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Human A673 Cells Secrete High Molecular Weight EGF-Receptor Binding Growth Factors That Appear to be Immunologically Unrelated to EGF or TGF-α

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Extracts of serum-free conditioned medium from human rhabdomyosarcoma A673 cells contain high molecular weight (HMW) transforming growth factors (TGFs) that can be partially purified by Bio-Gel P-100 and carboxymethyl (CM)-cellulose chromatography (Todaro et al: Proc Natl Acad Sci USA 77:5258, 1980). Reverse-phase high performance liquid chromatography (HPLC) revealed a principal peak of epidermal growth factor (EGF) radioreceptor assay (RRA) activity and anchorage-independent growth (AIG) activity that coeluted with 25–26% acetonitrile. If a trailing shoulder of EGF RRA activity from the CM-C chromatography was included in the material for HPLC analysis, additional active fractions were observed at 21–22% acetonitrile. Importantly, both active regions from HPLC failed to compete in radioimmunoassays under reduced and denatured conditions for human EGF (hEGF), human TGF-α (hTGF-α), or rat TGF-α (rTGF-α) and failed to give positive signals in Western blots under conditions in which TGF-α was readily detected when using an antisera raised against the 17 C-terminal amino acids of rTGF-α. Nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed EGF RRA and AIG activities in gel slices corresponding to M_r 15,000 and 22,000 in the 25–26% acetonitrile eluate and M_r 15,000, 20,000, 27,000, and 48,000 in the 21–22% acetonitrile eluate. The presence of multiple forms of EGF-receptor-binding peptides produced in vitro suggest size heterogeneity and possible immunologic diversity among high molecular weight members of the EGF/TGF-α family of growth-promoting polypeptides.

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Transforming growth factors (TGFs) are acid-stable growth regulatory polypeptides that confer anchorage-independent growth (AIG) on normal rat kidney (NRK) fibroblasts [1,2]. Epidermal growth factor (EGF) radioreceptor assay activity (RRA) and AIG activity were first identified in extracts of conditioned medium from cells transformed by a murine sarcoma virus [3]. Two classes of TGFs were subsequently defined depending on their ability to bind to the EGF membrane receptor and their AIG activity in NRK cells [4–6]. Unlike TGF-α, TGF-β does not compete with EGF for receptor binding sites and requires EGF or TGF-α to induce AIG in NRK cells in semisolid media [6,7]. The original active factor secreted by these cells was found to consist of both polypeptides [8]. The prototype, extracellular low molecular weight forms of TGF-α have been purified from human [9], rat [10,11], and mouse [12,13] transformed cell lines, and rTGF-α has been synthesized [14] and consists of single-chain polypeptides of approximately M_r 5,600 (5.6K), which are about 30% homologous with human EGF (hEGF) and mouse EGF (mEGF) [12,13]. TGF-β has been purified to homogeneity from normal human [15] and bovine [16] tissues, human tumor cells [17], and retrovirus-transformed rat cells [18] and consists of a dimer of 25K that on reduction yields two identical chains of 12.5K. Nucleotide sequences of complementary DNAs encoding mEGF [19,20], hTGF-α [21], rTGF-α [22], and human TGF-β [23], indicate that the functional low molecular weight form of each peptide is synthesized from a much larger precursor. Consequently, high molecular weight (HMW) forms of these growth factors might be found in vitro and in vivo.

Urine from patients with disseminated cancer appears to have increased concentrations of a HMW growth factor of approximately 30–35K with NRK AIG activity and EGF RRA activity [24,25]. When characterized from the urine of patients with malignant brain tumors, this tumor-associated HMW TGF appears to be a HMW form of hEGF and is present at a fourfold higher level than in the urine of normal individuals [26]. Both a low molecular weight form and HMW (>10K) forms of TGF-α-like growth factors have been described in conditioned medium from several human tumor cell lines [27–29], in a retrovirus-transformed rat cell line [30], in the urine of human tumor-bearing athymic mice [31], and in extracts of the same human tumor cells [17]. These latter two studies utilized human rhabdomyosarcoma cell line A673 [32], which unlike most human tumor cells line in long-term culture, release relatively large amounts of HMW growth factors with EGF RRA and AIG activities. In an effort to clarify the relationship between the HMW TGFs present in urine [24,25,31] and conditioned medium [27–30], and to compare these extracellular forms with the intracellular [17] form of HMW TGF-α, this report describes chromatographic properties, apparent molecular weights, and immunologic characteristics of HMW peptides with EGF-receptor-binding activity in the conditioned medium of human A673 cells.

MATERIALS AND METHODS

Conditioned Medium

Human rhabdomyosarcoma A673 cells [32] were grown as described [27] in roller bottles (850 cm^2; polystyerene, CORNING, Corning, NY) in 50 ml of DMEM with 10% fetal bovine serum (GIBCO, Grand Island, NY). Confluent monolayers

were rinsed twice with serum-free DMEM and incubated in 50 ml of serum-free Waymouth's medium for 8 hr. The medium was aspirated, and the A673 cells in each roller bottle were incubated in a second 50 ml of serum-free Waymouth's medium for 48 hr. The medium (conditioned medium) from several roller bottles was pooled, 1 μg per ml of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) was added, and the medium was centrifuged (10,000g for 30 min) and stored at −20°C. Thawed medium was concentrated 25-fold using a hollow fiber concentrator (DC-2; Amicon, Lexington, MA) extensively dialyzed (spectraphor tubing, molecular weight cutoff 3500, Spectrum Medical Industries, Inc., Los Angeles, CA) against 1% acetic acid (HOAc), lyophilized, and stored at −70°C.

Soft Agar Growth Assay

Aliquots of column eluate fractions or extracts of gel slices were examined for AIG activity in semisolid medium (Agar Noble, Difco Laboratories, Detroit, MI) [27] in DMEM containing 10% calf serum. Purified TGF-β (kindly provided by Dr. Michael B. Sporn), was added at 2 ng per ml over a 0.5% agar base layer in 35-mm dishes (Cat. No. 3001, Falcon, Oxnard, CA), and 1 ml of 0.3% agar was added, which contained the test sample in the presence of 3×10^3 NRK cells, clone SA_6, (kindly provided by Dr. Joseph E. DeLarco). On day 5 the dishes were overlaid with medium containing 0.3% agar, and incubation at 37°C in a humidified 5% CO_2 atmosphere continued until day 14. The number of colonies containing at least 20 cells or 50 cells or more in eight low-power fields were counted on day 14. SA_6 NRK cells, used between passages 12 and 16, do not form colonies in 0.3% agar except in the presence of clonogenic growth-promoting factors.

EGF Radioreceptor Assay

Samples were assayed for EGF RRA activity in 16-mm multiwell tissue culture plates (Linbro/Flow Labs, Hamden, CT) containing 10^4 A431 cells per well and highly purified mEGF as the ^{125}I-labeled tracer and reference standard [27]. More recently, a more rapid and convenient EGF RRA that uses isolated A431 cell membranes was employed [33]. Each assay included a standard curve of graded quantities of unlabeled mEGF with which to calculate the EGF equivalents in the test samples. Half-maximal inhibition of ^{125}I-mEGF binding to isolated A431 cell membranes is observed with 2–4 ng of competing unlabeled mEGF. In these two RRAs, hEGF and mEGF are equivalent.

Gel Filtration, Ion-Exchange, and Reverse-Phase High Performance Liquid Chromatography

Procedures for Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, CA) and CM-cellulose (CM-52, Whatman, Clifton, NJ) chromatography were performed [27]. Lyophilizates of 100 L (pool A) or 250 L (pool B) of conditioned medium stored at −70°C were reconstituted in 10 ml of 1 M HOAc and centrifuged (10,000g for 30 min), and the supernate was subjected to chromatography on a Bio-Gel P-100 (100–200 mesh) column (5 × 82.5 cm), equilibrated, and eluted with 1 M HOAc at 4°C; 12.4-ml fractions were collected.

Biologically active eluate fractions from the P-100 column (M_r approximately 15,000–20,000) were pooled, lyophilized, resuspended in 1 M HOAc, dialyzed (molecular weight cutoff 3,500) overnight against 5 mM ammonium acetate, pH 4.5,

at 4°C, and centrifuged at 100,000g for 30 min. The supernate was applied to a CM-cellulose column (1.5 × 3 cm) equilibrated with 5 mM NH_4OAc, pH 4.5 (starting buffer). A linear elution gradient formed of 200 ml of starting buffer and 200 ml of limit buffer (0.5 M NH_4OAc, pH 6.8) in a two-chamber, constant-level mixing device was applied at a flow rate of 80 ml per hr at 22°C. Fractions of 10 ml were adjusted to 1 M with respect to HOAc, and aliquots were concentrated by lyophilization prior to assay of biological activity.

Reverse-phase HPLC was performed with a C_{18}-μBondapak column (10-μm particle size, 0.39 × 30 cm, Waters Associates, Milford, MA) in a Waters Associate Liquid Chromatograph system equipped with two Model 6000 M solvent delivery pumps, a Model 660 solvent programmer, and a Model 450 variable wave-length UV detector set at 206 nm. A linear elution gradient of 18–35% acetonitrile in 0.05% trifluoroacetic acid was applied over 120 min at a flow rate of 0.7 ml per min at 22°C.

Protein concentration was determined by the method of Lowry et al [34] using bovine serum albumin as standard, or by amino acid analysis of hydrolyzed samples [35].

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

Samples were subjected to SDS-PAGE on 15% polyacrylamide slab gels according to the method of Laemmli [36] and sliced into 2-mm sections in a mechanical gel slicer (Model 190, Bio-Rad, Richmond, CA). Prestained molecular weight standards (Bethesda Research Labs, Gaithersburg, MD) were ovalbumen (43K), chymotrypsinogen (25.7K), β lacto-globulin (18.4K), lysozyme (14.3K), aprotinin (6.2K), and the β subunit of insulin (3.0K). Each test sample lane was bracketed by lanes of prestained standards to assist slicing and determination of molecular weight. For assay for biological activity, materials in individual SDS-PAGE slices were extracted by adding 1 ml of 1 M HOAc containing 100 μg of bovine serum albumin as carrier, crushing the gel slice in a conical tube with a glass rod, and incubating them for at least 24 hr at 4°C to permit polypeptides to leach out of the gel matrix. Following a 5-min centrifugation (Microfuge B, Beckman, Palo Alto, CA), 0.7 ml of supernate was aspirated and stored at either 4°C or −70°C prior to assay.

Radioimmunoassays for hEGF and TGF-α

Homologous hEGF radioimmunoassay (RIA) was performed by modifications [37] of a method [38] that uses an antiserum raised to highly purified hEGF that was generously provided by Dr. Yukio Hirata, National Cardiovascular Institute Research Center, Osaka, Japan. Highly purified hEGF [39] was used as ^{125}I-labeled tracer and reference standard. TGF-α RIA was performed using a commercial kit (Biotope, Inc., Seattle, WA), and as described [40], with bioactive synthetic rTGF-α (Peninsula Laboratories, Belmont, CA) used as radioiodinated tracer and reference standard. Antisera to the C-terminal 17-amino acid fragment (C17A) of rTGF-α were raised either in rabbits (Biotope, Inc.) or as an affinity-purified antiserum raised in sheep [40]. Half-maximal competition is observed in the Biotope system with about 0.5–1.0 ng native or reduced TGF-α. A noncommercial assay (Biotope, Inc.) with similar sensitivity was also used employing rabbit antiserum raised against synthetic human TGF-α (residues 1–50). All TGF-α RIAs using either rabbit or sheep antisera detected hTGF-α and rTGF-α equally well. Western immunoblots [40] for TGF-α were

carried out on the two biologically active HPLC components; the technique detected 50–75 ng of either rTGF-α or hTGF-α as reference standards. Synthetic hTGF-α was the gift of Dr. J.E. Tam, Rockefeller University (New York, NY).

RESULTS

The column eluate of Bio-Gel P-100 chromatography of 100 L of conditioned medium from A673 cells contained a 13–20K region of AIG and EGF RRA activity (Fig. 1). The elution profiles of both the 100-L pool and the 250-L pool (data not shown) of conditioned medium suggested the presence of at least two M_r species. The trailing shoulder with AIG and EGF RRA activities has previously been noted in medium from A673 cells [27, 41].

Column fractions comprising the entire region of TGF activity of the 100-L and 250-L pools were separately pooled, concentrated, and applied to a CM-cellulose column. There again appeared to be two components with EGF RRA activity in both the 100-L (Fig. 2, upper panel) and 250-L (Fig. 2, lower panel) pools, but neither was adequately resolved. Consequently, the earlier-eluting fractions 22–31 (Fig. 2,

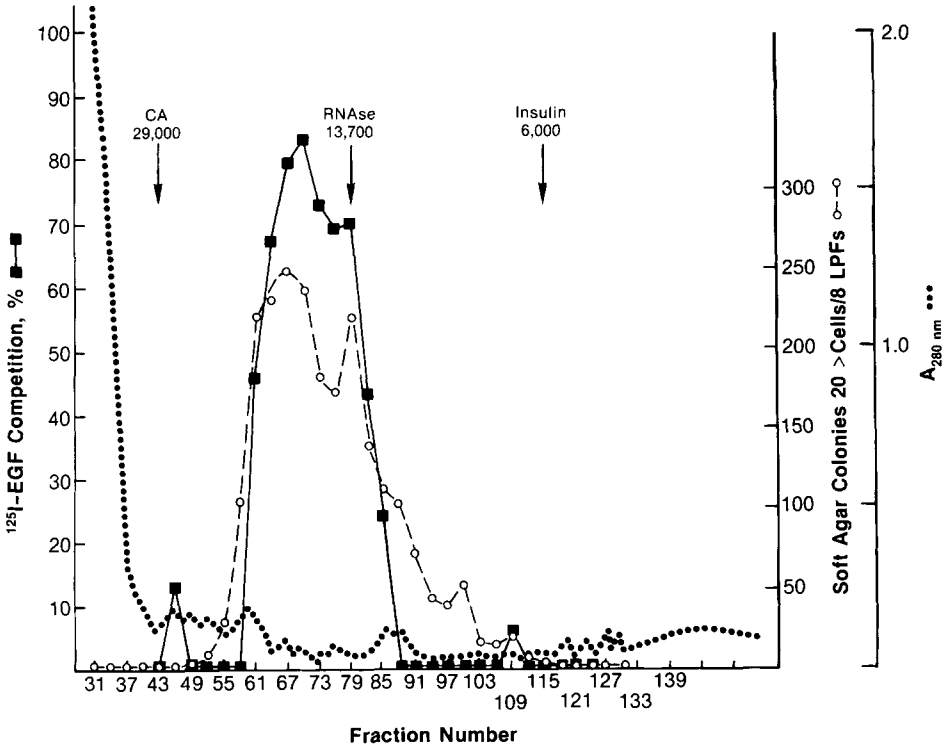

Fig. 1. Gel filtration chromatography of concentrated conditioned medium (100 L) from A673 cells. The lyophilized sample was dissolved in 1 M HOAc and centrifuged to remove insoluble material, and the supernate was applied to a Bio-Gel P-100 column (5 × 82.5 cm,100–200 mesh), equilibrated, and eluted with 1 M HOAc in 12.4-ml fractions. Protein was determined by absorbance at 280 nm (.....). A 100-μl aliquot of every third fraction was assayed for binding to EGF receptors (■ --- ■), and a 50-μl aliquot was assayed for NRK clonogenicity in semisolid medium (○ --- ○) [27]. Molecular weight markers were carbonic anhydrase, 29K; ribonuclease, 13K; and insulin, 6K.

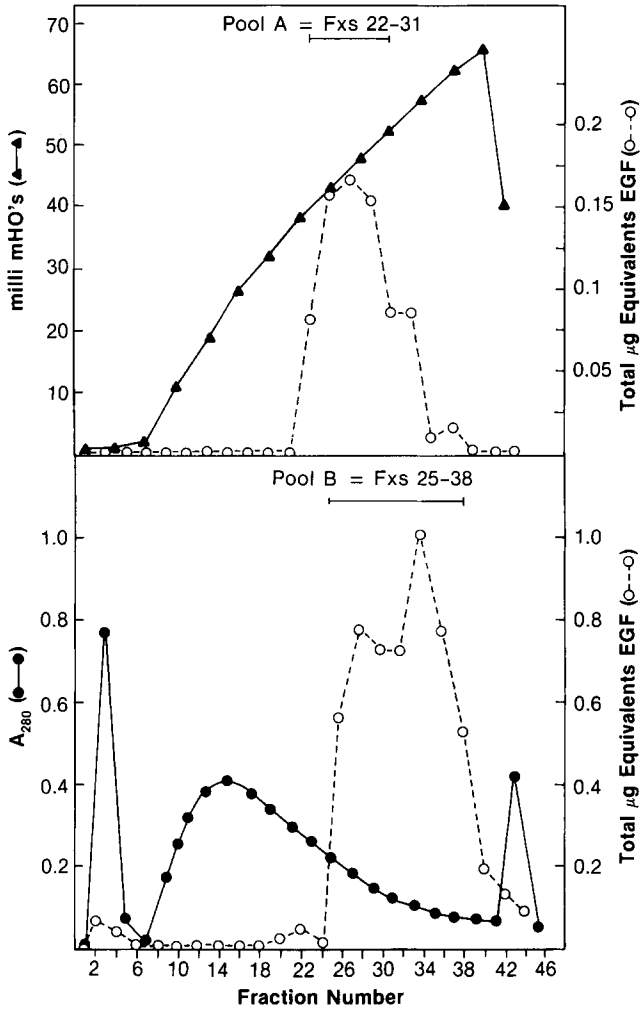

Fig. 2. Ion exchange chromotagraphy of pooled Bio-Gel P-100 fractions with TGF activity from 100-L (upper panel) (fractions 61–85, Fig. 1) or 250 L (lower panel) (P-100 chromatograph not shown) of A673 conditioned medium. The pooled fractions were lyophilized, reconstituted, dialyzed, applied to, and eluted from a column of CM-cellulose as described in "Materials and Methods." In the upper panel, conductivity (▲ -- ▲) is shown as a measure of the linearity of the pH elution gradient, while in the lower panel, absorbance at 280 nm (● -- ●) is a measure of protein concentration. RRA activity (○--○) was determined by competition of 200-μl aliquots from each 10-ml fraction with ^{125}I-EGF for binding to isolated A431 membranes as described [33]. Fractions containing the major peak of TGF activity in the upper panel (fractions 22–31, designated pool A) and all TGF activity in the lower panel (fractions 25–38, designated pool B) were pooled and lyophilized.

upper panel) from the 100-L pool were combined (pool A) and compared with all the TGF active fractions 25–38 (Fig. 2, lower panel) from the 250-L pool (pool B) by reverse-phase HPLC (Fig. 3). Pools A and B both contained a component with TGF activity that eluted with 25–26% acetonitrile (fractions 25–27, upper panel and fractions 20–22, lower panel, Fig. 3). Fractions 25–27 from pool A generated a linear dose-response curve in the EGF radioreceptor assay (inset, upper panel, Fig. 3). The

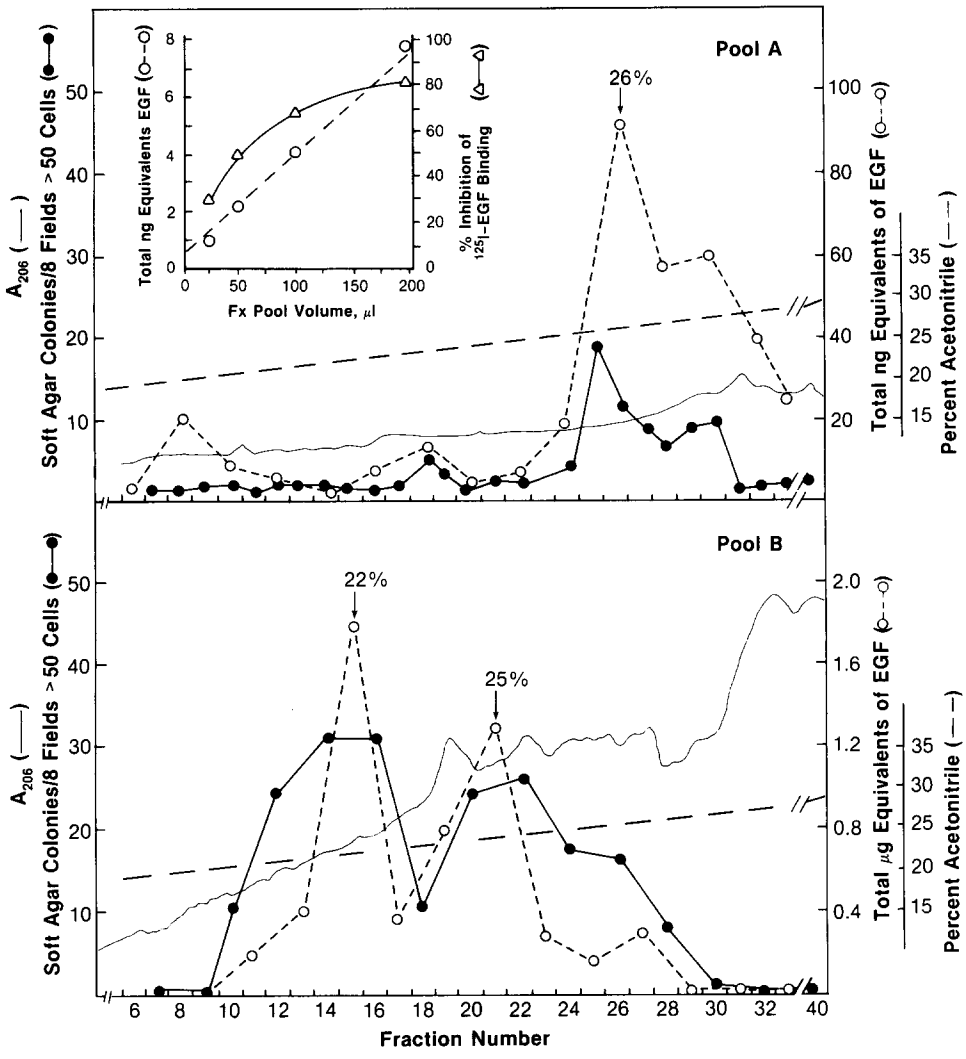

Fig. 3. Reverse-phase HPLC of Pool A (fractions 22–31) and Pool B (fractions 25–38) from CM-cellulose (Fig. 2). Each pool was lyophilized, resuspended in 0.05% trifluoroacetic acid, and subjected to HPLC; 100-μl aliquots of alternate fractions (1.8 ml total volume) were assayed in duplicate for EGF RRA activity (○ -- ○), and 50-μl aliquots were assayed for NRK AIG activity (● -- ●), as described in "Materials and Methods." Fractions 25–27 of pool A (upper panel) were pooled to evaluate the dose-response relationship in the EGF RRA assay (inset, upper panel). The arrows and percentages above the peaks of TGF activity indicate acetonitrile elution.

inclusion in pool B of the trailing shoulder of the larger peak of TGF activity from CM-cellulose chromatography generated an additional peak of AIG and RRA activity, which eluted with 21–22% acetonitrile (fractions 14–16, Fig. 3, lower panel). Because this latter activity was not observed in pool A, it appeared that it might represent components eluting in CM-C fractions 32–38 (Fig. 2, lower panel).

Aliquots of fractions 25–27 from pool A, which had the highest AIG and EGF RRA activity (Fig. 3, upper panel), were combined and subjected to SDS-PAGE under nonreducing conditions. Two TGF species of M_r 15,000 and 22,000 were identified by coincident peaks of AIG activity, EGF RRA activity, and mitogenic activity [41] (data not shown). An identical result (Fig. 4, upper panel) was obtained after SDS-PAGE of the peak activity fractions 20–22 from pool B. Comparable

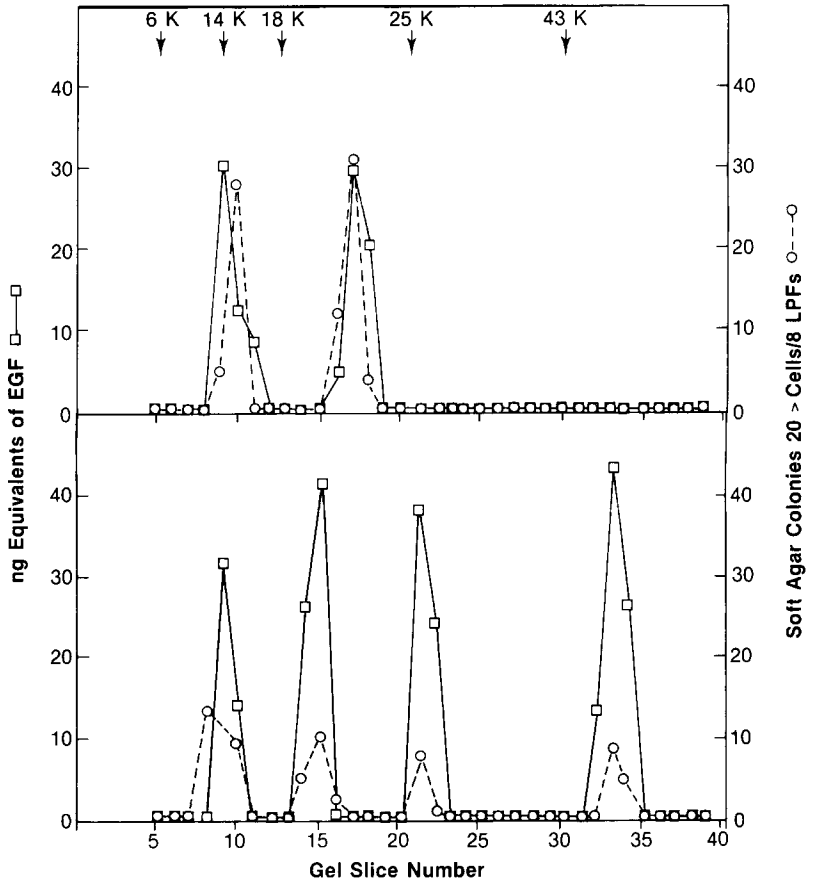

Fig. 4. SDS-PAGE of two peaks of TGF activity from pool B HPLC (Fig. 3, lower panel). Equal aliquots of fractions 14–16, representing the 21–22% acetonitrile elution region (lower panel),and fractions 20–22 from the 25–26% acetonitrile elution region (upper panel) were analyzed on 15% polyacrylamide slab gels under nonreducing conditions, and gel slices were eluted, as described in "Materials and Methods." A 200-μl aliquot of each 0.7-ml eluate was assayed for AIG activity (○ -- ○), and 500 μl was assayed in duplicate for EGF RRA activity (□ -- □). Prestained molecular weight standards were ovalbumin (43K), chymotrypsinogen (25.7K), β lactoglobulin (18.4K), lysozyme (14.3K), aprotinin (6.2K), and the β subunit of insulin (3.0K).

analysis of the more hydrophilic region of activity (fractions 14–16) from pool B revealed four peaks of coincident EGF RRA and AIG activity (Fig. 4, lower panel).

Pool B of A673 conditioned medium contained the most EGF equivalents (Figs. 2 and 3, lower panels). Aliquots of the two HPLC peaks of TGF activity from pool B were evaluated for their immunological relatedness to TGF-α [9–14] by RIAs and Western immunoblots (Table I). In both Biotope TGF-α RIAs using antisera raised in rabbits against either the C17A fragment of rTGF-α or the entire synthetic hTGF-α, and adding up to 100 ng of antigen as measured by the EGF RRA activity, neither of the HPLC peaks displaced ^{125}I-labeled rTGF-α from the antibodies. The same result using an affinity-purified sheep antisera raised against the synthetic C17A fragment of rTGF-α is shown in Figure 5. In this system, the TGF-α RIA detected hTGF-α activity in conditioned medium of human melanoma cells A-2058 [40]. Synthetic hTGF or synthetic rTGF-α in either its C-terminal (residues 34–50) or full length form (residues 1–50) did react fully [40] (Fig. 5), whereas highly purified hEGF did not react at all. Moreover, both of the HPLC peaks with AIG and EGF RRA activities failed to react in a sensitive RIA for hEGF [37,38]. Furthermore, as summarized in Table I, neither HPLC peak of AIG activity (added in an amount of 200 ng EGF equivalents by RRA) was detected by Western immunoblots under conditions in which 50–75 ng of synthetic rTGF-α or hTGF-α were clearly detected [40].

DISCUSSION

The A673 human cell line is unusual in its continued secretion of HMW forms of growth-promoting polypeptides with TGF-α-like biological activity even at a high

TABLE I. Immunologic Characterization of Partially Purified HMW TGFs Secreted by A673 Cells

	Immunologic assay		
	RIA		Western immunoblot
Growth factor	hEGF[a]	rTGF-α[b]	rTGF-α[c]
hEGF[d]	+	−	−
hTGF-α[e]	−	+	+
HPLC fraction[f]			
21–22% MeCN	<0.01 ng	<0.1 ng	−
25–26% MeCN	<0.01 ng	<0.1 ng	−

[a]hEGF RIA [37,38] in which detectability (10% displacement) is 0.1 ng highly purified hEGF reference standard [39], and 50% displacement occurs with 0.5 ng hEGF under reduced and denatured conditions.
[b]rTGF-α RIAs (Biotope, Inc., Seattle, WA) utilizing rabbit antisera against both C17A fragment of rTGF-α or entire synthetic hTGF-α. Detectability (10% competition) is 0.3 ng synthetic rTGF-α reference standard and in which 50% competition occurs with 0.5 ng competing synthetic rTGF-α or hTGF-α under reduced and denatured conditions.
[c]rTGF-α immunoblot [40] sensitive to detection of 50–75 ng of synthetic rTGF-α or hTGF-α.
[d]Highly purified hEGF [39].
[e]Synthetic hTGF was biologically active prior to testing.
[f]Equal aliquots of active HPLC fractions 14–16 (21–22% MeCN elution) or active HPLC fractions 20–22 (25–26% MeCN elution), as shown in Figure 3, lower panel, were combined to provide 30 ng of EGF RRA equivalents [33] for hEGF RIA, 100 ng of EGF RRA equivalents for either of the rTGF-α RIAs (see "Methods"), and 200 ng EGF RRA equivalents for Western immunoblots [40]. Both rTGF-α and hTGF-α were included at 75 ng as positive controls in each Western immunoblotting experiment.

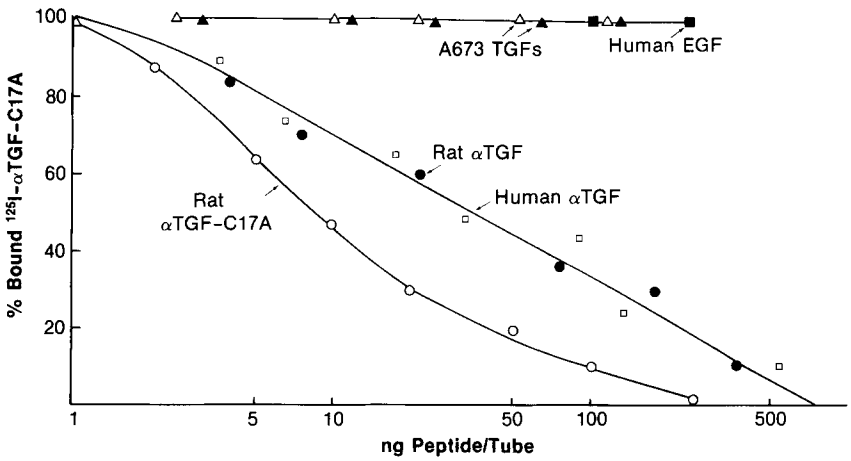

Fig. 5. Rat TGF-α RIA [40] for synthetic C17A fragment (○) and complete (●) rat TGF-α [14] and human TGF-α (□), purified [39] hEGF (■), and the two peak regions of TGF activity from HPLC (Fig. 3, lower panel) of A673 conditioned medium (△, ▲). The amount of A673-derived TGF-α activity added per RIA tube is expressed as ng equivalents of mEGF as measured in the EGF RRA. Each sample was reduced and denatured and was competed against ^{125}I-labeled rTGF-α (C-terminal 17-amino acid fragment) in the presence of affinity-purified sheep antisera raised against the same fragment of rTGF-α [40].

passage number. Accordingly, this human tumor line was selected to evaluate HMW polypeptides as a potential model for processing of TGF-α precursor species. Immunologic reagents developed against the C-terminal 17 amino acids of rTGF-α, a low molecular weight (5.6K) polypeptide [14], were reported in Western immunoblots to detect species of 22K, 42K, and 44K in medium conditioned by feline sarcoma virus-transformed rat cells [30]. Similarly, an affinity-purified sheep antiserum raised against the same C-terminal rTGF-α fragment detected a 20–22K TGF-α-like peptide in medium conditioned by the same transformed rat cell line in immunoblots [40]. More recently, an 18K intracellular form of hTGF-α-like biological activity was reported as the predominate species in A673 cell extracts, but was not immunologically characterized [17]. Other HMW species of extracellular peptides with TGF-α-like activity from tumor cells have been reported [28,29], but these too have not been immunologically characterized.

Fractionation of medium conditioned by A673 cells by sequential gel filtration, cation exchange, and reverse-phase high performance liquid chromatography revealed two components with TGF-α-like activity by assays for binding to EGF cell membrane receptors and NRK cell colony formation in soft agar. Immunological comparison to TGF-α by RIA and estimation of molecular weight by SDS-PAGE revealed a lack of immunological relatedness and size heterogeneity in both components. Besides failing to compete in RIAs under reduced and denatured conditions for hEGF or rTGF-α, neither active components from HPLC was detected in Western immunoblots under conditions in which rTGF-α and hTGF-α at about one-third the concentration were readily detected. Moreover, neither of these two active peaks in HPLC was detected in an ELISA assay for hTGF-α (R. Derynck and T.S. Bringman, unpublished data). Finally, precursor-specific antipeptide antibodies directed against the carboxy terminal amino acids of the rTGF-α precursor [22] were unable to

immune precipitate any peptides from ^{35}S-cysteine-labeled A673 cell cultures, in contrast to a readily detectable 14–17 K protein found in retroviral-transformed Fisher rat embryo cells (L.E. Gentry et al, submitted for publication).

Nonreducing SDS-PAGE of these two HMW growth-promoting components revealed EGF RRA and NRK cell AIG activities at 15K and 22K in the relatively hydrophobic materials eluting in HPLC with 25–26% acetonitrile, and 15K, 20K, 27K, and 48K in the relatively hydrophilic materials that eluted in HPLC with 21–22% acetonitrile. In addition to the major 18K species, growth factors of various molecular weights with TGF-α-like activity were also described in A673 cell extracts [17]. Thus, size heterogeneity of HMW EGF-receptor-binding growth factors may be characteristic of A673 cells. The predominant extracellular HMW forms that we observed were 15K and 20–22K. Our extracellular 20K form may correspond to the 18K moiety previously described in cell extracts [17], and the extracellular 15K species may result from glycosidase cleavage of the 20K species. Virally transformed rat cells contain a bioactive 17–19K TGF-α precursor, which after elastase treatment is converted to mature TGF-α, and after endoglycosidase treatment becomes a 14K moiety in Western immunoblots using an antisera raised against the synthetic rTGF-α C-terminal 20 amino acids ([42] and J. Massague, personal communication). In addition, tunicamycin treatment converts an 18K moiety of TGF-α produced by CHO cells transfected with a TGF-α expression vector into a 14K immunoreactive TGF-α species (R. Derynck, personal communication). The larger 27K and 48K species observed in nonreducing SDS-PAGE gels may represent HMW precursor forms or artifacts that are due to protein binding or aggregation of smaller species, as has been described for mEGF [43]. Similarly, in addition to secreted HMW TGF-α of 20K [30,40], 42K and 44K forms have also been observed in Western immunoblots of extracts of medium conditioned by feline sarcoma virus-transformed rat cells [30].

The urine of athymic mice bearing A673 cells is reported to contain a tumor-associated 20K complex of growth factors, which after resolution by HPLC includes a more hydrophilic component than mEGF that reacts with an antiserum raised against the C-terminal 17-amino acid fragment of rTGF-α [31]. Two RIAs using an antisera raised to the same antigen [40] (Biotope, Inc., Seattle, WA) detected HMW TGF-α-like factors in conditioned medium of the human breast carcinoma cell line MCF-7 (S. Bates and M.E. Lippman, personal communications) and in extracts of human milk and human mammary tumor tissue (D.S. Salomon and W.R. Kidwell, personal communications). In addition, we have detected hTGF-α in the urine of patients with disseminated breast carcinoma using the Biotope antiserum in a TGF-α RIA (K. Stromberg et al., submitted for publication). The fact that we have not detected similar HMW forms of immunoreactive TGF-α in A673 conditioned medium, even in an RIA using antisera raised against the entire low molecular weight hTGF-α, suggests several possibilities. First, it is possible that more specific immunologic reagents may be required to detect human HMW TGF-α-like growth factors of nonbreast origin. Second, the immunoreactive TGF-α-like component in athymic mouse urine [31] may be a murine growth factor, perhaps produced in response to the tumor. Third, the conditions under which A673 cells grow (ie, in vivo in the athymic mouse versus in vitro in cell culture) may influence the forms or relative amounts of the growth factors they produce. Fourth, there may be tissue-specific differences in the kinds of growth factors that are produced. Fifth, the inability of the antiserum to react may be due to sequestration of critical epitopes within these

possible TGF-α precursor molecules. The antiserum may also require the free C-terminal amino acid residue of TGF-α for reactivity, as has been described for other antisera [44,45] which are sometimes referred to as "wraparound" antisera. In support of this hypothesis, the synthetic hTGF-α C-terminal 17-amino acid fragment [14] that has a C-terminal residue blocked by an amide group is recognized only poorly by the Biotope antiserum (W.R. Hargreaves, unpublished data). Thus, hTGF-α precursors with C-terminal extensions [21,22] would probably not react with the Biotope antiserum. RIAs using antisera raised against various precursor forms of hTGF-α might identify the EGF-receptor-binding HMW growth factors secreted by A673 cells as immunologically related to hTGF-α. Alternatively, A673 cells may secrete HMW growth factors more closely related to the EGF-receptor-binding glycosylated growth factor of 23K released into conditioned medium of vaccinia virus-infected cells [46]. Last, and least likely, perhaps A673 cells produce immunologically distinct forms of HMW growth factors of the EGF/TGF-α family.

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REFERENCES

1. Todaro GJ, Marquardt H, Twardzik DR, Reynolds FH Jr, Stephenson JR: In Weinstein IB, Vogel HJ (eds): "Genes and Proteins in Oncogenesis." New York: Academic Press, 1983, pp 165–182.
2. Sporn MB, Anzano MA, Assoian RK, De Larco JE, Frolik CA, Meyers CA, Roberts AB: In Levine AJ, Van de Woude GF, Topp WC, Watson JD (eds): "The Transformed Phenotype." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1984, pp 1–4.
3. De Larco JE, Todaro GJ: Proc Natl Acad Sci USA 75:4001, 1978.
4. Frolik CA, Dart LL, Sporn MB: Fed Proc Fed Am Soc Exp Biol 41:855, 1982.
5. Anzano MA, Roberts AB, Meyers CA, Komoriya A, Lamb LC, Smith JM, Sporn MB: Cancer Res 42:4776, 1982.
6. Roberts AB, Anzano MA, Lamb LC, Smith JM, Frolik CA, Marquardt H, Todaro GJ, Sporn MB: Nature 295:417, 1982.
7. Sporn M, Roberts A, Shull J, Smith J, Ward J, Sodek M: Science 219:1329, 1983.
8. Anzano MA, Roberts AB, Smith JM, Sporn MB, De Larco JE: Proc Natl Acad Sci USA 80:6264, 1983.
9. Marquardt H, Todaro GJ: J Biol Chem 257:5220, 1982.
10. Twardzik DR, Todaro GJ, Marquardt H, Reynolds FH, Stephenson JR: Science 216:894, 1982.
11. Massague J: J Biol Chem 258:13606, 1983.
12. Marquardt H, Hunkapiller MW, Hood LE, Twardzik DR, De Larco JE, Stephenson JR, Todaro GJ: Proc Natl Acad Sci USA 80:4684, 1983.
13. Marquardt H, Hunkapiller MW, Hood LE, Todaro GJ: Science 233:1079, 1984.
14. Tam JP, Marquardt H, Rosberger DF, Wong TW, Todaro GJ: Nature 309:376, 1984.
15. Frolik CA, Dart LL, Meyers CA, Smith DM, Sporn MB: Proc Natl Acad Sci USA 80:3676, 1983.
16. Roberts AB, Anzano MB, Meyers CA, Wideman J, Blacher R, Pan YCE, Stein S, Lehrman SR, Smith JM, Lamb LC, Sporn MB: Biochemistry 22:5692, 1983.
17. Dart LL, Smith DM, Meyers CA, Sporn MB, Frolik CA: Biochemistry 24:5925, 1985.
18. Massague J: J Biol Chem 259:9756, 1984.
19. Gray A, Dull TJ, Ullrich A: Nature 303:722, 1983.
20. Scott J, Urdea M, Quiroga M, Sanchez-Pescador R, Fong N, Selby M, Rutter WJ, Bell GI: Science 221:236, 1983.

21. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV: Cell 38:287, 1984.
22. Lee DC, Rose TM, Webb NR, Todaro GJ: Nature 313:489, 1985.
23. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV: Nature 316:701, 1985.
24. Twardzik DR, Sherwin SA, Ranchalis J, Todaro GJ: J Natl Cancer Inst 69:793, 1982.
25. Sherwin SA, Twardzik DR, Bohn WH, Cockley KD, Todaro GJ: Cancer Res 43:403, 1983.
26. Stromberg K, Hudgins WR, Dorman LS, Henderson LE, Sowder RC, Sherrell BJ, Mount CD, Orth DN: Cancer Res (in press).
27. Todaro GJ, Fryling CM, De Larco JE: Proc Natl Acad Sci USA 77:5258, 1980.
28. Van Zoelen EJJ, Twardzik DR, Van Oostwaard TMJ, Van der Saag PT, De Laat SW, Todaro GJ: Proc Natl Acad Sci USA 81:4085, 1984.
29. De Larco JE, Pigott DA, Lazarus JA: Proc Natl Acad Sci USA 82:5015, 1985.
30. Linsley PS, Hargreaves WR, Twardzik DR, Todaro GJ: Proc Natl Acad Sci USA 82:356, 1985.
31. Twardzik DR, Kimball ES, Sherwin SA, Ranchalis JE, Todaro GJ: Cancer Res 45:1934, 1985.
32. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JJ, Dosik HK, Parks WP: J Natl Cancer Inst 51:1417, 1973.
33. Kimball ES, Warren TC: Biochim Biophys Acta 771:82, 1984.
34. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1956.
35. Cohen SA, Tarvin TL, Bidlingmeyer T: Am Lab 16:48, 1984.
36. Laemmli UK: Nature 227:680, 1970.
37. Oka Y, Orth DN: J Clin Invest 72:249, 1983.
38. Dailey GE, Kraus JW, Orth DN: J Clin Endocrinol Metab 46:929,1978.
39. Mount CD, Lukas TJ, Orth DN: Arch Biochem Biophys 240:33, 1985.
40. Hazarika P, Pardue R, Lorenzo I, Sawada T, Earls R, Dedman JR:(in press).
41. Iwata KK, Fryling CM, Knott WB, Todaro GJ: Cancer Res 45:2689, 1985.
42. Ignotz RA, Kelly B, Davis RJ, Massague J: Proc Natl Acad Sci USA 83:6307, 1986.
43. Taylor JM, Mitchell WM, Cohen S: J Biol Chem 249:2188, 1974.
44. Wilson, RE, Orth DN, Nicholson WE, Mount CD, Bertagna XY: J Clin Endocrinol Metabol 53:1, 1981.
45. Orth DN: "Methods of Hormone Radioimmunoassay," 2nd Ed. New York: Academic Press, 1979, pp 245–293.
46. Stroobant P, Rice AP, Gullick WJ, Cheng DJ, Kerr IM, Waterfield MD: Cell 42:383, 1985.