CONNECTIVE TISSUE ACTIVATION

XXVIII. A Connective Tissue Activating Peptide from Human Urine

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A protein factor in human urine which has the ability to activate connective tissue cells has been identified and partially purified; it appears to be different from epidermal growth factor and IgG. This urinary connective tissue activating factor (CTAP-U) is nondialyzable, labile to protease, stable to thiols, heat, and acid, and has an acidic isoelectric point. Purified preparations of CTAP-U have biologic activities that cause human connective tissue cells to synthesize incremental amounts of ^{14}C-hyaluronic acid, ^{35}S-proteoglycans, and ^{3}H-DNA in vitro. The cell spectrum responsive to this substance includes human synovial cells, human chondrocytes, and skin fibroblasts. CTAP-U does not react with antisera to connective tissue activating peptide–III or to antibodies against IgG or its Fc and Fab fragments. Furthermore, CTAP-U does not cross-react in a radioreceptor assay for insulin, basic somatomedin, or epidermal growth factor–urogastrone. Utilizing standardized isolation conditions, CTAP-U preparations with these properties have been isolated from the urine of 6 normal individuals.

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Human connective tissue cells are "activated" in vitro when incubated with extracts of human platelets, lymphocytes, and polymorphonuclear leukocytes (PMN) (1–3). Activation consists of a constellation of metabolic events including: stimulation of glycosaminoglycan (GAG) synthesis (both hyaluronic acid [HA] and sulfated GAG), increased glucose uptake, increased lactate production, and increased DNA synthesis. The activation process is mediated by peptides isolated from the cellular extracts and termed connective tissue activating peptides (CTAPs). CTAP-I from lymphocytes, CTAP-III and CTAP-P_2 from platelets, and CTAP-PMN from polymorphonuclear leukocytes have been at least partially characterized (4–9). CTAP-III has been sequenced (8), and antisera prepared in rabbits are available (10).

Evidence exists that cell activators, or growth factors, are excreted into the urine in a biologically active form. Holley and Kiernam found that a partially purified factor from urine stimulated growth in cultured 3T3 fibroblasts (11). Epidermal growth factor–urogastrone (EGF-URO) was isolated from human urine and its biologic activity verified (12–15). Among its numerous biologic effects, EGF-URO is mitogenic in various cell culture systems and may stimulate hyaluronic acid synthesis, glycolysis, and the uptake of sugars and amino acids (14). The gastric antisecretory hormone isolated from human urine by Gregory, β-urogastrone, has been shown to be the human equivalent of mouse epidermal growth factor (15); the combined term epidermal growth factor–urogastrone depicts the dual biologic activities that the peptide exhibits.

The present report describes another biologically active urinary protein fraction. These connective

tissue activating materials from urine (or CTAP-U) were found in the urine of 6 normal subjects. CTAP-U possesses biologic activities similar to the CTAP family of polypeptides isolated from other sources and appears to be different from EGF-URO.

MATERIALS AND METHODS

CTAP-U isolation methods. Urine samples (24–48-hour), from normal subjects, were collected without preservatives and stored at 4°C. Within 12 hours of collection, specimens were passed through a Prefilter (Millipore, Bedford, MA) to remove cellular debris, concentrated with a Pellicon cassette system (Millipore) to approximately 125 ml, and then dialyzed against 0.05*M* Tris, 0.1*M* KCl, pH 7.0, by continuous volume dialysis. The concentrated preparations were passed over a blue Sepharose CL-6B column (2.5 × 10 cm, Pharmacia, Uppsala, Sweden) equilibrated with 0.05*M* Tris, 0.1*M* KCl at pH 7.0. The unbound fraction was concentrated using the Pellicon cassette system, at which time 1 of 2 isolation methods was used, as shown in Figure 1.

In *method A*, samples were dialyzed against a mixture of 14 simple amphoteric and nonamphoteric buffers (0.025*M* "standard buffer") as described by Prestidge and Hearn (16). Preparative isoelectric focusing using the method of Prestidge and Hearn was carried out for 20–46 hours using Sephadex G-75 fine (Pharmacia), equilibrated with 0.025*M* standard buffers. Fractions with isoelectric points between 3.8 and 4.4 were pooled, concentrated with CX-10 immersible filters (Millipore), dialyzed against 1.0*M* acetic acid, pH 2.25, and fractionated with a Sephadex G-100 column (2.5 × 25 cm).

In *method B*, fractions were dialyzed against 0.05*M* citrate buffer, pH 3.0, then passed over a SP-Sephadex column (2.5 × 5 cm, Pharmacia). Fractions were eluted with

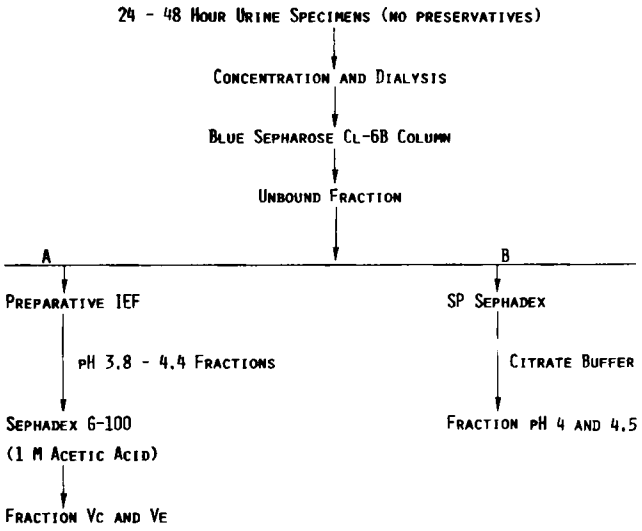

Figure 1. Outline of 2 isolation procedures yielding partially purified connective tissue activating materials from urine. IEF = isoelectric focusing.

0.05*M* citrate buffers, dialyzed against 0.1*M* NH_4HCO_3, lyophilized, and dialyzed against 0.15*M* NaCl in preparation for bioassay.

Fibroblast culture methods. Normal synovial cell strains were developed from explants obtained during amputation or arthrotomy as described previously (17). Cells were grown in T-75 flasks in medium consisting of 80% CMRL 1066, 10% fetal calf serum (FCS), 10% human serum supplemented with $NaHCO_3$, L-glutamine, HEPES buffer, penicillin, streptomycin, gentamicin, and erythromycin. Human dermal cells were obtained from explants (17) and chondrocytes by collagenase digestion of cartilage from human hip or knee surgery (18).

Glycosaminoglycan assays. The assay utilized measures ^{14}C-glucosamine incorporation into glycosaminoglycans (19). In brief, cells were trypsinized, counted with an electronic cell counter (Coulter Electronics, Hialeah, FL), and placed in microtiter polystyrene culture plates (Costar, Cambridge, MA) at a density of 2×10^4 cells/well per 0.1 ml of 99% Leibovitz-15 medium (Pacific Biologicals, Berkeley, CA) and 1% FCS. After 24 hours at 37°C, 100 μl of ^{14}C-D-glucosamine (specific activity 250 mCi/m*M*, New England Nuclear, Boston, MA) in 99% L-15, 1% FCS (0.5 μCi/ml) was added. After addition of the isotope, 20 μl of CTAP-U in 0.15*M* NaCl and control vehicles were added to the microcultures, and incubation continued at 37°C for 40 hours.

One hundred microliters of medium was spotted on Whatmann 3 MM filter paper, dried, immersed in a solution of 0.1% cetylpyridinium chloride (CPC), and washed 8 times with 0.1*N* NaOH to remove unincorporated ^{14}C-glucosamine. Isotope incorporation into GAGs was measured by liquid scintillation counting in a Beckman 7000 scintillation counter, using a toluene–POPOP-PPO system. All experimental groups were plated in quadruplicate and data were expressed as mean ± 1 standard deviation.

Sulfated glycosaminoglycans were measured by determining $^{35}SO_4$ incorporation into GAGs (5). Microcultures were handled as in the ^{14}C-glucosamine assay except that carrier-free $^{35}SO_4$ (50 μCi/ml) was the radioactive precursor. The GAG from 100 μl of media was precipitated on Whatmann 3 MM filter paper using 0.1% CPC solution, washed 4 times with 0.3*M* NaCl and 0.1% CPC, and counted in a Beckman 7000 scintillation counter.

Qualitative determination of the GAG types was accomplished by incubating 50 μl of media or control materials with fungal hyaluronidase, 50 TRU/ml (ex. *Streptomyces hyalurolyticus*) in 0.15*M* Na acetate, pH 5, chondroitinase ABC (2.5 units/ml) in 0.2*M* Tris, pH 7.5, and chondroitinase AC (2.5 units/ml) in 0.2*M* Tris, pH 7.5 for 150 minutes at 37°C, then subjecting the digested samples to the CPC-fixation wash procedure (19).

Chondrocyte proteoglycan monomer production. One million human chondrocytes in the eighth passage were incubated in T-25 flasks in 3.2 ml of 97% Eagles's synthetic medium (ESM), 3% FCS for 5 days. Then 0.3 ml of semipurified CTAP-U (835 μg/ml) or control vehicle was added along with $^{35}SO_4$ (100 μCi/ml, Amersham, England) and incubation continued for 48 hours at 37°C. The supernatant medium was removed, dialyzed against phosphate buffered saline (PBS), lyophilized, and redissolved in 1.5 ml of 0.5*M* Na acetate, pH 6.9. A 0.5-ml aliquot was subjected to gel

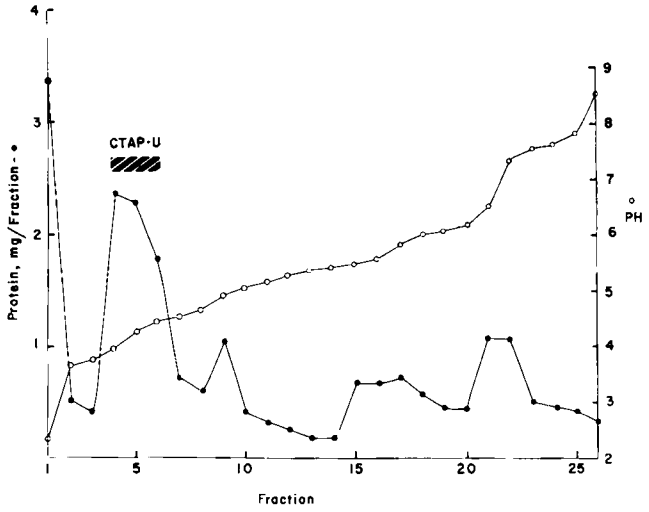

Figure 2. Preparative isoelectric focusing of urinary proteins not bound to blue Sepharose CL-6B. CTAP-U = connective tissue activating material from urine.

filtration on Sepharose 4B (1.5 × 16 cm) and Sepharose•2B (1.5 × 16 cm). The eluate was spotted on filter paper, dried, and counted in a scintillation counter.

Measurement of mitogenic activity. The rate of DNA synthesis by human synovial cells as measured by ^{3}H-methylthymidine incorporation has been previously described (4). Cells (1 × 10^{4}/well) were plated in microtiter wells (Costar) in 100 μl of 97% ESM and 3% FCS. After 24 hours at 37°C, 5 μl of CTAP-U or control material was added and incubated for 24 hours at 37°C. Fifteen microliters of ^{3}H-methylthymidine (1.5 μCi/well) in 100% ESM was added. After 24 hours, the incubation medium was discarded and the cell sheet was washed twice with PBS, 5% trichloroacetic acid, and 100% methanol and then allowed to dry. Following cell lysis with 50 μl of 0.3*N* NaOH, extracts were spotted on glass filter paper and counted in a Beckman 7000 liquid scintillation counter.

Other procedures. Polyacrylamide gel electrophoresis was performed using 15% gels in sodium dodecyl sulfate (SDS) and glycine-Tris buffers (20). Proteins were measured by a spectrophotometric method (21). Double immunodiffusion was performed using the Ouchterlony method. Mouse EGF (100 μg/ml) was purchased from either Collaborative Research (Waltham, MA) or ICN (Cleveland, OH) or was isolated from mouse submaxillary glands according to the method of Savage and Cohen (22). A sample of β-urogastrone was kindly provided by Dr. H. Gregory, Cheshire, UK. Rabbit anti-human IgG, goat anti-human Fc, and Fab fragments of IgG were purchased from Miles, Yeda, Israel. Anti-mouse EGF antisera (B/Bo = 0.35 at 1/18,000 dilution of antiserum) were raised in rabbits, using mouse EGF-URO, prepared according to the method of Savage and Cohen (22). This antiserum (MDH-150) cross-reacted weakly with human EGF-URO (B/Bo = 0.35 at a dilution of 1/300 of antiserum).

The radioreceptor assays (RRA) for epidermal growth factor, basic somatomedin (equivalent to somatomedin-C or insulin-like growth factor–I [23,24]), and for insulin were performed essentially as previously described (25). Antiserum and radiolabeled basic somatomedin were kindly provided by Drs. B. Bhaumuck and R. M. Bala, Department of Medicine, University of Saskatchewan, Saskatoon, Canada. Antibody to CTAP-III was raised in rabbits as previously described (10).

RESULTS

Isolation of CTAP-U. Urine specimens from 6 normal subjects were studied separately for their CTAP-U activity. A Pellicon cassette system with a molecular weight cutoff of 10,000 daltons concentrated and dialyzed 48-hour collections to a volume of 100 ml in 2–3 hours. In 15 experiments these crude concentrates stimulated ^{14}C-glucosamine incorporation into GAG at rates 1.6 to 7.0 times that of control; in 9 experiments the concentrates enhanced tritiated thymidine incorporation into DNA at rates 1.3 to 5.2 times control rates.

Albumin, a major contaminating protein, was removed without significant loss of biologic activity by passage over a blue Sepharose CL-6B column. The albumin-free unbound fraction was then fractionated

Table 1. Partial purification of connective tissue activating material from urine

Total fraction	Protein (mg)	Δcpm in ^{14}C-glycosaminoglycan assay*	Specific activity†
Unbound fraction from blue Sepharose CL-6B	47.5	2,393	75
pH 4.0 fraction from SP-Sephadex	6.8	2,744	221
pH 4.5 fraction from SP-Sephadex	7.3	3,250	216

* Δcpm refers to difference in ^{14}C-hyaluronic acid (cpm/100 μl media) between test and vehicle treated assay wells.

† Specific activity = Δcpm/μg protein in assay wells.

Table 2. Characteristics of connective tissue activating material from urine (CTAP-U)

Fraction	cpm, ^{14}C-glycosaminoglycan	Experimental: control*
0.15*M* NaCl	1,203 ± 180	–
0.15*M* NaCl + 10^{-3}*M* dithioerythritol (DTE)	1,423 ± 100	–
CTAP-U	3,661 ± 293	2.8
CTAP-U (heat)	3,733 ± 235	2.9
CTAP-U (acid)	4,617 ± 444	3.5
CTAP-U (2-mercaptoethanol)	4,058 ± 317	3.1
CTAP-U (DTE)	3,556 ± 132	2.5
CTAP-U + pronase	1,055 ± 108	0.8
CTAP-U + saline	4,154 ± 583	3.2
Pronase + saline	1,205 ± 216	0.9

* Ratio of cpm in experimental and control wells. See Materials and Methods for procedures used.

by isoelectric focusing; typical running voltages started near 170V and gradually increased to 1,100V over 20–40 hours. Protein recovery from the gel bed was approximately 50%.

Two major protein peaks were present, one at pH 2.3 and a second with a pH between 3.9 and 4.6 (Figure 2). Fractions from the second peak showed a four- to eightfold increase in ^{14}C-glucosamine incorporation into GAG, compared with saline controls. These fractions were free of CTAP-III, human IgG, human Fc, and Fab fragments of IgG, as determined by double immunodiffusion. Subsequent isoelectric focusing experiments were carried out for 46 hours, and lactic and proprionic acids were added to the buffer mixture to enhance the acidic end of the gel bed. This provided a reproducible linear pH gradient between 2.0 and 8.0. ^{14}C-glucosamine incorporation into GAG was again stimulated by the large protein peak between pH 3.9 and 4.6. In addition, minor stimulatory activity (experimental:control ratios 1.3–2.0) was present in fractions 12–26 (pH 5.1–7.7). These fractions formed precipitin lines with rabbit anti-human IgG and goat anti-human Fc and Fab fragments of IgG, but not with CTAP-III.

The second peak (pH 3.9–4.6) was further fractionated with Sephadex G-100 under dissociative conditions using 1.0*M* acetic acid. Two fractions (Vc and Ve) eluting near cytochrome C (molecular weight 12,384 daltons) yielded experimental:control ratios of 33.6 and 41.3, respectively ($P < 0.001$) in the ^{14}C-glucosamine assay. One of these fractions (Ve) was tested for basic somatomedin content by radioimmunoassay (RIA) and radioreceptor assay; insulin content was measured by RRA. No reaction was detected in the basic somatomedin or insulin assays in which 2 μg of protein from fraction Ve was added to assays sensitive to 0.4–1 ng of either insulin or basic somatomedin.

Since the isoelectric focusing experiments suggested that the pI of CTAP-U activity was between 3.9 and 4.6, anion exchange experiments were performed using SP-Sephadex to fractionate material which did not bind to blue Sepharose. The unbound material was applied using 0.05*M* citrate buffer, pH 3.0, then eluted batchwise with increasing pH, using 0.05*M* citrate buffers. Potent stimulators of GAG synthesis were eluted at pH 4.0 and 4.5. The specific activity, as defined in the ^{14}C-glucosamine assay, was increased by this procedure (Table 1). Fractions eluting at pH 4.0 and 4.5 did not react with antisera to CTAP-III, IgG, or Fc and Fab fragments of IgG.

Polyacrylamide gel electrophoresis of the pH 4.5 fraction using 15% gels in SDS buffer showed 2 major bands at 35,000 daltons and 25,500 daltons, a minor band at 14,000, and 4 faint bands between 50,000 and 78,000. The electrophoretic patterns for CTAP-U (pH 4.0 and 4.5 fractions) from 6 normal subjects yielded identical band patterns (data not shown).

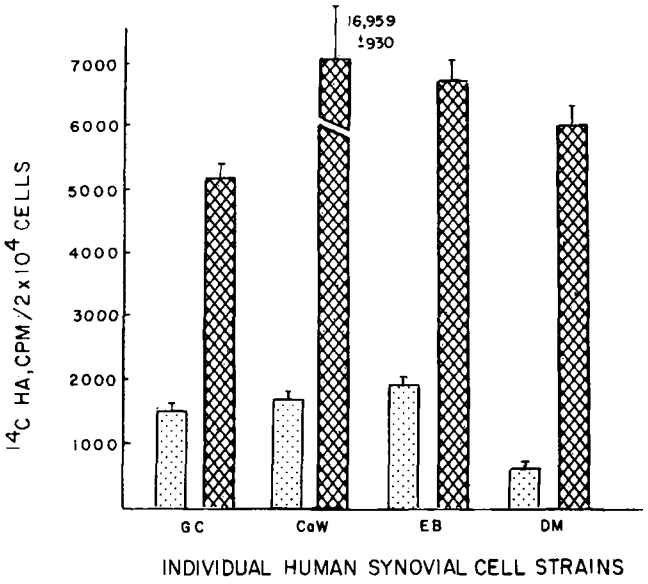

Figure 3. Partially purified connective tissue activating material from urine (CTAP-U) stimulates ^{14}C-hyaluronic acid (^{14}C-HA) synthesis in 4 different normal adult human synovial cell strains. Hatched bars = CTAP-U (fraction Vc); dotted bars = control: $P < 0.001$.

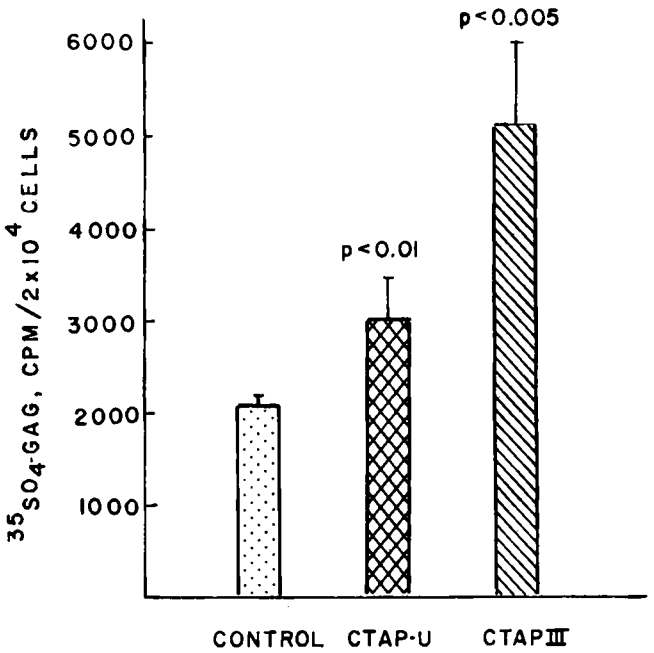

Figure 4. Partially purified connective tissue activating material from urine (CTAP-U) stimulates ^{35}S-glycosaminoglycan (GAG) synthesis by human synovial cultures. Stimulation by CTAP-U is less potent than that by CTAP-III.

Characteristics of CTAP-U. CTAP-U, like the other CTAP mediators, was inactivated by protease digestion. One milliliter of pH 4.5 fraction (734 μg/ml) was incubated with either pronase (200 μg in 0.05*M* Tris, pH 7.9) or 0.15*M* NaCl at 37°C for 4 hours, dialyzed against 0.15*M* NaCl overnight, and then tested in bioassay. As shown in Table 2, pronase completely abolished the GAG stimulatory activity of CTAP-U. Heating partially purified CTAP-U to 100°C for 10 minutes had no effect on incremental hyaluronate synthesis. Incubation of CTAP-U with either 0.001*M* dithioerythritol and 0.15 NaCl at 4°C or 1% 2-mercaptoethanol, or 1*M* acetic acid (pH 2.25) for 24 hours at 4°C, followed by dialysis against 0.15*M* NaCl for another 24 hours at 4°C, did not impair the ^{14}C-HA stimulating capacity of CTAP-U.

Effect of CTAP-U on GAG formation. CTAP-U was a potent stimulator of glycosaminoglycan formation. In 10 synovial cell strains, CTAP-U at various stages of purification and at concentrations from 1 to 830 μg/ml markedly stimulated the incorporation of ^{14}C-glucosamine into GAG. Experimental:control (E:C) ratios ranged from 2.3 to 10.0 with a mean of 5.1 ± 2.7 in different experiments. A representative experiment comparing the responses of 4 normal synovial cell strains is shown in Figure 3. Enzyme digestion of GAGs in the cell media showed that 78% was hydrolyzed by fungal hyaluronidase, while 81% was hydrolyzed by both chondroitinase ABC and chondroitinase AC. Thus, virtually all of the ^{14}C-GAG produced by the synovial cells was hyaluronic acid.

Stimulation of incorporation of $^{35}SO_4$ into GAGs produced by human synovial cells has been demonstrated previously with CTAP-III (5). Partially purified CTAP-U at a concentration of 15.4 μg/ml stimulated $^{35}SO_4$ incorporation into GAG to a modest extent ($P < 0.01$) in synovial cultures. CTAP-III (12.3 μg/ml) was a more potent stimulator of $^{35}SO_4$ GAG formation than CTAP-U (Figure 4).

Experiments with human dermal fibroblasts showed that CTAP-U stimulated ^{14}C-glucosamine incorporation into GAG. Addition of increasing amounts of partially purified CTAP-U resulted in increasing synthesis of hyaluronic acid (Figure 5). At concentrations of 60 μg/ml, maximal stimulation of hyaluronate synthesis was noted. Partially purified CTAP-U also stimulated $^{35}SO_4$ incorporation into GAG in dermal fibroblasts (E:C ratio 1.4).

Effect of CTAP-U on proteoglycan monomer formation by chondrocytes. Medium from human chondrocytes incubated with $^{35}SO_4$ and fractionated over Sepharose 4B showed a radioactive peak in the void

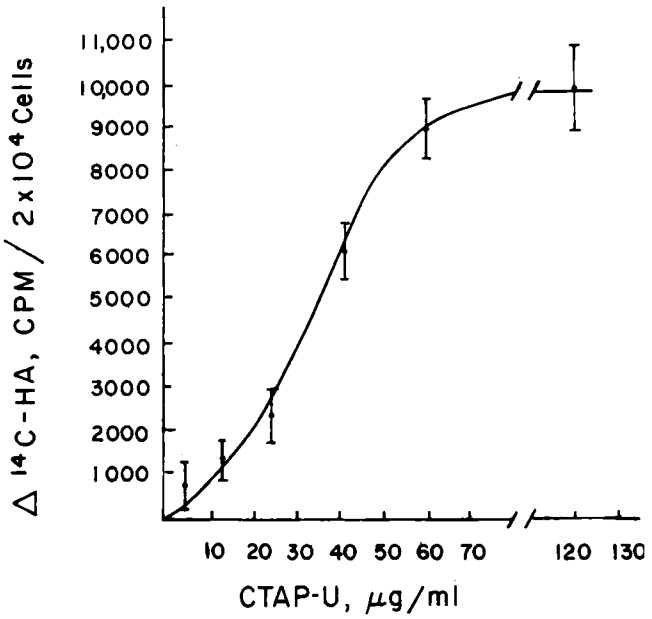

Figure 5. Dermal fibroblasts show increasing ^{14}C-hyaluronic acid (^{14}C-HA) synthesis in response to increased amounts of partially purified connective tissue activating material from urine (CTAP-U).

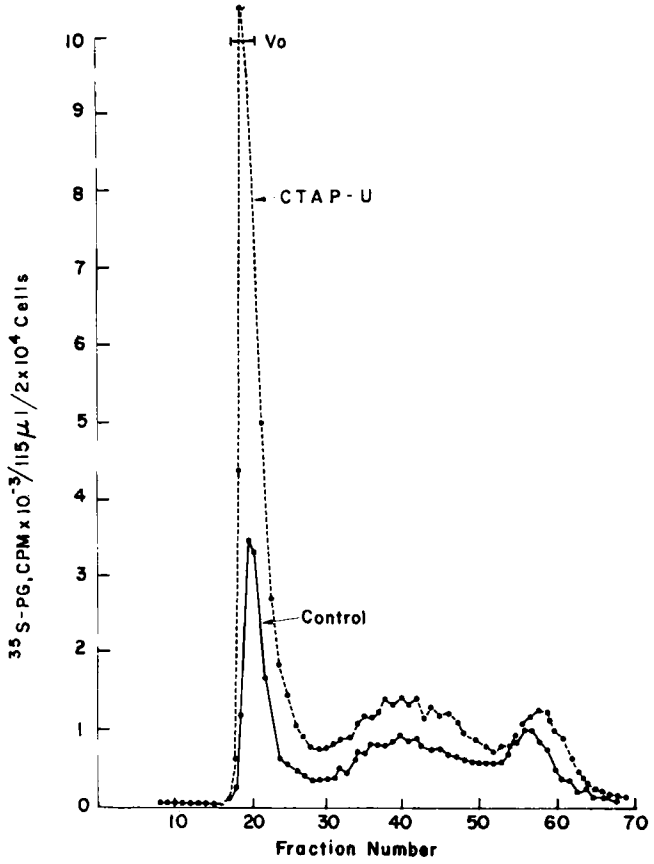

Figure 6. Connective tissue activating material from urine (CTAP-U)–stimulated human chondrocytes secrete ^{35}S-proteoglycan, which elutes in the void volume (Vo) of a Sepharose 4B column.

volume. The size of these macromolecules is presumably greater than 3 million daltons and corresponds with the size of proteoglycan (PG) monomer. CTAP-U (71 µg/ml) stimulated increased incorporation of $^{35}SO_4$ into ^{35}S-PG in chondrocyte cultures (Figure 6). Fractionation of ^{35}S-PG from chondrocyte media on Sepharose 2B (data not shown) showed a peak of radioactivity in the internal volume of the column, indicating that these cells form little proteoglycan aggregate. These data suggest that CTAP-U is a potent stimulator of proteoglycan monomer formation by human chondrocytes in vitro.

Effect of CTAP-U on DNA synthesis. CTAP-U stimulated ^{3}H-methylthymidine incorporation into DNA in human synovial cells. A dose-response curve for partially purified CTAP-U is shown in Figure 7. Maximal stimulation of DNA synthesis in synovial cells was reached with concentrations of 40 µg/ml of CTAP-U.

Can EGF-URO account for the activity of CTAP-U? The possible contamination of CTAP-U by EGF-URO was evaluated by a radioreceptor assay with a lower limit of sensitivity of 0.4 ng EGF-URO. In the RRA for EGF-URO, a small amount of competition was observed for fraction Ve, corresponding to a maximum of 0.38 ng of EGF-URO for each 1 µg of fraction Ve. Further, the negative reaction of 5.3 µg of the pH 4.5 CTAP-U fraction in the RRA assay made it possible to assign a similar limit for the potential content of EGF-URO with this particular CTAP-U preparation.

To ascertain whether the amounts of EGF (0.4 ng per 1.0 µg CTAP-U) which might contaminate the CTAP-U fraction could account for the observed biologic activity, mouse EGF (and in one experiment, urogastrone) was tested for biologic activity at concentrations similar to those that may have contaminated the purified CTAP-U fractions. From the experiments shown in Table 3, it was clear that 1–30 µg/ml of CTAP-U caused substantial stimulation of GAG syn-

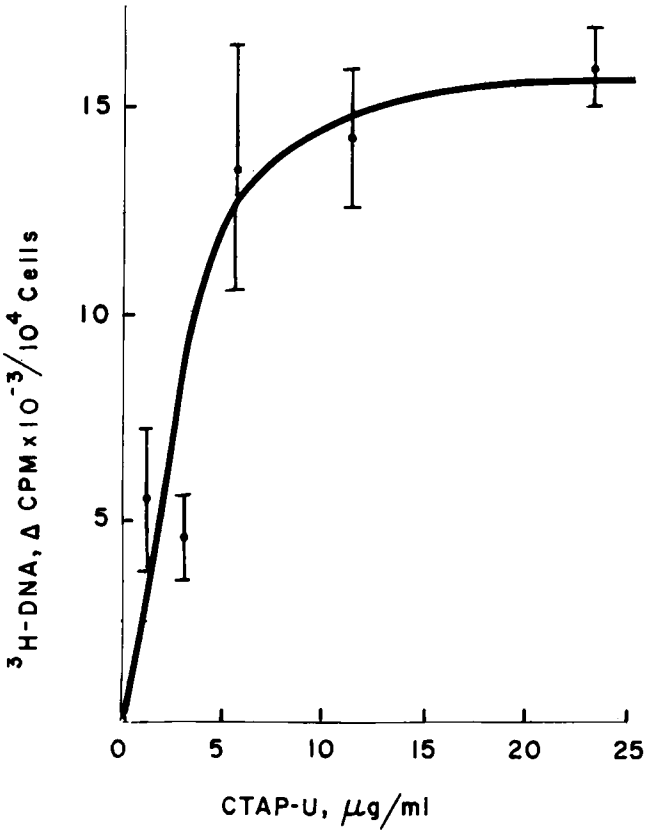

Figure 7. Increasing amounts of purified connective tissue activating material from urine (CTAP-U) (fraction at pH 4.0) stimulates increasing ^{3}H-DNA synthesis.

Table 3. Comparison of biologic activity of connective tissue activating material from urine (CTAP-U) and epidermal growth factor (EGF)

Experiment no.	Agonist*	Concentration	Experimental:control ratio	
			^{14}C-glycosaminoglycan	^{3}H-DNA
2663	CTAP-U	2 μg/ml	1.4	
		8 μg/ml	3.1	
		30 μg/ml	9.7	
	EGF-1	5 ng/ml	0.8	
		50 ng/ml	1.0	
2665	CTAP-U	2 μg/ml	2.5	
	EGF-1	200 ng/ml	1.4	
		40 ng/ml	0.9	
2667	CTAP-U	1 μg/ml		3.4
		2 μg/ml		5.9
		30 μg/ml		14.4
	EGF-1	1 ng/ml		1.7
		2 ng/ml		1.7
		20 ng/ml		4.2
		200 ng/ml		12.3
2670	CTAP-U	4 μg/ml		6.4
	EGF-2	5 ng/ml		2.4
2674	CTAP-U	1 μg/ml		3.9
	EGF-3	1 ng/ml		4.0
2676	CTAP-U	40 μg/ml	6.9	
	EGF-3	50 ng/ml	2.1	
2688	Urogastrone	50 ng/ml	3.0	1.8

* CTAP-U was a highly purified fraction eluted at pH 4.0 from SP-Sephadex; EGF-1, 2, and 3 were mouse EGF obtained from 3 different sources.

thesis in human synovial cells. Although with these concentrations of CTAP-U one could not, by the radioreceptor assay alone, rule out the presence of a maximum of 2.4 ng/ml EGF-URO, the simultaneous assay of mouse EGF-URO showed that up to 50 ng/ml EGF-URO caused little stimulation of HA synthesis in synovial cell cultures (Table 3). Thus, EGF-URO, if present, could not account for the ability of CTAP-U to stimulate ^{14}C-HA synthesis by synovial cells. However, the concentration of CTAP-U (fraction at pH 4.0, 5–10 μg/ml; Figure 7) that caused near maximal stimulation of thymidine incorporation might possibly have contained sufficient EGF-URO (up to 0.8 ng/ml) to account for some of the mitogenic activity of CTAP-U.

DISCUSSION

The data presented here indicate that human urine contains a factor or factors (denoted here collectively as CTAP-U) that activate human connective tissue cells in vitro. The protein fractions isolated from urine were able to stimulate glycosaminoglycan synthesis (both hyaluronate and SO_4-GAG) in human synovial and dermal cell cultures and to stimulate ^{3}H-thymidine incorporation in synovial cell cultures. In addition, the CTAP-U fractions stimulated $^{35}SO_4$-PG formation in human chondrocyte cultures, probably as proteoglycan monomer. The CTAP-U activity was nondialyzable, sensitive to proteases, and stable in the presence of thiols and acid. Most of the biologic activity was contained in protein fractions having apparent isoelectric points between 4.0 and 4.5.

An important issue in the present work was to determine whether the connective tissue activating factor(s) present in human urine might correspond to other characterized "growth factors" known to alter connective tissue metabolism (26). The growing list of such factors includes substances that have been, in large part, isolated from blood, blood products, and glandular tissue; little is known about the metabolic fate and urinary excretion of these substances.

One growth factor known to be excreted in human urine is the well-characterized EGF-urogastrone (isoelectric pH 4.5), which in normal adults is excreted in the urine in amounts ranging from 120–1,360 ng/kg/24 hours (27,28). In addition, human urine is known to contain components (molecular weights 28K and 33K) that are much larger than EGF-URO, but that cross-react with EGF-URO in radioreceptor and radioimmunoassays (29). Although it was not possible to exclude unequivocally the presence of a small amount (less than 0.001%) of EGF-URO in the CTAP-U preparations, the radioreceptor assay demonstrated clearly that the CTAP-U preparations could not have contained sufficient EGF-URO to account for the marked stimulation of GAG synthesis in synovial cells.

Our "negative" result with highly purified EGF-URO can be contrasted with work with human fibroblasts wherein EGF-URO (6 ng/ml, or $10^{-9}M$) has been reported to stimulate incorporation of ^{3}H-glucosamine into putative extracellular GAG (30). Moreover, unlike EGF-URO, which has been reported not to stimulate $^{35}SO_4$ incorporation into human fibroblast GAG (14), the CTAP-U clearly did stimulate $^{35}SO_4$ incorporation into GAG. Taken together, the data indicate that the connective tissue activating activity of human urine described here does not represent EGF-URO. Likewise, the RRA and RIA analyses indicate that the CTAP-U preparations did not contain

appreciable amounts of either basic somatomedin (isoelectric pH 8.6) or insulin (isoelectric pH 5.30 to 5.35).

Immunoglobulin G and some of its components have been shown to stimulate HA synthesis in synovial cells (31). A large number of serum proteins are detectable in normal urine and, in general, clearances of serum proteins tend to follow their molecular weights. IgG is the second most prevalent serum protein excreted; Fc and Fab fragments of IgG are present in lesser concentrations. In this study, urinary fractions containing IgG and IgG fragments showed stimulation of ^{14}C-GAG synthesis (1.3–2 times control values). Urinary IgG and its fragments probably account for some of the biologic activity seen in concentrated urine specimens; however, the stimulatory activity of the CTAP-U fractions was clearly independent of IgG.

The nature of the connective tissue activating material in urine is uncertain. CTAP-U may represent an excretory form of members of the CTAP family or of other endogenous growth factors; alternatively, it may be a substance unique to the urinary tract. CTAP-U differs from CTAP-III, since it did not cross-react with CTAP-III antibodies; chemical characteristics and biologic activities appear to distinguish CTAP-U from CTAP-I, which is not mitogenic in synovial cell cultures (4). CTAP-U might be related to the platelet-derived CTAP-P_2, since CTAP-P_2, like CTAP-U, is stable to heat and thiols and is able to stimulate both GAG and DNA synthesis in synovial cell cultures (6). Because there are several protein constituents in the fractions from human urine that activate connective tissue cells, it will be necessary to obtain each of these components in homogeneous form in order to establish the relationship of the CTAP-U polypeptide(s) to other known growth factors.

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