CONNECTIVE TISSUE ACTIVATION

XXXVI. The Origin, Variety, Distribution, and Biologic Fate of Connective Tissue Activating Peptide–III Isoforms: Characteristics in Patients with Rheumatic, Renal, and Arterial Disease

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Objective. **To determine the origin, distribution, and biologic fate of platelet-derived connective tissue activating peptide–III (CTAP-III), to define the relative amounts of the antigen forms (CTAP-III, beta-thromboglobulin [β-TG], neutrophil activating peptide–2 [NAP-2]) in plasma of normal persons and those with rheumatic or end-stage renal disease, and to define the isoforms of CTAP-III in platelets, plasma, transudates, and tissue deposits.**

Methods. **CTAP-III in plasma was measured by enzyme-linked immunosorbent assay, and growth promoting activity of CTAP-III isoforms was tested in synovial and peritoneal cell cultures by measuring increased synthesis of ^{14}C-glycosaminoglycan (^{14}C-GAG) and ^{3}H-DNA. Isolated CTAP-III was characterized by Western blotting, microsequencing, and mass spectrometry.**

Results. **CTAP-III was the primary isoform of this antigen family in normal platelets and platelet-rich plasma; β-TG and NAP-2 accounted for <1% of CTAP-III isoforms. Previously undescribed isoforms, i.e., CTAP-III des 1, des 1-2, des 1-3, and a phosphate adduct of CTAP-III, were observed in varying amounts. Elevated plasma levels of CTAP-III antigen were found in a substantial fraction of rheumatic disease patients: 24% of those with rheumatoid arthritis, 36% of those with systemic sclerosis, and 15% of those with systemic lupus erythematosus. All 10 patients with end-stage kidney disease had marked elevations of plasma CTAP-III levels, which stimulated DNA and GAG synthesis by peritoneal cells in culture. Only large isoforms (such as CTAP-III) were detected in venous plasma of normal subjects, rheumatic disease patients, and patients receiving long-term dialysis. Normal human spleen and kidney contained substantial (μg/gm) amounts of CTAP-III and traces of an isoform with the electrophoretic mobility of CTAP-III des 1-15/NAP-2. Liver, lung, and urine contained lesser (ng/gm) amounts of CTAP-III.**

Conclusion. **These data show that, among the 10 known isoforms, intact CTAP-III itself was the major circulating isoform (>90%), and β-TG was the most rare (0–1%). Deposition of CTAP-III in tissues, such as synovium, spleen, and kidney, is associated with partial processing to NAP-2–like isoforms and the potential to induce neutrophil and fibroblast activation in patients with rheumatic or end-stage renal disease.**

Connective tissue activating peptide–III (CTAP-III) is a human platelet α-granule–derived growth factor that is more than 1,000 times as abundant as other growth factors in platelets. The amino acid sequence of CTAP-III has been determined and confirmed by cloning the gene (1–3). Early studies of a CTAP-III isoform, β-thromboglobulin, (β-TG, identical to CTAP-III des 1-4) did not attribute biologic activities to this entity. Radioimmunoassay (RIA) of β-TG indicated that it has a plasma half-time of 100

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minutes and that it equilibrates with synovial and amniotic fluids but not cerebrospinal fluid; urine levels of β-TG antigen are ~0.5% of venous plasma levels (4). Protein sequence homology studies show CTAP-III and its isoforms to be members of a family of proteins that includes neutrophil activating peptide–1 (NAP-1)/interleukin-8, melanoma growth-stimulating activity, human GRO, 9E3, γ-IP–10, and platelet factor–4, entities thought to be associated with growth, wound repair, inflammation, and neoplasia (5). Others have included these proinflammatory agonists in a supergene "intercrine" cytokine family (6).

Many of the biologic activities of CTAP-III are anabolic; CTAP-III stimulates synthesis of DNA, hyaluronic acid (HA), sulfated glycosaminoglycan (GAG) chains, proteoglycan monomer, and proteoglycan core protein in human synovial fibroblast cultures (1,7–10). CTAP-III also stimulates glucose transport, formation of prostaglandin E_2, HA synthetase activity, and plasminogen activator activity (11–14). Elevated plasma levels of CTAP-III/β-TG antigen have been found by RIA in patients with rheumatoid arthritis (RA), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), and forms of vasculitis (15,16). Immunoassay of CTAP-III in human serum shows concentrations ranging from 5,000 to 35,000 ng/ml; such levels stimulate DNA and GAG synthesis in vitro and may resemble the amounts seen near connective tissue cells during blood clotting in microenvironments where platelet α-granule release occurs (17).

Isoelectric focusing of CTAP-III from platelets stored frozen for years revealed marked structural microheterogeneity; subsequently, several amino-terminal cleavage products were found, which appear to result from proteolytic activity in platelet preparations (17–19). Three isoforms, CTAP-III des 1-13, des 1-14, and des 1-15/NAP-2, stimulate ^{14}C-GAG synthesis; the latter 2 isoforms also promote ^{3}H-DNA synthesis in human fibroblast cultures (17,20). Recent data show that CTAP-III causes histamine release from basophil granules and that the CTAP-III (des 1-15)/NAP-2 isoform is chemotactic for human neutrophils (21,22). A related study showed that CTAP-III and its isoforms compete with NAP-1 for a receptor on human neutrophils (23).

Both CTAP-III and an isoform with the electrophoretic mobility of CTAP-III/NAP-2 have been detected in synovial tissue from patients with osteoarthritis (OA) and RA (24). The finding of CTAP-III isoforms in diseased tissue and data showing that some cleavage isoforms acquire new and potent biologic activities highlight the importance of studying qualitative and quantitative differences and distribution of CTAP-III isoforms in health and disease states. All CTAP-III isoforms (CTAP-III, CTAP-III des 1-4/β-TG, CTAP-III des 1-13, CTAP-III des 1-14, and CTAP-III des 1-15/NAP-2) are equally well detected by Western blotting using polyvalent antisera raised against CTAP-III (17,18). The present study examined the quantity and isoforms of CTAP-III in platelets from circulating blood, plasma, urine, tissues, and organ storage/sequestration sites, thus providing a clearer idea of the relative amounts and distribution of specific CTAP-III isoforms. The investigations were performed using Western blotting and, where possible, microsequencing and/or mass spectrometry.

PATIENTS AND METHODS

Patient and control groups. All studies were carried out with protocols previously approved by the Institutional Review Board, with the informed consent of participating individuals. The normal control group comprised healthy volunteers from hospital personnel.

Patients with diabetes, rheumatic disease, or renal disease. To clarify the relationship of CTAP-III/β-TG antigen to diabetes, we studied patients with type I diabetes who were less than 40 years old. Patients were excluded if they had hypertension, vascular occlusive disease, diabetic ulcers, renal insufficiency, or proliferative retinopathy, or if, in the prior week, they had taken a medication known to interfere with platelet function.

Patients with rheumatic diseases met the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA, SLE, or SSc (25–27). A brief history, physical examination findings, and relevant laboratory data were recorded on a standardized form for each rheumatic disease patient, and a score thought to reflect the level of active inflammation was assigned based on the clinical data. Asymptomatic patients were given a score of 0, patients whose clinical symptoms had a minimal effect on functional status were given a score of 1, patients whose functional status was significantly impaired were given a score of 2, and those whose symptoms were so debilitating as to require maximal therapeutic intervention were given a score of 3. Virtually all patients were receiving antirheumatic drugs at the time of blood sampling.

Ten patients undergoing maintenance dialysis treatment for irreversible chronic renal failure were studied; 5 were receiving hemodialysis and 5 were receiving long-term peritoneal dialysis. Samples from hemodialysis patients were obtained before dialysis, after 30 minutes of dialysis and at the end of the procedure. Blood samples from patients treated with peritoneal dialysis were obtained at random times during dialysis, and peritoneal dialysate fluid was collected after 3 hours of equilibration.

Patients undergoing coronary angioplasty. Fifteen patients were referred for elective coronary angioplasty for treatment of angina. The group included 13 men and 2 women (mean ± SD age 60 ± 8 years); 10 had unstable angina pectoris. Coronary angioplasty was performed utilizing standard techniques, including femoral artery cannulation with 8 or 9 French catheters to allow placement of 8 or 9 French heparin-bonded coronary guiding catheters to instrument the coronary arteries. Patients received 325–975 mg of aspirin for at least 2 days prior to the procedure; heparin (10,000 units) was infused as an intravenous bolus at the beginning of the procedure, and then 5,000 units was administered every 60 minutes thereafter. Several patients were receiving calcium channel blockers or nitrates.

Collection and processing of blood samples for enzyme-linked immunosorbent assay (ELISA). Peripheral venous blood was drawn from each subject's arm through a 21-gauge butterfly needle. The initial 2–3 ml was discarded and the subsequent 3 ml was placed in a tube with 300 μl of a platelet-stabilizing solution (Thrombotect, consisting of 2.5% EDTA, 0.025% 2-chloroadenosine, 7% procaine HCl), mixed by gentle inversion, and placed on ice. Plasma was separated from whole blood by centrifugation at 1,500*g* for 30 minutes at 4°C and stored at −20°C.

In the patients undergoing angioplasty, arterial and central venous blood samples for ELISA were obtained from the aorta, coronary arteries, inferior vena cava, and coronary sinus with a heparin-bonded balloon catheter, and anticoagulated as noted above. A 2.5–3.5-mm USCI LPS 2 or Profile Plus balloon catheter was advanced to the tip of the guiding catheter to obtain the first sample for measurement of CTAP-III. Samples were obtained with slow aspiration into Thrombotect and heparin–containing syringes and placed on ice. The stenosis to be treated was then crossed with a 0.14″ flexible, steerable guidewire which was used to support and direct the balloon catheter. The balloon catheter was centered on the stenosis and inflated to 4–10 atmospheres as many times as necessary (usually 2–6) to achieve an adequate posttreatment result. Following the final inflation, the guidewire was removed from the balloon catheter and another plasma sample was obtained. The balloon catheter was left across the stenosis and serial samples were obtained, usually at 5, 10, and 20 minutes after the final balloon inflation. The balloon catheter was then withdrawn proximal to the stenosis and further samples obtained.

Measurement of CTAP-III in platelet-pack plasma. Platelet packs were obtained 1 day after the date of expiration for human use; after centrifugation at 1,375*g* for 20 minutes at 4°C, supernatants were pooled and filtered through 0.65 μ*M* and 0.22 μ*M* Millipore filters. CTAP-III was measured by radial immunodiffusion (RID); measurements of ring diameters were made at 48 hours and compared with known standards. Five observations were made on pools of 10 or more platelet units; 3 measurements were from individual bags containing 1, 4, or 6 units per bag.

Isolation of CTAP-III from human platelets, tissues, plasma, and other biologic fluids. CTAP-III was isolated from human platelets by acid–ethanol extraction, acetone precipitation, gel filtration, heparin affinity chromatography, and immunoaffinity chromatography, as reported previously (18,28). Other tissue and fluid samples were also extracted with acid–ethanol and processed for Western blotting; in some instances, additional purification was carried out to facilitate structural studies. The extraction protocol utilized 10 volumes of acid–ethanol (95% ethanol, 5% 1.25*N* HCl) per ml or gm of sample (plasma, platelet-pack plasma, peritoneal dialysis fluid, or tissue homogenates); after mixing, extraction was continued for 24–48 hours at 4°C with slow stirring. Preparations were centrifuged at 20,000*g* for 10 minutes; the supernatant fluid was dialyzed against water, lyophilized, and reconstituted in a smaller volume for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). For structural studies, CTAP-III was isolated from outdated platelet-pack plasma (~6 days post–blood donation) by acid–ethanol extraction, further simplified by agarose chromatography (CM Bio-Gel; Whatman, Hillsboro, OR), and subsequently isolated by immunoaffinity chromatography (24). Human spleen and other tissues were homogenized as earlier reported for synovial tissue (24), extracted by acid–ethanol, and CTAP-III was then isolated (when CTAP-III concentrations permitted) in the same manner as noted above for platelet-pack plasma.

Protein measurement and antiserum development. Protein was measured by a colorimetric method (29) and/or UV absorption (30). Antiserum to native CTAP-III and recombinant CTAP-III (rCTAP-III) was raised in rabbits. Male New Zealand white rabbits (8–10 weeks old) were immunized with 500 μg of CTAP-III in 0.15*M* NaCl in Freund's complete adjuvant. Booster injections with antigens in Freund's incomplete adjuvant were given at 6 and 12 weeks. Animals were bled at 4 weeks and then biweekly.

Immunologic measurement of CTAP-III isoforms. The ELISA used in these studies employs a double-antibody sandwich procedure using affinity-isolated anti–CTAP-III antibody and its biotinylated counterpart with an avidin–biotin–peroxidase detection system (Vector Laboratories, Burlingame, CA) (28). Measurement of CTAP-III by RID utilized filtered, heat-inactivated rabbit anti-human CTAP-III antiserum; this method is effective over a range of 2–50 μg/ml (31).

Analytic polyacrylamide gel electrophoresis. CTAP-III and its isoforms were analyzed by SDS-PAGE in 8*M* urea/8% total acrylamide (28,32). Proteins separated by SDS-PAGE were detected by either silver or Coomassie brilliant blue R-250 staining (28,33). Proteins separated by SDS-PAGE were prepared for Western blotting by transferring onto Immobilon-P using a semi-dry blotter (Polyblot; American Bionetics, Hayward, CA) and then identified by Coomassie brilliant blue R-250 and immunostaining with antisera to rCTAP-III (28,32). Alternatively, Western blots of proteins, following electrophoretic separation, utilized immobilization in a nitrocellulose membrane (28). Membrane-bound antigens were probed with antisera to rCTAP-III (1:500 or 1:2,000) and the complexes were detected with a Bio-Rad immunoblot assay kit (horseradish peroxidase–conjugated goat anti-rabbit IgG; Bio-Rad, Richmond, CA) or by staining for alkaline phosphatase.

Amino acid sequence determination. Aqueous protein solutions were applied to a glass fiber filter pretreated with 2.0 mg of Biobrene and subjected to NH_2-terminal sequencing using an Applied Biosystems (Foster City, CA) 470A gas

phase protein sequencer interfaced with an Applied Biosystems 120A analyzer according to standard protocols (34).

Mass spectrometric measurements. Mass measurements were made on a Vestec (Houston, TX) single quadruple mass spectrometer with a Vestec electrospray interface (35,36). The protein concentration utilized in these studies was 100 ng/ml in 4% acetic acid, 50% acetonitrile. The flow rate was 6 μl/minute. Data were acquired using a Teknivent Vector-2 acquisition system. The data were further processed, and the multiply charged ion series observed for each component were deconvoluted and the calculated values determined using PROCOMP 1.2 (37).

Cell culture methods. Cultures of human synovial cells from patients with OA were derived from explants obtained at arthrotomy (7,8). Monolayer cultures were grown in T-75 flasks in CMRL 1066 medium (Gibco, Grand Island, NY) supplemented with 5% human serum (HS), 10% neonatal calf serum (NCS), and 5% fetal calf serum (FCS), sodium bicarbonate, L-glutamine, 0.02*M* HEPES buffer, penicillin, streptomycin, and gentamicin. Cell strains were studied at the second through fifth passages after removal from explants. Cells from 500 ml of peritoneal dialysis fluid were pelleted by gentle centrifugation (~1,000 revolutions per minute, in a model HN-S centrifuge; IEC, Needham Heights, MA) for 10 minutes. The cell pellet was resuspended in 1.0 ml of peritoneal fluid and plated in a 10-mm well of a 24-well culture dish (Costar; Cambridge, MA). After 48 hours of incubation at 37°C in 95% air, 5% CO_2, adherent cells were observed; the medium was then changed to CMRL 1066_{80} $NCS_{15}HS_{15}FCS_5$ (supplemented as above) and incubation continued. After 9 days, there was confluent growth of fibroblast-like cells; these were passaged with trypsin to a 35-mm well of a 6-well culture dish for subsequent study.

Measurements of growth parameters by isotope incorporation. Measurement of ^{14}C-glucosamine incorporation into ^{14}C-GAG, mostly ^{14}C-HA, was effected by plating cells in 96-well microtiter plates, 10^4 cells/well in 200 μl of semisynthetic medium (Leibovitz medium, L-15; Hazelton Biologics, Lenexa, KS), with 1% FCS, L-glutamine, penicillin, gentamicin, streptomycin, sodium carbonate, and 0.02*M* HEPES buffer, pH 7.6 (7,28). Test materials and vehicles had contact with cells for ~44 hours before medium was harvested for assay. Incorporation of isotope into secreted ^{14}C-HA was measured by scintillation counting following the cetylpyridinium chloride–fixation wash procedure (7,28).

To measure ^{3}H-thymidine incorporation into synovial cell DNA, cells were plated, 10^4 cells/microtiter well, in 100 μl of Eagle's synthetic medium (ESM) supplemented with 1% FCS, antibiotics, L-glutamine, and HEPES buffer and incubated in a humidified chamber at 35–37°C (28,31). After 20 hours of incubation, test samples or vehicles were added (5–15 μl/well) and incubation continued for 24 hours; ^{3}H-methylthymidine (1.5 μCi/15 μl ESM/well) was then added and incubation resumed for 24 hours. Medium was aspirated, discarded, and cell sheets were washed, first with phosphate buffered saline (pH 7.0), 5% trichloroacetic acid and then with absolute methanol. After drying at 35°C, cells were lysed for 1 hour at 37°C with 50 ml of 0.3*N* sodium hydroxide. Fifty microliters of cell lysate was pipetted into a counting vial containing Ecolume cocktail, and radioactivity was measured in a scintillation counter.

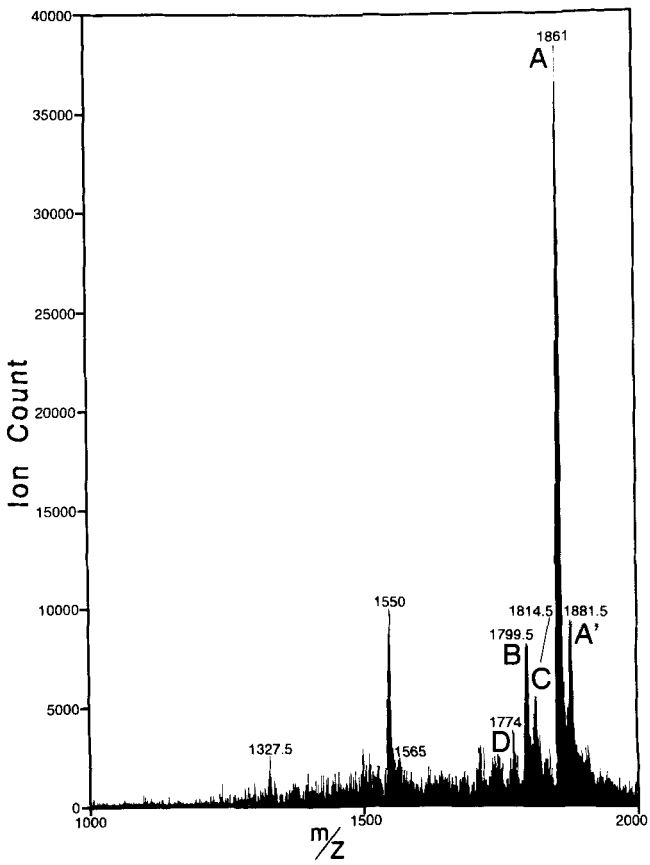

Figure 1. Electrospray mass spectrum analysis of immunoaffinity-purified connective tissue activating peptide-III (CTAP-III) isolated from human platelets, showing a cluster of +5 charged species ranging from m/Z 1,774 to 1,881.5. The mass peaks correspond to intact CTAP-III (A), the noncovalent phosphate adduct of CTAP-III (A'), CTAP-III des 1-3 (B), CTAP-III des 1-4/beta-thromboglobulin (β-TG) (C), and an unidentified component (D). Electrospray mass spectrometry generates protein ions having multiple charges by spraying them into a vacuum region at high electric potential. The mass:charge ratio of the ions is measured, allowing the mass of the compounds to be calculated from the mass:charge ratio for each charge state and the ionic charges themselves. The ion peaks at m/Z 1,550 and m/Z 1,327.5 represent the +6 and +7 charge states, respectively.

RESULTS

Intact CTAP-III is the primary isoform present in platelets. Earlier we estimated that CTAP-III isolated from washed platelets was ~86% intact CTAP-III, 4% β-TG, and 10% CTAP-III des 1-15/NAP-2 (17). Since earlier preparations used platelets that had been stored frozen for many years, it was possible that

Table 1. Connective tissue activating peptide III (CTAP-III) isoforms isolated from 6 different platelet preparations*

Preparation no.	Isoform, %						Relative specific activity†	
	Deamidated	des 1	des 1-2	des 1-3	des 1-4	Intact CTAP-III	GAG	DNA
P2011	57	NF	10	27	Trace	64	11.0	780
P2012	53	NF	10	28	NF	63	68.7	956
P2017	70	14	15	22	Trace	50	99.2	1,049
P2027	29	NF	5	14	Trace	81	6.1	258
P2028	25	NF	6	14	Trace	81	41.7	1,881
P2030	34	NF	7	Trace	Trace	93	37.7	1,324

* Quantitation of the various forms of CTAP-III is based on the quantities of the respective PTH–amino acids in the second sequenator cycle. The first cycle was not used for quantitation, to avoid interference from any contaminating free amino acids which might have been present. Specific activity was not correlated with age of preparation or the percentage of deamidated or proteolytic isoforms. The percent deamidated CTAP-III is based on the quantities of PTH-Asp and PTH-Asn observed in the first sequenator cycle. The presence of the des 1 form was indicated by a high level of PTH-Leu in the first sequenator cycle and PTH-Ala in the second. NF = not found; "trace" indicates <1%.
† Defined as increase in cpm/μg/ml of CTAP-III in the culture well, determined during the initial portion of the dose-response curve. GAG = glycosaminoglycan.

isoforms formed during prolonged cold storage. In the present study we isolated CTAP-III from recent platelet packs and used microsequencing and mass spectrometry to determine the CTAP-III isoform content. CTAP-III isolated from frozen human platelets ranged from 195 to 455 μg/gm of wet platelets (mean ± SD 333 ± 98 μg/gm). Current CTAP-III preparations showed only a trace of CTAP-III des 1-15/NAP-2–sized isoform with silver stains or Western blots, suggesting that this isoform represents <1% of the applied protein.

Mass spectrometric analysis of CTAP-III isolated from platelets revealed mostly intact CTAP-III and traces of several cleavage isoforms; these included β-TG, CTAP-III des 1-3, and a phosphate adduct of CTAP-III (Figure 1). Neither of the latter 2 isoforms has previously been reported. Separation by reverse-phase high performance liquid chromatography showed a large CTAP-III peak and several small peaks; these were shown by Western blotting to be reactive with anti rCTAP-III. Sequence analysis confirmed that intact CTAP-III was the dominant species and also verified the presence of CTAP-III des 1-3; the other small peaks were apparently blocked and did not sequence.

To better define the frequency and quantity of amino-terminal isoforms, 6 CTAP-III preparations were used for amino-terminal sequencing in amounts sufficient to permit measurement of secondary and tertiary sequences. The results are shown in Table 1. These data revealed the presence of the des 1 and des 1-2 isoforms and confirmed the presence of the des 1-3 isoform; very little β-TG was found. The 3 oldest preparations (isolated between June 1991 and February 1992) exhibited the most amino-terminal modification; the 3 most recent preparations (isolated between June 1992 and August 1992) contained 81–93% intact CTAP-III. Neither the degree of amino-terminal deamidation nor amino-terminal proteolytic cleavage had a clear effect on the anabolic activities of these preparations.

Molecular isoforms of CTAP-III in plasma. CTAP-III released from the platelet α-granule into the plasma may be enzymatically converted to smaller isoforms by proteolytic enzymes, including the tryptic, chymotryptic, and elastase-like enzymes found in the platelets themselves (38,39). To evaluate this we examined platelet-pack plasma, which contained platelet releasate 5–6 days of age, as well as fresh, conventional plasma samples. In platelet-pack plasma the large concentration of CTAP-III (Table 2) is material present at the time of blood donation in addition to any contributed by platelets releasing α-granule components on activation by ex vivo storage and/or preparative procedures.

Table 2. Plasma concentrations of connective tissue activating peptide–III (CTAP-III)*

Source (no. of samples)	CTAP-III, ng/ml
Peripheral veins, healthy adults (26)	54.0 ± 10.7
Peripheral veins, diabetes patients (26)	55.4 ± 19
Inferior vena cava and coronary sinus (4)	60.8 ± 18
Aorta and coronary arteries (10)	88.4 ± 30.1
Plasma from platelet packs (8)	51,374 ± 15,910

* Measurements in the first 4 plasma groups were carried out by enzyme-linked immunosorbent assay; CTAP-III levels in platelet-pack plasma were measured by radial immunodiffusion. Values are the mean ± 1 SD.

In earlier studies, CTAP-III isolated from platelet-pack plasma showed only the amino-terminal sequence of CTAP-III; no β-TG was detected (24). One could argue, however, that selective extraction had occurred with salt fractionation; therefore, we reexamined the CTAP-III isoform content of platelet-pack plasma by extracting it with acid–ethanol (the standard extraction method in this study) and then isolating CTAP-III by sequential cation-exchange and immunoaffinity chromatography. Fourteen micrograms of this material was submitted to 4 cycles of amino-terminal sequencing: the sequence detected was N/D-L-A-K-, characteristic of CTAP-III; no secondary sequence was seen, again indicating that neither β-TG nor CTAP-III des 1-15/NAP-2 was present in significant amounts in plasma. We previously reported the frequent partial deamidation of the NH_2-terminal asparagine of CTAP-III isolated directly from platelets (18). This modification has no effect on anabolic biologic activities. Although not detected by sequencing, it was clear that trace amounts of small CTAP-III isoforms are present in such plasma, as shown by Western blot (Figure 2, lane 3).

The electrophoretic mobility of CTAP-III isoforms in plasma samples of 4 healthy volunteers (3 men, 1 woman) is shown in Figure 3. All contained a large CTAP-III isoform compatible with the intact form. Not even trace amounts of the small isoforms were detected.

Molecular isoforms of CTAP-III at sites of platelet sequestration. Since certain organs, such as liver and spleen, have been considered to be sites of pooling, sequestration, and/or destruction of platelets, we studied CTAP-III isoforms detectable in spleen, liver, lung, and kidney. Two normal human spleens were extracted with acid–ethanol; these preparations contained 25–35 μg of CTAP-III isoforms/gm wet weight of tissue, as measured by RID. This concentration is consistent with what would be expected if 10% of the total platelet population (a normal value) were pooled in the spleen. One normal spleen preparation was further processed by cation-exchange and immunoaffinity chromatography; 70 μg of CTAP-III isoforms was recovered. Amino-terminal sequencing confirmed the presence of the intact form of CTAP-III. A Western blot showed molecular weight species like those found in platelet-pack plasma (Figure 2, lane 4). Acid–ethanol extracts of liver (Figure 2, lane 5) and lung (Figure 2, lane 6) contained trace (nanogram) amounts of CTAP-III isoforms; Western blots (Figure 2) showed CTAP-III primarily in the large parental isoform in association with unique large forms which, based on size, may be disulfide-linked multimers.

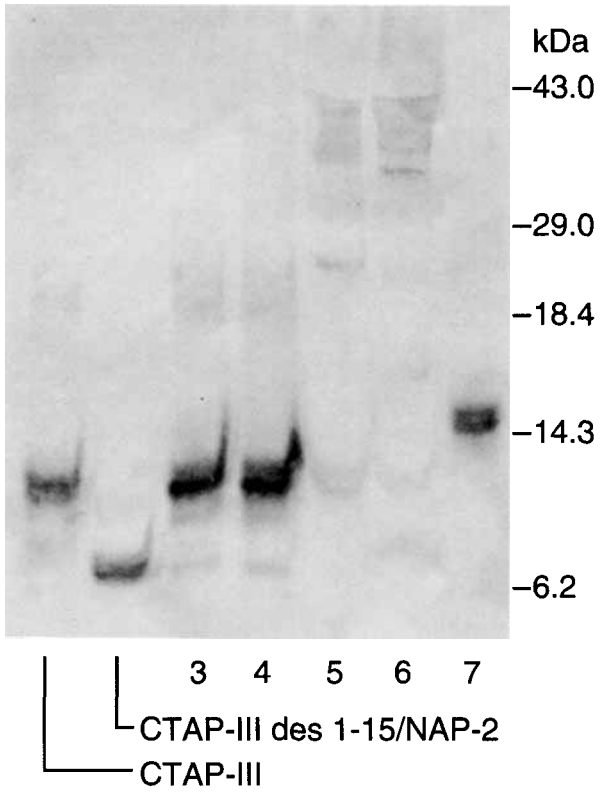

Figure 2. Western blot comparing immunoaffinity-isolated connective tissue activating peptide–III (CTAP-III) and CTAP-III des 1-15/neutrophil activating peptide–2 (NAP-2) with material isolated from platelet-pack plasma (lane 3), normal human spleen (lane 4), normal human liver (lane 5), normal human lung (lane 6), and a 24-hour collection of human urine (lane 7). See text for details.

CTAP-III isoforms in urine and kidney tissue. Only nanogram amounts of CTAP-III isoforms were detected in a 24-hour urine sample by Western blot; the isoform in urine appeared to be larger than the parental form of CTAP-III (Figure 2, lane 7). Since the mobility of this isoform was not affected by dilution, it is doubtful that the apparent larger size was an electrophoretic artifact related to total protein in the lane. The apparent molecular size of the urinary antigen may be related to dimerization of an NAP-2–sized isoform or a disulfide interchange linking NAP-2– or CTAP-III–sized isoforms and unrelated proteins. Kidney tissue from 2 individuals with normal serum creatinine levels was shown to contain microgram amounts of CTAP-III. Cortical and medullary components were extracted with acid–ethanol for CTAP-III isoforms. In 1 patient, the renal cortex contained 2.3

μg of CTAP-III/gm of tissue and the medullary fraction had 1.8 μg of CTAP-III/gm of tissue; the second patient had a larger concentration in the renal medulla than in the cortex. Western blot indicated that much of the antigen was compatible with intact CTAP-III and a lesser amount was the size of CTAP-III des 1-15/NAP-2 (Figure 4). Nothing resembling the size found in urine was seen.

Plasma concentrations of CTAP-III antigens in control and patient groups. Plasma CTAP-III concentrations are shown in Table 2. Peripheral venous plasma concentrations of CTAP-III were similar in the healthy controls and in the group of young patients with uncomplicated type I diabetes. Since the mean age in these 2 groups was 28 years and 24 years, respectively, it is reasonable to question whether data from older persons would be substantially different. The results seen in Figure 5 show that the majority of the patients with RA (mean age 50.7 years) had CTAP-III values within the range found for the younger groups. Venous plasma concentrations of CTAP-III from central locations, such as the inferior vena cava and coronary sinus, were not significantly different from peripheral venous values.

Mean arterial plasma concentrations of CTAP-III (Table 2) were somewhat greater than venous values ($P < 0.001$). Arterial blood samples were obtained from 10 patients just prior to balloon angioplasty; CTAP-III concentrations ranged from 43 ng/ml to 144 ng/ml, and values for 4 of the 10 patients were

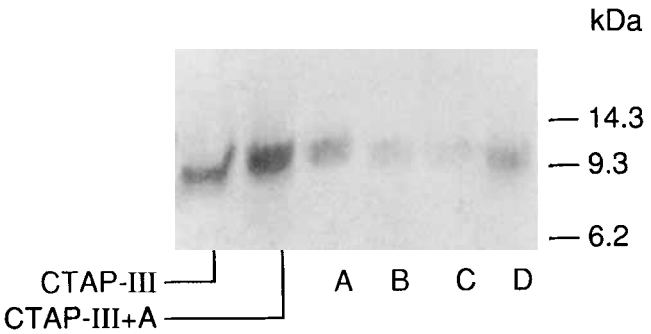

Figure 3. Western blot comparing 50 ng of authentic platelet-derived connective tissue activating peptide–III (CTAP-III) with immunostained substances extracted from the plasma of 4 normal subjects. The first lane shows the electrophoretic mobility of purified CTAP-III applied in a physiologic buffer; the second shows the slight retardation in mobility when the peptide is in the more protein-rich environment found in acid–ethanol extracts (A). The 4 plasma samples had the mobility of CTAP-III. Lanes A, B, C, and D contain samples from a 66-year-old man, a 41-year-old man, a 62-year-old man, and a 42-year-old woman, respectively.

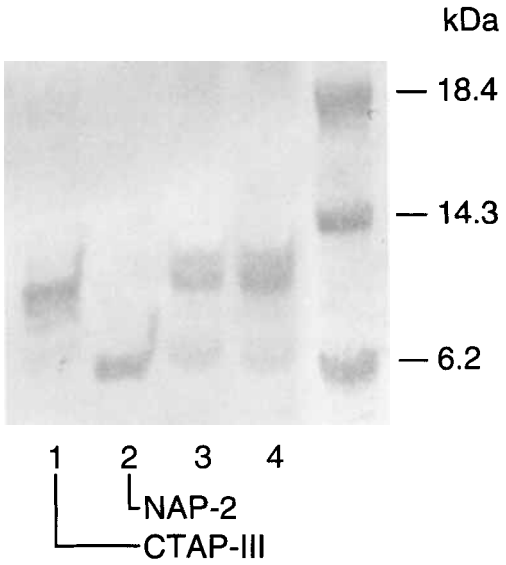

Figure 4. Western blot comparing immunoaffinity-isolated connective tissue activating peptide–III (CTAP–III) and neutrophil activating peptide–2 (NAP-2) with CTAP-III antigen extracted from kidney tissue from 2 subjects with normal creatinine levels. CTAP-III and NAP-2 sized isoforms were found in both the renal cortex (lane 3) and the renal medulla (lane 4).

within the normal range for peripheral veins. It is not clear whether the higher arterial measurements in the remaining 6 patients reflect intrinsic abnormalities of the arterial intima that induce local platelet activation detected by the catheter, or whether trauma inflicted by the sampling catheter induced local platelet release of CTAP-III.

Figure 5 compares plasma CTAP-III values in RA, SSc, and SLE patients with values in controls. The 42 patients with RA included 36 women and 6 men. Ten of the 42 (24%) had plasma CTAP-III values that exceeded the normal range. Two patients whose disease activity status was judged to be 0 had normal plasma CTAP-III values. CTAP-III values were elevated in 50% of the patients with an activity status of 1, in 30% of patients with an activity status of 2, and in 1 of 2 with an activity status of 3. Not only was there no consistent relationship between global estimates of disease activity and elevated CTAP-III values, but individuals with clearly active disease frequently had normal CTAP-III levels.

Four of 11 patients with progressive systemic sclerosis (SSc) had abnormal CTAP-III plasma values. Two samples had markedly elevated levels. Both patients were judged to have a disease activity status of 3; 1 patient was receiving prednisone, the other

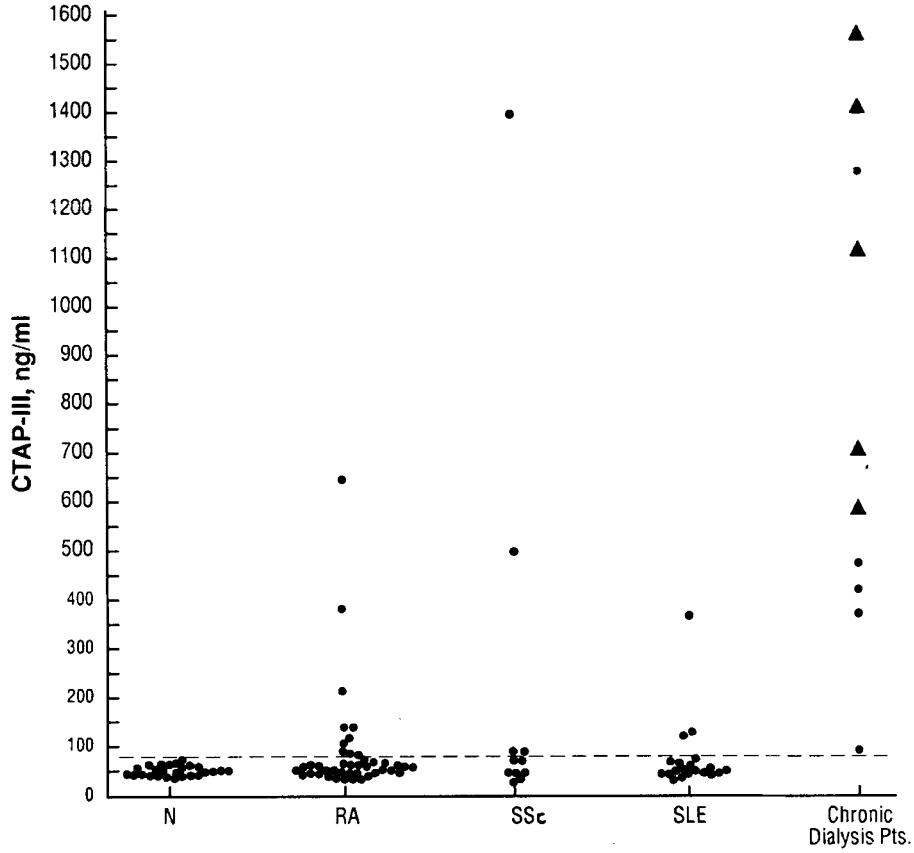

Figure 5. Peripheral venous plasma values for CTAP-III/β-TG/NAP-2 isoforms, as measured by enzyme-linked immunosorbent assay, in normal subjects (N), patients with various inflammatory rheumatic diseases (rheumatoid arthritis [RA], systemic sclerosis [SSc], and systemic lupus erythematosus [SLE]), and dialysis-dependent patients with chronic renal disease. In the latter group, 5 were treated with hemodialysis (circles) and 5 with peritoneal dialysis (triangles). See Figures 1 and 2 for other definitions.

penicillamine. The 2 SSc patients with slightly elevated plasma CTAP-III values had less active disease (activity status of 1 and 2). Among the 7 SSc patients with normal plasma CTAP-III values, all were judged to have an activity status of 1 or 2.

Three of 20 patients with SLE had abnormal plasma CTAP-III values; all 3 had disease activity status scores of 3. One had proteinuria, and 1 had mesangial proliferation, but none had renal failure. Of the 17 SLE patients with normal plasma CTAP-III measurements, only 2 were judged to have inactive disease; the remaining 15 had variable activity values, ranging from scores of 1 in 4 patients to scores of 3 in 6 patients.

Molecular isoforms of CTAP-III in patients with RA. Western blotting of plasma extracts from 4 women with active RA and elevated plasma CTAP-III values showed only a large molecular isoform of CTAP-III (results not shown). This contrasts with the finding of small isoforms in the synovial tissue itself in this disease (28).

Plasma CTAP-III antigen concentrations in patients with severe renal disease. Strikingly elevated concentrations of plasma CTAP-III antigens were found in patients with chronic renal failure (Figure 5); the values in the figure reflect CTAP-III concentrations measured just prior to hemodialysis or at random times during ongoing peritoneal dialysis. After 30 minutes of hemodialysis, values decreased in 3 patients, and increased in 2. Values at the conclusion of dialysis were increased over initial values in 3 patients (47%, 71%, and 140%), unchanged in 1, and decreased in 1 (33%). Hemodialysis patients had lower plasma levels of CTAP-III, on average, than did the peritoneal

dialysis patients (mean ± SD 530 ± 399 ng/ml versus 1,090 ± 386 ng/ml). The lower levels of CTAP-III in the hemodialysis patients are probably not attributable to clearance of CTAP-III by the hemodialysis membranes, since the levels of CTAP-III did not consistently fall during the 3–4 hours of hemodialysis.

Three of the 4 patients with the highest CTAP-III values (1 receiving hemodialysis and 3 receiving peritoneal dialysis) had recently had infection requiring parenteral antibiotics; however, no other underlying renal or systemic diagnoses were associated with consistently high CTAP-III values. Furthermore, patients with high CTAP-III levels had no consistent differences in recent laboratory values (e.g., levels of blood urea nitrogen, serum creatinine, electrolytes, or calcium, or findings on liver function tests), hematologic indicators (e.g., white blood cell count, platelet count, hemoglobin level), or use of specific medications (e.g., erythropoietin, vitamin D analogs, antihypertensive agents, or antibiotics). Peritoneal dialysis fluid was studied for CTAP-III content by ELISA after 3 hours of equilibration in the peritoneal cavity; the mean ± 1 SD level in the 5 samples was 47 ± 2.9 ng/ml, averaging 5.0 ± 1.9% of the concurrent plasma values. This is consistent with equilibration data determined for other substances of similar molecular size, such as beta-2-microglobulin (40).

CTAP-III isoforms in patients with severe renal disease. Western blots of extracted plasma samples from dialysis patients showed a large CTAP-III isoform compatible with the 85-residue parental protein (Figure 6). A sample of peritoneal dialysis fluid from 1 of these patients was lyophilized and processed in a manner similar to plasma. Western blot showed only the large CTAP-III isoform (results not shown). There was no evidence of a proteolytic cleavage isoform consequent to transport across the peritoneal membrane.

Sensitivity of peritoneal cells to CTAP-III in vitro. Since we were able to propagate fibroblast-like cells released into the peritoneal dialysis fluid, it was possible to test whether cells derived from the peritoneum were metabolically responsive to CTAP-III at the concentrations (1–2 μg/ml) shown to be present at the capillary–peritoneal interface. A comparison of the stimulatory effect of CTAP-III on peritoneal and synovial cells in monolayer culture is shown in Figure 7. CTAP-III, 1–2 μg/ml, stimulated ^{14}C-GAG synthesis by 50–100% ($P < 0.001$) in peritoneal fibroblast cultures. As seen in Figure 7, this level of responsiveness was similar to that of human synovial cells; however, synovial cells were clearly more responsive with

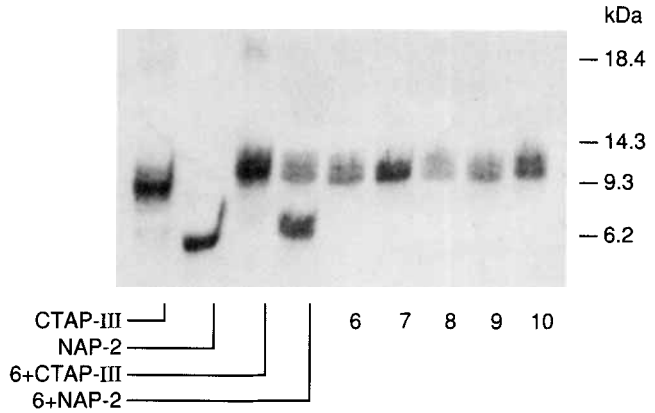

Figure 6. Western blot showing the mobility of the CTAP-III isoforms found in the plasma of patients with renal disease receiving long-term peritoneal dialysis (lanes 6–10). Lanes 4 and 5 show that the CTAP-III isoform from patient 1 (lane 6) comigrates with CTAP-III but not with NAP-2. No small CTAP-III isoforms were seen in the plasma from these 5 patients; the increased band density reflects the higher plasma levels of CTAP-III antigen found by enzyme-linked immunosorbent assay. Western blots of plasma from 5 patients treated with hemodialysis yielded results similar to those seen here.

higher concentrations of CTAP-III. Basal synthesis of ^{3}H-DNA in peritoneal fibroblasts was much lower than that in synovial cells; nevertheless, the peritoneal cells were stimulated to synthesize 50–60% more ^{3}H-DNA by the lower concentrations of CTAP-III ($P < 0.005$).

Arterial plasma CTAP-III antigens at angioplasty sites. The mean ± SD degree of coronary artery stenosis prior to angioplasty was 66 ± 10%, as judged by caliper measurement. Post-angioplasty measurement showed that the stenosis had been reduced to 40 ± 9%. Intracoronary plasma CTAP-III antigen measurements before and after angioplasty are shown in Table 3. The data suggest that intracoronary CTAP-III may rise early after angioplasty, with a peak seen within the first few minutes, concordant with the timing of platelet deposition noted in other studies (41).

DISCUSSION

The pathophysiologic significance of increased amounts of CTAP-III in plasma and tissues derives from the known proinflammatory activities of CTAP-III isoforms. The CTAP-III des 1-15/NAP-2 isoform is a potent stimulator of neutrophil activation and chemotaxis, and the isoforms that have been most studied (CTAP-III the des 1-13, des 1-14, and des 1-15/NAP-2) activate connective tissue cells to stimulate mitogene-

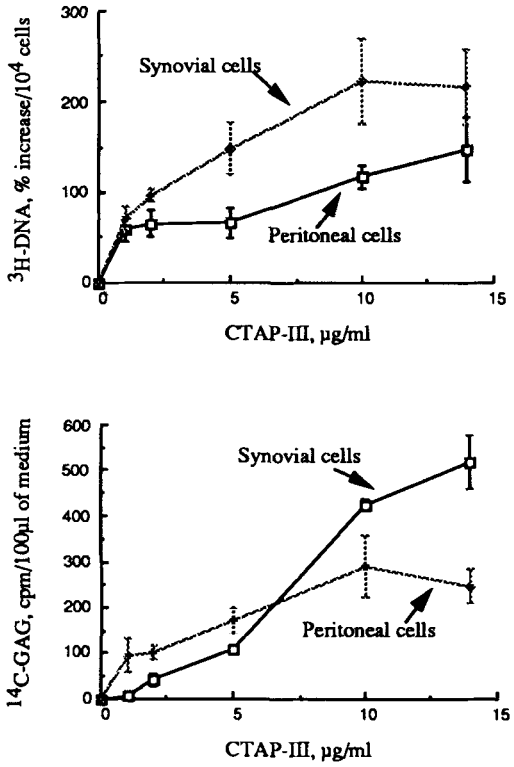

Figure 7. Comparison of the stimulatory effects of connective tissue activating peptide-III (CTAP-III) in synovial and peritoneal connective tissue cells in monolayer culture. Synovial and peritoneal cells both responded to the anabolic effects of CTAP-III at concentrations of 1–2 μg/ml, levels similar to those found in the plasma of patients with end-stage renal disease. ^{14}C-GAG = ^{14}C-glycosaminoglycan.

sis and extracellular matrix synthesis. It is possible that the heparin-binding capability of these isoforms may serve to concentrate them in inflammatory tissue and amplify focal biologic activity.

The relative abundance of CTAP-III provides a unique opportunity to study the distribution, the in vivo processing, and possibly the ultimate fate of a potent cytokine. The total circulating platelet population of ~1.42 × 10^{12} platelets contains ~130 mg of CTAP-III; if one postulates a platelet survival time of 10 days, then ~13 mg is released from platelets each day. The total CTAP-III found in the plasma amounts to only 120 μg, while that detected in the urine is only ≤100 ng per day. Measurements of CTAP-III in joint fluid, which may be considered a specialized interstitial space, are similar to levels in concurrently obtained plasma (42). If the plasma half-time for the CTAP-III/β-TG antigen is 100 minutes (4), the steady-state concentration of the CTAP-III antigen in extracellular water (plasma plus interstitial fluid) can be estimated to be 101 ng/ml. This value exceeds the measured mean value in normal individuals (54 ± 10.7 ng/ml [±SD]), possibly indicating that the actual half-time operative under physiologic conditions is somewhat shorter (~50 minutes) and/or that a substantial proportion of the CTAP-III released daily by spent platelets never reaches the plasma due to destruction and/or restriction to limited cellular compartments. The surprisingly high concentrations of CTAP-III in the kidney may be an example of this latter possibility.

We have shown, by Western blots, sequence analysis, and mass spectrometry, that intact CTAP-III is the dominant isoform in this antigen family; this was true in the platelets of origin, in platelet-pack plasma, and in the spleen. While there was <1% β-TG, we found larger amounts of amino-terminal deamidated CTAP-III, as reported earlier (18), as well as CTAP-III des 1, des 1-2, and des 1-3, all isoforms not previously recognized. These data contradict the earlier belief that β-TG/CTAP-III des 1-4 was the naturally occurring entity in platelets and plasma. The proportion of smaller isoforms (such as NAP-2) found in platelets, plasma, and spleen was small (<1%) and detected only on Western blots; this contrasts with data from RA and OA synovial tissue extracts, where small isoforms accounted for 30–100% of the antigen (24).

Elevated plasma CTAP-III values in patients with RA, SLE, and SSc were found with approximately the same frequency as in earlier studies (15,16). Abnormal values were not present in all patients with active disease; however, all elevated values occurred in patients with clinical evidence of disease activity. Increased plasma levels of CTAP-III may result from episodic release due to intravascular platelet activation induced by multiple substances (such as DNA, IgG, or complement components), and in the periphery, at sites of recent microvascular lesions. The

Table 3. Intracoronary connective tissue activating peptide III (CTAP-III) values after percutaneous transluminal coronary angioplasty (PTCA)*

Time	CTAP-III, ng/ml
Baseline	68 ± 8
1 minute post-PTCA	94 ± 24
5 minutes post-PTCA	140 ± 39†
10 minutes post-PTCA	120 ± 26‡
15–20 minutes post-PTCA	111 ± 40

* Values are the mean ± 1 SD.
† P = 0.07 versus baseline, by Mann-Whitney U test.
‡ P = 0.09 versus baseline, by Mann-Whitney U test.

degree to which medications (such as glucocorticoids or nonsteroidal antiinflammatory drugs) modify platelet activation is uncertain.

While only nanogram amounts of CTAP-III isoforms were detected in normal 24-hour urine samples, normal renal cortex and medullary tissue had microgram amounts of CTAP-III per gram of tissue. Much of the antigen was intact CTAP-III, and part was the size of CTAP-III des 1-15/NAP-2. These values are roughly 40-fold the values in normal plasma 500–1,000 times our estimates for liver, lung, and aorta. If the 9-kd plasma CTAP-III (50 ng/ml) is freely filtered through the glomerulus and reabsorbed by the tubules, more than half of the CTAP-III disposed of each day (~8.5 mg) is delivered to the tubules, but only traces appear in the urine. Thus, it is not unreasonable to anticipate substantial amounts of extractable CTAP-III in the renal parenchyma; present measurements of renal tissue account for 7–8% of the postulated filtered load of cytokine. Alternative explanations for the "missing" portion of the renal CTAP-III include the possibilities that 1) it may be rapidly destroyed by proteolysis following tubular absorption, or 2) less likely, the cytokine may not be completely filtered.

The physiologic significance of renal localization of CTAP-III may lie in certain structural characteristics of the kidney. Our studies of the glycosaminoglycans of canine kidney showed the cortex to be rich in heparitin sulfate, with lesser amounts of dermatan sulfate; the medulla, on the other hand, contains mostly hyaluronic acid (43). Among adult tissues, only joint fluid and cartilage have higher concentrations of hyaluronate than are found in the renal medulla. One might speculate that delivery of an "HA synthesis–stimulating cytokine" (such as CTAP-III) is important for maintenance of the high medullary concentrations of HA; the level of CTAP-III that we detected is adequate to stimulate GAG (especially HA) synthesis by many cell types. In patients with end-stage renal disease, where elimination and storage of CTAP-III was impaired, plasma CTAP-III values rose to levels of 300–1,800 ng/ml. CTAP-III in such elevated levels was shown to be capable of promoting DNA and GAG synthesis in vitro in peritoneal fibroblast-like cells.

Several studies have provided transport data showing that patients receiving long-term peritoneal dialysis frequently show decreased peritoneal membrane permeability and histologic evidence of membrane thickening and collagen deposition (44–47). It is possible that high levels of CTAP-III (and other cytokines) may be critical to these changes.

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