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Connective Tissue Activation. XXX: Isoelectric Point Microheterogeneity of CTAP-III, a Human Platelet-Derived Growth Factor[1] (42292)

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Abstract. Connective tissue activating peptide (CTAP-III) is one of the growth factors found in the α granules of human platelets. This small cationic platelet protein shows several apparent isoelectric point variants after the preparative isoelectric focusing step in large scale preparations from multiple donors. Analytical isoelectricfocusing techniques under denaturing conditions, coupled with Western blotting and immunodetection, now show that CTAP-III prepared rapidly from single donors also has multiple isoelectric point variants, suggesting that this finding is more likely related to post-translational modification than preparative artifact or genetic polymorphism.

Connective tissue activating peptide-III (CTAP-III) is a small protein (mol wt 9278) growth factor from human platelets, one of a small group of human growth factors which has been sequenced (1). The effects of CTAP-III on cultured fibroblasts have been studied in detail; in addition to its mitogenic effect, CTAP-III has a range of "activating" effects, including stimulation of glycosaminoglycan, proteoglycan and prostaglandin E_2 synthesis, stimulation of glycolysis, glucose transport, and secretion of plasminogen activator (2, 3). These biologic activities are consistent with the possibility that CTAP-III is involved *in vivo* in stimulating both synthesis and degradation of extracellular matrix components. Plasma levels of CTAP-III have been found to be elevated in patients with rheumatoid arthritis; the level of CTAP-III appeared to be correlated with disease activity (4, 5). In addition, plasma CTAP-III was elevated in patients with several types of active vasculitis and in some persons with chronic vascular disease.

Earlier we isolated CTAP-III from pooled outdated human platelets by a scheme which involved preparative isoelectric focusing (IEF). Biologically active CTAP-III which appeared homogeneous by sodium dodecylsulfate polyacrylamide gel electrophoresis was found in at least three distinct isoelectric point zones on IEF (6). We undertook to study the isoelectric point variants of CTAP-III by analytical IEF in both preparations from pooled outdated platelets and fresh preparations from individual donors. If the microheterogeneity seen in the multidonor preparations was an artifact of prolonged preparation procedures, or was due to allelic variations in the population, CTAP-III prepared quickly and gently from individuals might be expected to show fewer isoelectric point variants.

The results demonstrate the multiple isoelectric point variants of CTAP-III in both fresh, individual preparations and in preparations from pooled, outdated platelets.

Materials and Methods. *Preparation of CTAP-III.* Preparations from pooled, outdated human platelets were prepared as previously described (1, 7). Briefly, CTAP-III, isolated by acid–ethanol extraction, gel permeation and ion exchange chromatography, appeared homogenous by acid–urea polyacrylamide electrophoresis and SDS polyacrylamide gel electrophoresis. This was used to immunize rabbits as reported (8); antiserum from week 31 was passed over a column of CTAP-III antigen coupled to activated Sepharose CH-4B (Pharmacia), and the specific anti-CTAP-III IgG was eluted with 0.1 *M* glycine, pH 3.0. The specific anti-CTAP-III IgG was conjugated to Sepharose CH-4B, and an affinity column was prepared and used to isolate CTAP-III directly from acid–ethanol extracts of human platelets. Affinity-isolated CTAP-III was then separated by preparative isoelectricfocusing. Preparative isoelectricfocusing to purify CTAP prepara-

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tions was carried out using the Pharmacia Flat Bed Apparatus FBE 3000 and the Electrophoresis Constant Power Supply ECPS 3000/150. Sephadex G-75M was washed with 2× deionized water and dried. A stable pH gradient was achieved using a system of amphoteric and nonamphoteric buffers according to the method of Prestidge and Hearn (9). Samples were eluted from 26 separate lanes across the pH gradient for subsequent study.

Preparation of CTAP-III. To prepare CTAP-III from fresh platelets from individual donors, 40 ml of blood were collected into heparin-containing vacutainers; for thrombin extrusion studies, 1 ml 3.5% sodium citrate in 0.015 *M* theophylline was added immediately to each 10-ml vacutainer. Blood samples were centrifuged at 1000 rpm for 10 min and the platelet-rich plasma (supernatant) was collected. Centrifugation and collection were repeated three times. Platelet-rich plasma was then centrifuged in a refrigerated centrifuge at 4000 rpm for 20 min at 10°C; the supernatant (platelet-poor plasma) was removed, and the platelet pellet was resuspended in 4 ml phosphate-buffered saline (PBS: 0.001 *M* phosphate, pH 7.4, 0.15 *M* NaCl). Platelets were then either lysed by freezing and thawing six times, or aggregated with thrombin (10 units/ml). The aggregated platelet mass was centrifuged at 4000 rpm at 10°C for 10 min and the supernatant fluid collected. The CTAP-III in the supernatant from each releasing procedure was immediately isolated by passage over an immunoaffinity column; the sample was applied and the column washed in PBS; bound protein (CTAP-III) was then eluted with 0.1 *M* glycine–HCl, pH 3.0. Column fractions were dialyzed overnight against 12 liters of distilled water, and concentrated by lyophilization. Total protein was determined by a colorimetric method (10); CTAP-III was specifically measured by radial immunodiffusion. Small immunoaffinity columns were prepared by coupling affinity-purified anti-CTAP-III to cyanogen bromide-activated Sepharose beads (Sepharose 4B, Pharmacia). Anti-CTAP-III (20.5 mg) was coupled to 1.5 g of Sepharose 4B in 0.2 *M* citrate, pH 6.5, for 19 hr at 4°C. The gel was blocked and washed following the manufacturer's recommendations.

Electrophoresis. Analytical isoelectricfocusing was carried out on samples from multiple individual donors. Final concentrations of the gel components were: pH 3–10 Servalyte ampholytes, 5%; glycerol, 10%; acrylamide, 6%; urea, 8 *M;* ammonium persulphate, 0.1 mg/ml; riboflavin, 5.0 μg/ml; and TEMED, 0.063 μl/ml. Urea (electrophoresis grade) was dissolved in 25% glycerol and acrylamide solution at 56°C. Ampholytes and double distilled water (to volume) were added, and the mixture degassed for 10 min. Catalysts were added, and the mixture cast with fluorescent lamps illuminating the gel. Vertical slab equipment was a Bio-Rad Model 220. Gels were 1.5 mm thick and accommodated 15 samples. A Pharmacia FBE 3000 power supply and Lauda RM2 cooler were used; gels were run at 4°C at 1000 V max., 150 mA max., and 10 W max.

Fifteen micrograms of total protein were applied to each lane; samples which were too dilute were lyophylized and taken up in 40 μl of 8 *M* urea. Diluent consisted of 5 *M* urea, ampholytes, 2%, and glycerol, 50%; this was added to samples to double the original volume. After prefocusing until the voltage reached 1000 V (1 hr), samples were applied and focusing was carried out until the current dropped to 4 mA (3 hr). The gel was removed, blotted as described below, and stained by a silver method (11). Pharmacia basic IEF standards were used to calculate the isoelectric points of samples. Polyacrylamide gel electrophoresis (PAGE) in SDS was carried out as previously described (12). Gels were stained by the silver method or blotted (see below) and stained by an immunoperoxidase procedure.

Protein transfer and immunodetection. The isoelectric point heterogeneity of these preparations could not be studied by conventional methods. CTAP-III appeared to be soluble in fixatives normally used to stain analytical gels; even use of harsh fixatives did not yield adequate results. To circumvent this problem, we transferred the protein to nitrocellulose membranes by a modification of the method of Southern (13). The immobilized CTAP-III was then detected with specific rabbit anti-CTAP-III followed by an anti-rabbit IgG conjugated to horseradish peroxidase. The final result was a "blot" of the gel, with bands of specific antigen visible. The procedure of Southern (13) for DNA transfer was modified as described

below. Immediately after focusing, the gel was transferred to a filter paper bed soaked in "20×" TBS (40 m*M* Tris base, 3 *M* NaCl; adjusted to pH 7.5 with HCl). Nitrocellulose membrane soaked in "2×" TBS (20 m*M* Tris base, 0.3 *M* NaCl, adjusted to pH 7.5 with HCl) was laid upon the gel, with the edges resting on spacers with the same thickness as the gel. A piece of filter paper of slightly larger dimensions than the nitrocellulose, soaked in 2× TBS, followed by ten pieces of dry paper, was laid on top of the nitrocellulose. The gel was blotted for 1 hr; it was then fixed and stained as previously reported (11). The blot was dried and developed with rabbit anti-CTAP-III and the Biorad HRP kit, following Biorad's recommended procedure.

Results. Preparative IEF resolved affinity isolated CTAP-III from pooled outdated human platelets into at least three species focussing at pH 6.8, 7.6, and 9.3. (Fig. 1). N-Terminal amino acid determination indicated that the N-terminal tetrapeptide was intact in all forms. The highest pI variant of CTAP-III (9.3) had the highest specific activity with respect to synthesis of [^{14}C]hyaluronic acid ([^{14}C]HA) and [^{3}H]DNA (data not shown).

Characterization of fresh CTAP-III isolates from individuals. Individual preparations demonstrated only albumin (mol wt 66,000) and CTAP-III (mol wt 9000) by SDS–PAGE. CTAP-III was present in fresh preparations in amounts ranging from 186–314 μg/ml as determined by radial immunodiffusion. The preparations stimulated synthesis of glycosaminoglycans, determined by incorporation of [^{14}C]glucosamine into cetylpyridinium chloride precipitable polymer (14).

Transfer of protein from the gel to nitrocellulose was shown to be effective for molecules at least as large as albumin by reaction of blots with antialbumin as well as anti-CTAP-III. Presumably, smaller molecules would be transferred even more completely. The specificity of the antisera used was demonstrated by reaction with "blots" of SDS–PAGE of fresh preparations, where the antibody reacted specifically with a single 9000 Da band (Fig. 2).

Isoelectric point heterogeneity. Multiple isoelectric point variants were demonstrated in both individual and pooled preparations; a species with a p*I* of 7.6 appeared most abundant (Fig. 3). All but the most basic band found in the older pooled preparations existed in the fresh preparations, although some bands

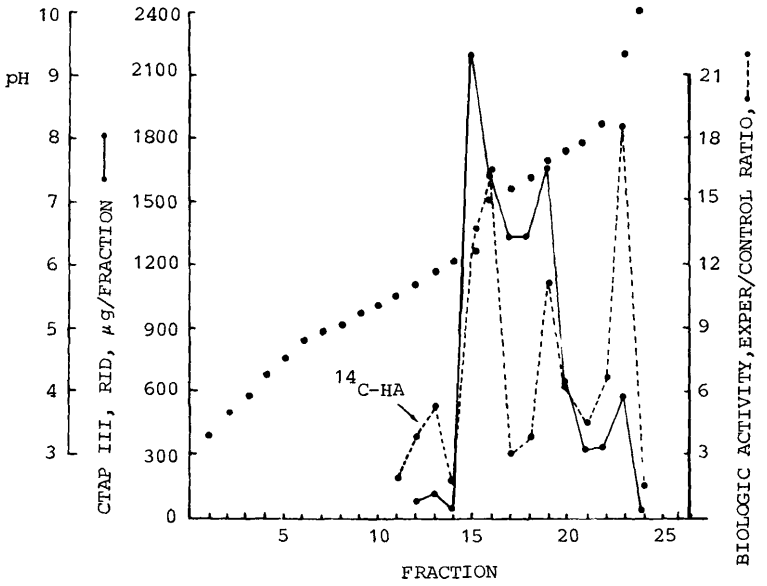

FIG. 1. This figure shows the localization of CTAP-III, measured by radial immunodiffusion in different zones of the pH gradient; the corresponding biologic activity was measured in terms of stimulated synthesis of [^{14}C]hyaluronic acid ([^{14}C]HA, dotted line).

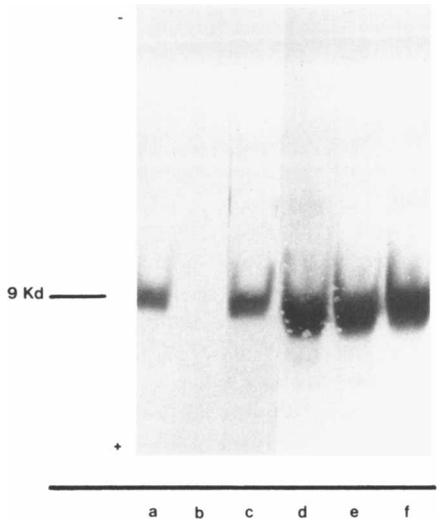

FIG. 2. An SDS–PAGE Western blot of mercaptoethanol reduced CTAP-III from four individuals, a control, and an example of unreduced CTAP-III, was visualized with rabbit AntiCTAP-III and goat anti-rabbit IgG–peroxidase conjugate. (a) Fresh CTAP-III (subject 1). (b) Control material not bound to immunoaffinity column. (c) Unreduced CTAP-III (subject 1). (d) Fresh CTAP-III (subject 2). (e) Fresh CTAP-III (subject 3). (f) Fresh CTAP-III (subject 4).

were so faint that comparison was difficult. The most basic band, with a p*I* of 9.9, was found in aged individual preparations but not in fresh ones. There was no significant difference between preparations prepared by thrombin extrusion and those prepared by freezing and thawing, nor were any anomalous bands found in preparations made from non-extruded material. There were differences between stored individual preparations and fresh preparations; the prominent band of p*I* 7.6 being fainter, while bands more basic than pI 7.6 appeared more abundant; a very basic (p*I* 9.9) band was also found. Conversely, after strong reduction with 2-mercaptoethanol, bands more basic than p*I* 7.6 disappeared, and more acidic bands were found (data not shown).

Discussion. The data confirm the existence of multiple isoelectric point variants of CTAP-III and indicate that the microheterogeneity is not an artifact of preparation, nor due to allelic variation in the donors. In the denaturing isoelectric focusing system, polypeptides, unless they contain disulfide bonds, are thought to be unstructured and exhibit an isoelectric point close to that calculated from the amino acid sequence (15). Although CTAP-III contains four cysteine residues, the major band found has a pI close to the calculated value of 7.65. Several of the prominent bands appear in doublets separated by about 0.1 pH unit. These may represent β-thromboglobulin (β-TG), a biologically inactive degradation product of CTAP-III found after loss of the aminoterminal tetrapeptide (1). The calculated p*I* of β-TG is 0.1 p*I* unit more acidic than that of CTAP-III.

These results are consistent with conformational isomerism or post-translational modification as possible causes of the observed microheterogeneity. Multimer formation was not considered likely, as we have not observed species corresponding to the expected higher molecular weight forms. The presence of the same IEF pattern of CTAP-III in acid extracts of platelets (presumably derived from unreleased α granules) as found in the thrombin-released product raises the possibility that this diversity of forms dates back to the time of synthesis and storage in granule form. The possibility that β-TG is responsible for half of the heterogeneity deserves consideration, al-

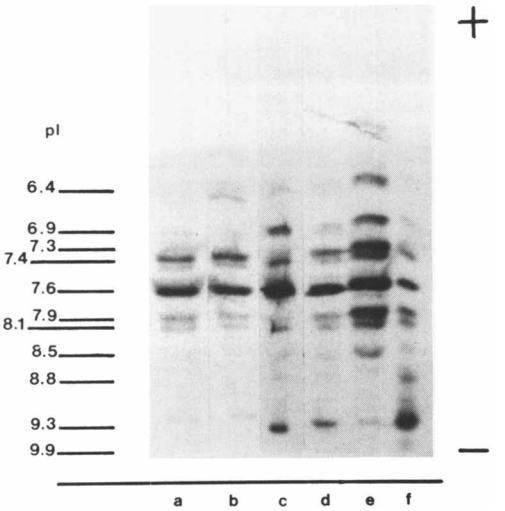

FIG. 3. Analytical IEF western blot, stained with monospecific AntiCTAP-III. (a) CTAP-III, fresh (subject 2). (b) CTAP-III, fresh (subject 4). (c) CTAP-III, fresh (subject 1). (d) CTAP-III, fresh thrombin extrusion (subject 1). (e) Preparative fraction from p*I* 7.3 zone. (f) Preparative fraction from p*I* 9.3 zone.

though it is presently difficult to test. Other possible explanations for isoelectric point heterogeneity include deamidation, phosphorylation, and variable degrees of glycosylation.

The isoelectric point heterogeneity of CTAP-III may have important physiologic ramifications, since the preparative experiments to date have shown significant quantitative differences in growth factor activities in the different isoelectric zone fractions (6). Although the possible significance of the heterogeneity is unclear, examination of large-scale preparations of the individual variants should help explain the structural basis of the heterogeneity and clarify its possible biological significance.

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