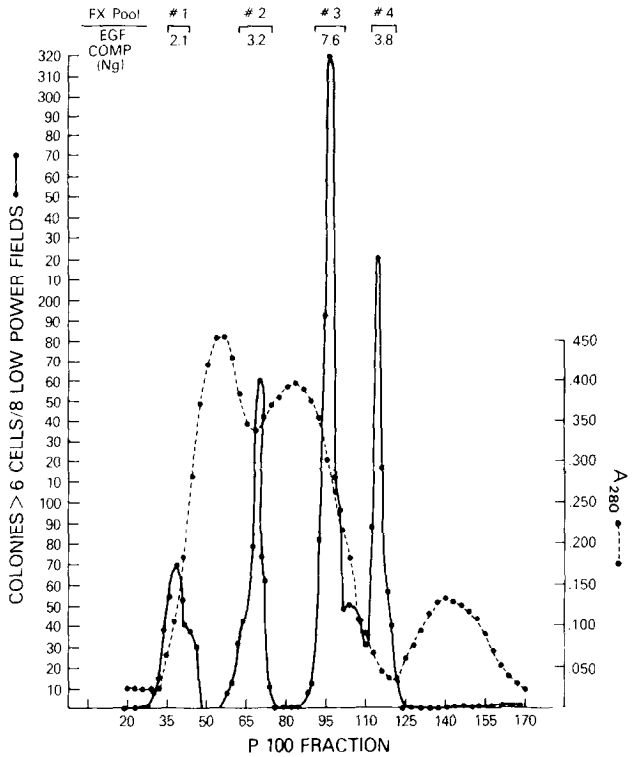

Figure 1. Bio-Gel P-100 column chromatography of acid-thanol solubilized peptides (60 mg of protein) from term human placentas. Fractions of 12.5 ml were collected and 0.5 ml aliquots of alternate fractions were assayed for anchorage-independent growth of NRK cells in soft agar (●—●—●), and A_{280} reading (●-●-●). The fractions representing the four peak soft agar activities were pooled (shown in brackets at the top of the graph) and after lyophilization assayed for EGF-competing activity (results shown below each bracket in ng equivalents of EGF). Molecular weight markers on the P-100 column included BSA (68,000 MW) Carbonic Anhydrase (29,000 MW) Cytochrome C (12,500 MW), and Insulin (6,000 MW).

scored on the indicated day, usually day 12, for the number of colonies six cells or larger per eight low power fields. Plates were overlaid with media containing 0.3% agar on day 5.

RESULTS

Three major and one minor peak of growth factor activity was present in human term placenta after acid-ethanol extraction and P-100 column chromatography (Figure 1). Each of these peak activities have the two characteristic properties of transforming growth factors (TGFs) in that each stimulated anchorage-independent growth of normal rat fibroblasts and each competed with ^{125}I-EGF for receptor binding to EGF receptor rich human carcinoma cells. Moreover, each peak fraction scored in the radioreceptor assay in proportion to its NRK growth-promoting activity in soft agar. For

example, fraction pool #3 of approximately 10,000 molecular weight had about twice the activity in both the soft agar growth-stimulatory and EGF-receptor assays as did fraction pool #2 of 20,000 molecular weight. Peaks #3 and #4 of approximately 10,000 and 6,000 molecular weight respectively produced very large (often irregular) colonies in soft agar of perhaps 50 to 100 NRK cells, while peaks #2 and #1 of 20,000 and over 30,000 molecular weight gave much smaller colonies of about 10 to 15 cells. Because peak #4 of 6,000 molecular weight was assumed to be EGF, which alone does not stimulate growth in our soft agar assay, this result was surprising. Consequently, the two lower molecular weight peaks were tested in a radioimmunoassay for human EGF by a reported procedure (14). In this highly sensitive assay for immunoreactive human EGF, capable of detecting as little as 5 picograms, neither peak was active when tested at three dilutions equivalent to 10, 50 and 250 pg/tube based on estimated EGF radioreceptor activity (Dr. David Orth, personal communication). An additional line of evidence that the 6,000 molecular weight peak is not human EGF is that it binds to carboxymethyl cellulose equilibrated with 5 mM Ammonium Acetate, pH 5.0 which is a characteristic of TGFs (15). The peak in excess of 30,000 molecular weight did not score in a colony-stimulating assay for human granulopoiesis (Dr. Rachel Huot, personal communication). This suggests it is different from the more acid-labile growth factors of approximately 27,000 and 50,000 molecular weight which are found in the conditioned medium from human placental tissue (16).

A dilution curve of each of the soft agar stimulatory peaks from P-100 chromatography is shown in Figure 2. The relative homogeniety and high concentration present in the smaller two peaks is indicated in their near-linear dose-response curves, and, as suggested from the A280 readings (Figure 1), the two larger size activities of soft agar growth appear heterogenous and plateau after the addition of about 0.7 mg of protein by Lowry measurement (17).

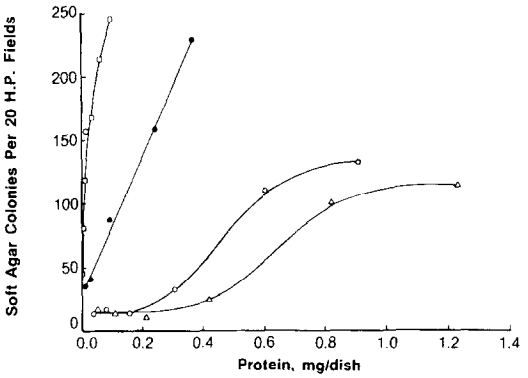

Figure 2. Dilution curves of the soft agar colony-forming activities separated by P-100 chromatography. Fractions from the P-100 column showing soft agar activity were pooled and aliquots for the dilution series were lyophilized. Soft agar assays were carried out as described in Materials and Methods. A colony is >6 cells. >30K (Δ - Δ), 20K (o-o), 10K (□ - □) 6K (•-•).

The effect of various treatments on the stability of the soft agar stimulatory activity of the four peaks is presented in Table 1. Each peak of activity requires intact disulfide bonds and withstands exposure to urea and repeated lyophilization. The persistence of anchorage-independent growth after DTT exposure to the peak in excess of 30,000 molecular weight is perplexing, and requires further evaluation. Heating to 100°C in 1 M acetic acid enhances activity, particularly in the 20,000 molecular weight peak, which suggests removal of an inhibitor of NRK soft agar colony formation. The larger three size classes of growth factor activity were clearly trypsin sensitive and therefore were protein in nature. An unexpected result was the several fold increases in activity after the 6,000 molecular weight factor was treated with trypsin. Evidence that this TGF activity, however, is due to a protein was provided by the fact that the protease papain did greatly reduce the soft agar stimulating activity. A likely explanation for the trypsin insensitivity is that a contaminant trypsin inhibitor, perhaps the 6,500 molecular weight Aprotinin present during extraction, is responsible. An additional alternative is that this 6,000 molecular weight region contains a TGF which also acts as a trypsin inhibitor.

TABLE 1. Stability Characteristics of the Transforming Growth Factors as Assessed by Growth in Soft Agar

Treatment	Soft Agar Colonies/60 mm Dish >30K	20K	10K	6K
Trypsin control	347	122	229	54
Trypsin	4	8	26	139
Dithiothreitol control	81	96	118	44
Dithiothreitol	446	15	20	6
Urea control	254	55	385	58
Urea	641	64	517	90
Acetic acid control	576	50	167	70
Acetic acid (100°C, 10 min)	628	398	654	197
Repeated lyophilization (three times)	709	32	108	177

Aliquots of the soft agar active pools following P-100 chromatography were lyophilized for trypsin, dithiothritol and urea treatment after being dissolved in 0.1 M NH_4HCO_3. For trypsin treatment a control solution and one containing trypsin treated with L-tosylamido-1 phenylethyl chloromethyl ketone at 50 ug/ml were incubated at 37°C for 2 hr, at which time soybean trypsin inhibitor was added to give 100 ug/ml. For dithiothreitol treatment, a control solution and one containing 0.1 M dithiothreitol were incubated at room temperature for 1 hr. For urea treatment a control solution and one containing 8 M urea were incubated at room temperature for 16 hr. Each of these solutions was then dialyzed extensively against 0.2 M acetic acid and lyophilized. Acid stability studies were carried out on samples in 1 M acetic acid and treated as described before lyophilization. Soft agar assays were carried out as described.

Roberts, et al recently reported that acid-ethanol extracts of normal mouse tissues which contain little EGF (such as liver, muscle, brain, or heart) comprise a new class of cellular TGF's which are potentiated by the addition of exogenous EGF (2). The role of endogenous placental EGF and its contribution to TGF activities was not examined in our present study.

DISCUSSION

The finding of several size classes of TGFs in normal term placenta presents numerous opportunities for further characterization. Besides being the perhaps the most readily available of all human tissues, placental tissue can also be grown in both cell and organ culture (18). Consequently this normal human tissue source, and the carcinogenic progression of its abnormal counterparts of hydaditaform mole and choriocarcinoma, provide a model system to examine the role of TGF's in embryonic development and carcinogenesis. In addition, the placenta should be useful in in vitro studies of growth factor synthesis and processing similar to studies which examined processing of other placental hormones (19).

Preliminary studies have indicated that conditioned medium from first-trimester human placental organ culture does not contain an extracellular TGF-like activity, although of course the very process of short term organ culture may terminate this expression in human placenta. Thus our present finding of intracellular TGF activities in placental extracts after homogenization suggests a function related to endogenous growth modulation. In contrast, a factor inhibiting anchorage-independent growth of human malignant cells is secreted by normal first trimester placenta in vitro. A possible interpretation of these results is that the placenta, with its rich EGF receptor concentration (12), acts as a sponge for TGFs produced by the developing fetus. In line with this the importance of dosage in TGF expression recently has been emphasized (2). Consequently, we hypothesize that the high concentration of EGF membrane receptors in placental trophoblast might serve to reduce the potential

deleterious consequences of fetal-derived TGF's in the material circulation. Furthermore, besides placental EGF receptor binding of fetal TGF's, trophoblast production of a factor inhibitory to anchorage-independent cell growth might provide an additional mechanism to maintain maternal homeostatis.

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