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Transforming Growth Factor (TGF) Activity in Human Urine: Synergism Between TFG-Beta and Urogastrone

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Human urine contains an acid and heat stable peptide with an apparent molecular weight of 8,000–10,000 that, in the presence of urogastrone (EGF), induces the anchorage-independent growth of nontransformed cells in semisolid media. This nonmitogenic growth-modulating activity does not compete with EGF for binding to EGF membrane receptor sites and can be resolved from EGF by high-performance liquid chromatography. The urine-derived growth factor has been purified more than 10,000-fold and shares many biochemical properties with and is functionally related to the B class of TGFs isolated from transformed cells and nonneoplastic tissues. The low molecular weight anchorage-independent growth-stimulating activity universally present in human urine is a result of the synergistic interaction of this urine-derived TGF-beta and urogastrone.

Key words: transforming growth factor, alpha and beta human urine

Transforming growth factors (TGFs) are a family of low molecular weight acid and heat stable growth regulatory peptides that induce anchorage-independent cell growth in nontransformed cells [1,2]. Functionally, two classes of TGFs (alpha and beta TGF) have been described based on their interactions with epidermal growth factor (EGF). Type alpha TGFs, which compete with EGF for membrane receptor binding sites, have been purified to homogeneity from the conditioned media of both rodent and human tumor cells [3–5]. TGF-alpha shares amino acid homology with EGF [6] and requires TGF-beta in order to induce the formation of progressively growing colonies in soft agar [7]. A second class of TGFs, designated TGF-beta, are nonmitogenic, do not bind to EGF receptor sites, and demonstrates an absolute requirement of an EGF receptor ligand such as EGF or TGF-alpha to stimulate nontransformed cells to grow as colonies in semisolid media [8,9]. This latter subclass of TGFs has been identified and purified to homogeneity from a variety of neoplastic and normal tissues, including platelets [10] and human placenta [11].

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We have previously shown that the urine of normal, pregnant, and tumor-bearing humans contains a common 8,000 M_r low molecular weight transforming growth factor activity that competes for binding to EGF receptors and stimulates the anchorage-independent growth of nontransformed cells [12,13]. In this study we show that this low molecular weight TGF activity we observed is due to the synergistic interaction of urogastrone and a urine-derived peptide functionally related to the beta class of TGFs.

MATERIALS AND METHODS

Urine Specimen Collection

Urine was collected over a 24-h period from normal male and female controls and selected cancer patients with disseminated disease. Specimens were processed immediately following collection without prior freezing.

Extraction of Urine Specimens

Normal control urine was pooled from 12 donors and processed utilizing a modification of the acid-ethanol extraction of Roberts et al [14] and as reported previously [15]. Clarified urine was extracted with acidified 95% ethanol containing phenylmethyl sulfonyl fluoride and aprotinin as protease inhibitors, centrifuged to remove acid-insoluble material, and the pH of the supernatant was adjusted to 4.0 by addition of ammonium hydroxide. Peptides were precipitated by the addition of four volumes of cold anhydrous ether and two volumes of cold ethanol. The resulting precipitate was collected by centrifugation, redissolved in 1 M acetic acid, dialyzed against 0.2 M acetic acid, and chromatographed on a Bio-Gel P-10 system (2.5 × 75 cm) at room temperature with 1 M acetic acid as the eluent. Column fractions of 3.5 ml were collected, and appropriate aliquots were lyophilized and tested for the presence of soft agar growth-promoting activity and EGF-competing activity as described below.

Soft Agar Growth Assay

Column fractions were tested for the presence of factors capable of stimulating nontransformed normal rat kidney (NRK, clone 49F) fibroblasts to grow as colonies in soft agar as described previously [16]. A single-cell suspension of approximately 10^4 NRK cells was mixed with the lyophilized sample to be tested and seeded in 2.0 ml of Dulbecco's modified Eagle's medium containing 10% calf serum (Grand Island Biological Co., Grand Island, NY) plus 0.3% agar (Difco Laboratories, Detroit, MI) into 60-mm Costar petri dishes containing a base layer of the same medium plus 0.5% agar. Plates were re-fed with 2.0 ml of the same medium plus 0.3% agar at 7 days. Colonies consisting of more than 20 cells were scored in 8 random low-power fields at both 7 and 14 days. NRK cells do not grow as colonies in 0.3% agar except in the presence of soft agar growth-promoting factors. High-performance liquid chromatography (HPLC)-purified mouse EGF was added to plates at a concentration of 1 ng/ml, an amount predetermined by titration to support minimal colony formation when used with a preselected lot of calf serum. This addition was required to detect TGF-beta activity following HPLC purification.

EGF-Binding Competition Assays

Aliquots of column fractions were also tested for the presence of factors capable of competing with ^{125}I-radiolabeled mouse EGF for binding to receptors on formalin-fixed human carcinoma A-431 cells according to methods published previously [16].

HPLC

High-performance liquid chromatography of the peak anchorage-independent growth-stimulating activity from Bio-Gel P-10 columns was performed as previously described [5]. Separations were achieved utilizing a u Bondapak C_{18} column (10-mm particle size, 0.39 × 30 cm, Waters Associates, Milford, CT). The mobile phase was 0.05% trifluoroacetic acid, and the mobile-phase modifier was either acetonitrile or n-propanol containing 0.045% trifluoroacetic acid.

Growth Factors

Mouse submaxillary gland EGF was purchased from Collaborative Research (Waltham, MA) and purified to homogeneity by HPLC; rat TGF was purified from Snyder-Theilen feline sarcoma virus-transformed Fisher rat embryo cells [17] as previously described [3]. Urogastrone was purified from human urine as described [18] and followed by HPLC. Platelet-derived growth factor (PDGF) was purified as described [19].

RESULTS

Acid-ethanol solubilized polypeptides from urine pools derived from healthy individuals were chromatographed on a Bio-Gel P-10 column equilibrated in molar acetic acid. Aliquots of individual column fractions were tested for competing with [^{125}I]EGF for binding to EGF membrane receptors and for stimulating NRK fibroblasts to form progressively growing colonies in soft agar. As shown in Figure 1, the major EGF-competing activity (fractions 95–120) elute in the region of the 6,000 M_r insulin marker; a minor, albeit reproducible, peak of activity (fractions 75–85) is also found eluting slightly before the 13,700 M_r RNase marker. When aliquots of corresponding column fractions were tested for their ability to stimulate the growth of NRK fibroblasts in soft agar, a distinct peak of activity eluting in the range of 8,000–10,000 M_r was seen. Reassay of column fractions 75–90 at a tenfold dilution required the addition of exogenous EGF in order to observe colony formation in soft agar.

The major peak of NRK colony-stimulating activity from gel filtration columns (fraction 92 and 93) was further purified on a C_{18} u Bondapak column. The results of this experiment are shown in Figure 2. A single peak of anchorage-independent growth-stimulating activity (fraction 38) eluting at 40% acetonitrile well after the majority of urinary peptides was seen, which did not compete with ^{125}I]EGF binding to receptor-rich human epidermoid carcinoma (A431) cells. No NRK colony formation in soft agar was noted when this fraction was assayed without the addition of exogenous EGF. Contaminating urogastrone (fractions 39–44) eluted as a doublet in the concentration range of 42% acetonitrile, similar to the urogastrone standard. TGF-alpha elutes from this column at approximately 20–22% acetonitrile. Again,

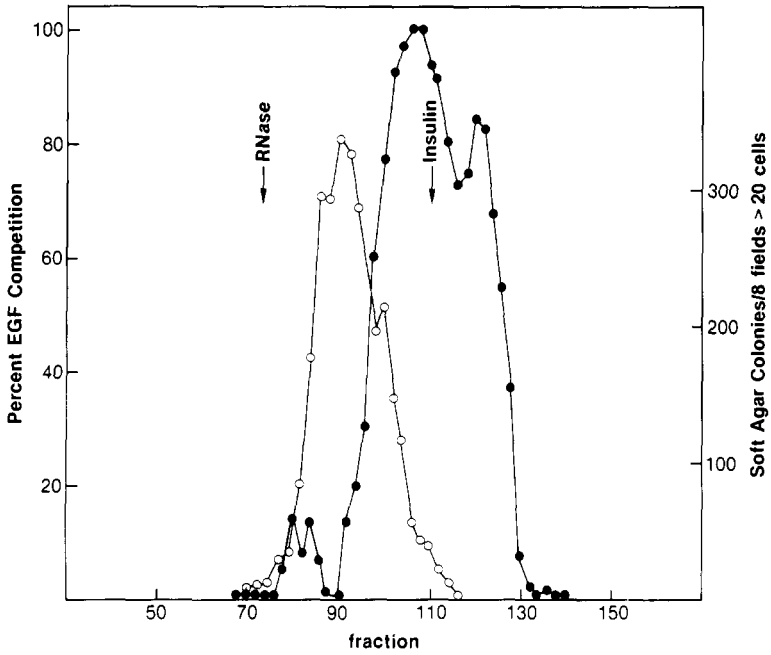

Fig. 1. Bio-Gel P-10 chromatography of acid-ethanol solubilized peptides from human urine. A sample containing 42 mg of acid-ethanol solubilized urine peptides was applied to a Bio-gel P-10 column (100–200 mesh, 2.5 × 75 cm) equilibrated in acetic acid. Aliquots of each 3.5-ml fraction were lyophilized and tested in duplicate for EGF competition on receptor-rich A431 human carcinoma cells and for stimulating NRK cells to form progressively growing colonies in soft agar in the presence of exogenous EGF [16] as described in Materials and Methods. Molecular weight markers included carbonic hydrase, 29,000 M_r; and insulin, 6,000 M_r. ^{125}[I]EGF elutes with the insulin marker.(○—○), number of soft agar colonies; (●—●), present EGF competitive.

these fractions, in contrast to fraction 38, supported the growth of only small NRK colonies 4–6 cells in size in soft agar assays. Reconstitution experiments, in which fractions 38 and 40 were mixed, induced a strong response in the soft agar assay and demonstrated the requirement of both components for maximum stimulation of anchorage-independent cell growth. Rechromatography of the peak of activity functionally related to TGF-beta (fraction 38) on a u Bondapak C_{18} column and elution with n-propanol is shown in Figure 2 (panel B). The absorbance profile monitored at A_{206} demonstrates a major peptide eluting in the range of 29–30% n-propanol; the nonsymmetrical absorbance profile (shoulder to the right of the main peak) indicates heterogeneity and appears to be a function of proteolysis occurring during urine processing, as first-voided morning urines collected on ice and immediately processed appear to be more homogenous for TGF-beta and urogastrone. When tested in the presence of exogenous EGF, the NRK colony-forming activity coeluted with the major absorbance peptide, eluting at 29–30% n-propanol. Again, no EGF-competing activity in a sensitive receptor assay (1 ng = 50% competition) could be detected in this fraction.

As shown in Figure 3, urine-derived TGF-beta was purified approximately 10,000-fold relative to acid-ethanol solubilized urine polypeptides. Relative to unfractionated urine (7–10 mg of protein/ml), the purification is in excess of 1:10,000 as acid-ethanol extraction removes over 80% of extraneous Lowry positive material.

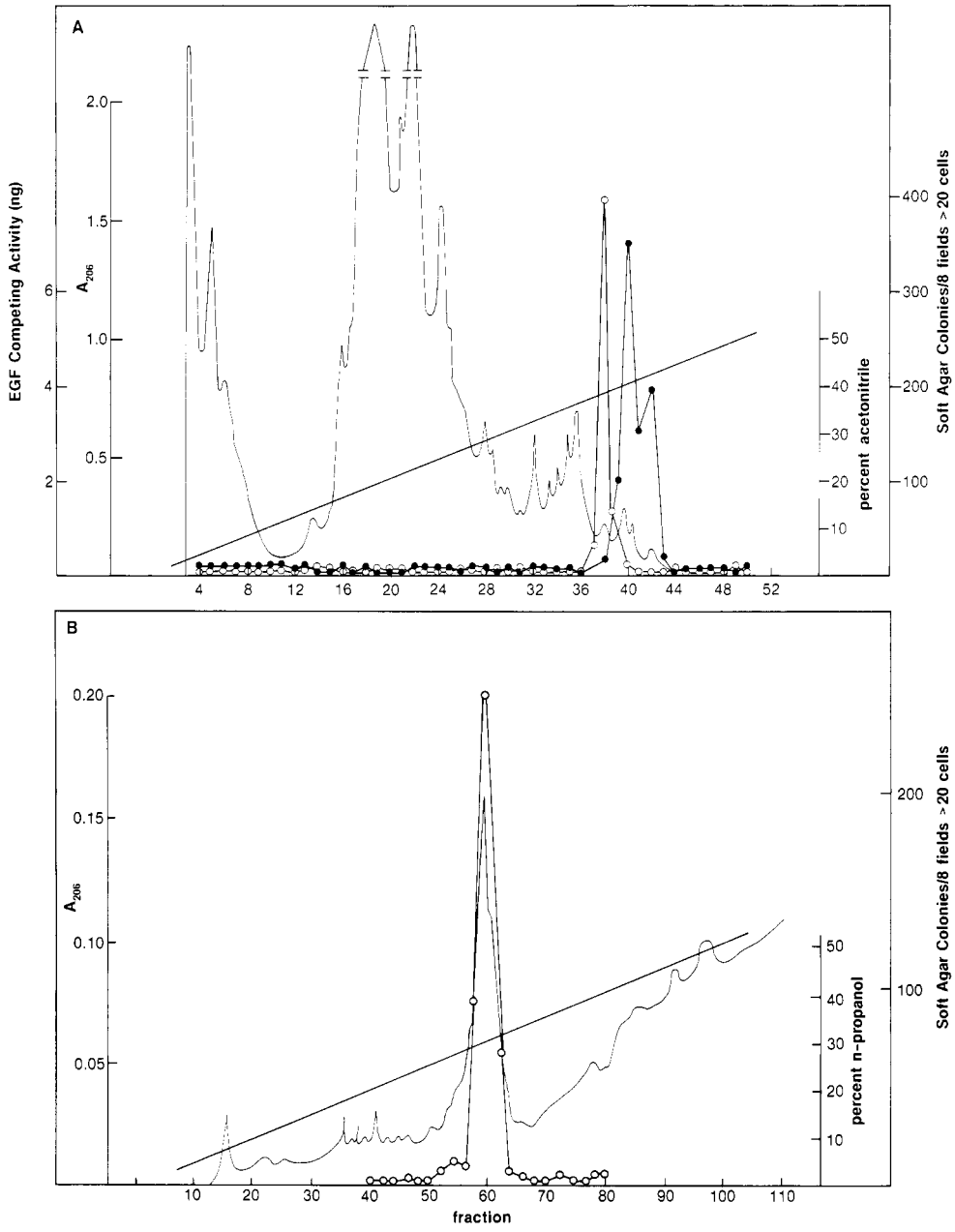

Fig. 2. Reverse-phase high-performance liquid chromatography of the urine-derived TGF-beta. The peak fractions of anchorage-independent growth-stimulating activity from a Bio-Gel p-10 column (fractions 92,93) were lyophilized and resuspended in 0.045% trifluoroacetate, HPLC grade, in H_2O and injected into a u Bondapak C_{18} column as described in Materials and Methods (Panel A). Elution was achieved with a linear 0–60% gradient of acetonitrile (Panel A) or n-propanol (Panel B) in 0.045% TFA at 22°C at a flowrate of 1 ml/min. Aliquots of each fraction were assayed in duplicate for stimulating colony growth in soft agar and for competition with [^{125}I]EGF binding to A431 cells; the solid line denotes absorbance at 206 nm. (○—○), number of soft agar colonies; (●—●), ng of EGF competing activity; (—), absorbance at 206 nm.

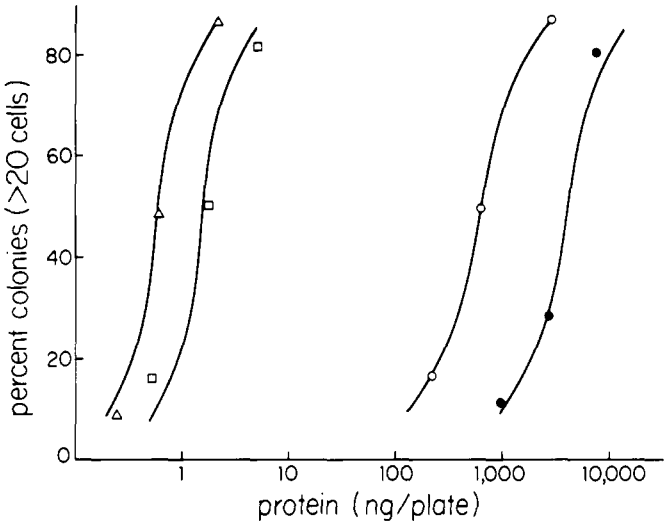

Figure 3. Dilution curves of urine-derived TGF-beta activity in soft agar following purification steps. Aliquots of peak TGF-beta fractions were assayed for NRK colony stimulation, as described in Materials and Methods, at 1:3 dilutions in the presence of 2.5 ng of EGF per plate: acid-ethanol solubilized urine peptides (●—●); after Bio-Gel P-10 (○—○); after HPLC on u Bondapak C_{18} columns; acetonitrile elution (□—□); n-propanol elution (△—△).

Less than 500 pg/ml of purified TGF-beta following n-propanol elution was needed to induce NRK colony formation in soft agar in the presence of 1 ng/ml of EGF. Yield of TGF-beta was 200–400 ng/l of urine.

The requirement of the urine-derived TGF-beta for an EGF receptor ligand in order to stimulate NRK colony formation is shown in Table I. Following purification by HPLC and resolution of the TGF-beta activity from contaminating urogastrone, urine TGF-beta when tested alone does not support NRK colony formation. Soft agar plates containing TGF-beta supplemented at the time of seeding with HPLC-purified EGF from both human urine and mouse submaxillary gland, however, do support rigorous anchorage-independent cell growth. Large, round, compact colonies of NRK cells exceeding 100 cells with concomitant changes in the media phenol red indicator (red to yellow) are seen 10 days postseeding. At a constant EGF concentration, the addition of HPLC-purified urine-derived TGF-beta stimulates colony formation in a dose-dependent manner.

Both human and mouse EGF are equally effective in acting synergistically with urine TGF-beta when tested at equivalent nanogram levels. Colony morphology, however, does differ; whereas mouse submaxillary gland EGF induces compact round colonies in the present of TGF-beta, EGF purified from either human or mouse urine induces diffuse elongated colony formation.

TGF-alpha, purified from retroviral-transformed Fischer rat embryo cells [3], can also substitute for EGF at similar concentrations in the TGF-beta-dependent anchorage-independent cell growth assays. Whereas PDGF alone did not stimulate anchorage-independent growth, it did significantly enhance the activity of TGF-beta. In contrast, highly purified EGF, urogastrone, and TGF-alpha stimulate poor colony formation when tested alone in soft agar colony assays and require TGF-beta in order

TABLE I. Stimulation of Anchorage-Independent Cell Growth by Urine-Derived Beta Transforming Growth Factor

Growth factor	Addition	Soft agar colonies[a] >20 cells: eight random low power fields
TGF-beta[b]	None	<20
	Urogastrone[c] (1 ng)	242
	EGF (mouse)[d] (1 ng)	300
	TGF (rat)[e] (1.2 ng)	285
	PDGF (human)[f] (5 ng)	>350
None	Urogastrone (1 ng)	<20
	EGF (mouse) (1 ng)	<20
	TGF (rat) (1.2 ng)	<20
	PDGF (human) (5 ng)	<20

[a]NRK cells were seeded in soft agar as described in Materials and Methods; concentrations of growth factor represent amounts per ml. Number of colonies were scored in duplicate and are presented as average values.
[b]TGF-beta was HPLC-purified from urine and was used at a concentration of 5 ng/plate.
[c]Urogastrone was purified from urine by HPLC and contains two forms of EGF-competing activity.
[d]Mouse EGF was purified from mouse urine as described [12].
[e]TGF was purified from serum-free media conditioned by Snyder-Theilen feline sarcoma virus-transformed Fisher rat embryo cells [13].
[f]Purified PDGF was a gift from Dr. E. Raines.

to stimulate the formation of large progressively growing colonies. At high concentration of EGF ligand (>20 ng/ml), the endogenous TGF-beta present in the calf serum used in the soft agar assays does support intermediate NRK colony formation (unpublished observation). Purified preparations of the urine-derived TGF-beta was not effective in stimulating [^{125}I]deoxyuridine incorporation into rodent monolayer cells (data not shown).

DISCUSSION

Human urine contains a peptide that, in the presence of an EGF receptor ligand such as EGF or TGF-alpha, reversibly stimulates the anchorage-independent growth of nontransformed cells in semisolid media. This modulator of anchorage-independent cell growth was not mitogenic on either rodent or human fibroblasts and is thus functionally related to TGF-beta isolated from neoplastic and non-neoplastic tissues, including kidney [22], placenta [11], and human platelets [10]. It also shares many similar biochemical properties with TGF-beta [1], including heat and acid stability and the requirement of disulfide bonds for maintenance of activity (data not shown).

The urine-derived TGF-beta elutes from gel filtration columns with an apparent molecular weight of 8,000–10,000. Since TGF-beta from both human platelets [10] and kidney [22] elutes with an aberrantly low molecular weight during gel filtration, the elution of urine-derived TGF-beta may also be retarded. TFG-beta purified from human platelets is a two-chain polypeptide of 25,000 daltons consisting of two disulfide-linked identical peptide chains of 12,500 daltons each [10]. Experiments to determine the relationship of urine-derived TGF-beta to TGF-beta isolated from other sources are in progress. Homogenous purified preparations of TGF-beta will be required to confirm preliminary results, which suggest that urine TGF-beta may be a degradation product of platelet TGF-beta.

In previous reports we have described a low molecular weight 8,000–10,000 M_r "transforming growth factor" activity in human urines from both normal [12] and cancer patients [13] with disseminated disease. This activity was detected in Bio-Gel filtration column fractions, which as we have shown in this study, contain both TGF-beta and urogastrone. This activity, then, is not due to TGF-alpha, as all detectable EGF-competing activity observed in this region elutes on HPLC analysis in the range of EGF (38–40% acetonitrile), rather than at the 20–24% acetonitrile concentration where human melanoma TGF-alpha elutes. Urine therefore contains TGF-beta or a degradation product thereof, possibly derived from either human platelet [10], or kidney TGF-beta [22], or both. Recently, Assoian et al [23] have shown that the induction of anchorage-independent growth of the NRK cell line we used in this study is due to the concomitant action of PDGF, TGF-beta, and an analogue of epidermal growth factor. The PDGF enhancement of urine-derived TGF-beta colony formation we have observed in our studies also indicates this to be the case. Studies are now in progress to identify a PDGF related component in urine specimens.

We conclude that the low molecular weight "transforming activity" found in human urine samples which stimulates anchorage-independent cell growth, is a result of the synergistic complementation of a urine-derived TGF-beta, urogastrone, and possible other growth modulators such as PDGF.

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