# CONNECTIVE TISSUE ACTIVATING PEPTIDE III INDUCTION OF SYNTHESIS AND SECRETION OF PLASMINOGEN ACTIVATOR BY SYNOVIAL FIBROBLASTS

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**Connective tissue activating peptide III (CTAP-III) is a platelet factor that induces, in cultured connective tissue cells, activities observed in chronic inflammation. In this study we measured plasminogen activator secretion by synovial fibroblasts after stimulation by CTAP-III. Increased plasminogen activator secretion was observed 24–48 hours after stimulation. Induction was prevented by dexamethasone ($10^{-9}$–$10^{-7}M$), cycloheximide (1 μg/ml) and, variably, by actinomycin D (0.3 μg/ml), but not by cytosine arabinoside ($10^{-4}M$). This is the first evidence that CTAP-III induces degradative as well as proliferative activity by connective tissue cells.**

Human platelets contain, and release during degranulation, a 9,278 dalton cationic protein, connective tissue activating peptide III (CTAP-III), which activates normal synovial and skin fibroblasts and chondrocytes (1,2). Such activation results in metabolic and secretory activities characteristic of inflammatory states and detectable in rheumatoid synovial cells in culture. Among its established actions, CTAP-III is mitogenic and stimulates glycosaminoglycan formation, glycolysis, cyclic AMP accumulation, and prostaglandin $E_2$ secretion (2–4).

Increased neutral protease activity is commonly observed in rheumatoid synovium, and its presence correlates with inflammatory injury (5). In the present study, we sought to determine whether synovial activation by CTAP-III caused neutral protease induction. We report that this peptide enhanced secretion of plasminogen activator (PA) in both rheumatoid and non-rheumatoid cells. CTAP-III, heretofore recognized primarily as a "growth factor," may play a role in tissue remodeling and promote tissue degradation in inflammatory sites.

## MATERIALS AND METHODS

**Fibroblast cultures.** Human synovial membrane was obtained at operation, minced, and tissue fragments distributed in explant cultures in order to establish synovial fibroblast cell lines (6). Some human synovial and skin fibroblast lines were recovered after storage at −80°C. Cell lines were confluent at passage and had been passaged at least 3 times before use or storage. In patients from whom cell lines were obtained, the diagnosis of rheumatoid arthritis or other rheumatic diseases was confirmed by record review using American Rheumatism Association (7) or conventional clinical criteria.

For each experiment, $2 \times 10^4$ fibroblasts were added to each well of a microtiter plate (Costar) in a volume of 0.1 ml L-15 medium containing 3% plasminogen-depleted fetal calf serum. In some experiments, wells were precoated with $^{125}$I-fibrin. Cultures were set up in replicates of 8; cells were permitted to attach and spread, and then mediators were added. In some experiments, drugs or latex particles (0.8μ) were added at the same time. Fibroblast cultures were incubated at 37°C for 4–48 hours; monolayers were then washed and the medium replaced with Dulbecco's modified Eagle's medium supplemented with 0.05% lactalbumin hydrolysate (DME-LH) in preparation for PA assay.

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Supported by US Public Health Service grants AM-20557 and AM-10728 and by the Michigan Chapter, Arthritis Foundation.

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Submitted for publication August 8, 1983; accepted in revised form January 13, 1984.

**Table 1.** Plasminogen-dependent fibrinolysis by synovial fibroblasts*

| Patient cell line (diagnosis)† | Stimulus | | |
|---|---|---|---|
| | None | CTAP-III (n) | MNL-CM (n) |
| DM (N1) | 8.6 ± 8.5 | 32.1 ± 20 (5) | 43.8 (2) |
| CB (OA) | 6.0 | 31.8 (1) | 50.9 (1) |
| GJ (PA) | 15.0 | 29.5 (2) | 38.6 (1) |
| EG (RA) | 15.1 ± 5.6 | 47.6 ± 5.4 (4) | 64.3 ± 11.6 (3) |
| All | 10.9 ± 8.7 | 34.4 ± 17.9 | 43.9 ± 23.9 |

* Synovial fibroblasts were plated onto $^{125}$I-fibrin substrates and incubated in the presence or absence of connective tissue activating peptide III (CTAP-III, 20 μg/ml) or mononuclear leukocyte–conditioned medium (MNL-CM, 1:10) for 48 hours. Then plasminogen-dependent fibrinolysis was measured as described in Materials and Methods. Values are % $^{125}$I-fibrin solubilized, mean ± SD.
† N1 = normal; OA = osteoarthritis; PA = psoriatic arthritis; RA = rheumatoid arthritis.

**Preparation of mediators.** CTAP-III was purified from outdated blood bank platelets and characterized as previously described (1). All preparations used contained a single protein as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

For preparation of mononuclear leukocyte–conditioned medium (MNL-CM), mononuclear leukocytes were purified from heparinized whole blood by Ficoll-Hypaque centrifugation and incubated at a concentration of $5 \times 10^6$ cells/ml in serum-free RPMI 1640 medium (Gibco, Grand Island, NY) for 2 days at 37°C in 5% $CO_2$. This conditioned medium was stored at −20°C until use at a 1:10 dilution as a positive control to stimulate fibroblast secretion of PA.

**Plasminogen activator assay.** Fibroblast monolayers were washed after 4–48 hours of culture and the medium replaced with 0.2 ml DME-LH. PA activity of intact cells or fibroblast-conditioned medium (F-CM) was assayed using a $^{125}$I-fibrin plate method as described below.

When fibroblasts were cultured directly on the iodinated fibrin substrate, cell-associated PA activity was measured over the 3-hour period following the change of medium. Plasminogen (2 μg) was added to half the wells, and culture plates were incubated at 37°C. If PA was present, plasminogen was converted to plasmin which solubilized the $^{125}$I-fibrin substrate. After 3 hours, the assay mixture containing iodinated fibrin split products was recovered and counted. The substrate in 12 wells that had contained only medium was digested with trypsin and counted to determine total radioactivity. Fibrinolysis was expressed as the percentage of the substrate solubilized during the assay period and was corrected for radioactivity solubilized in the absence of enzyme (1–3%). Plasminogen, in the absence of PA, produced no added fibrinolysis, nor did streptokinase in the absence of plasminogen. Streptokinase-activated plasminogen solubilized more than 90% of the substrate. Fibrinolysis of replicate cultures varied by 15%.

When fibroblasts were cultured in uncoated wells, it was possible to assay secreted PA in F-CM. Prior to the assay, cultures were incubated in DME-LH for 24 hours at 37°C, then the CM was collected. Aliquots of 10–50 μl were added to $^{125}$I-fibrin–coated wells and diluted to a total volume of 0.2 ml in 0.1*M* Tris–HCl, pH 8.0, with or without 2 μg plasminogen. Plates were then incubated for 3 hours and fibrinolysis was determined as described for the assay using intact cells.

Human fibrinogen (Calbiochem, San Diego, CA) was purified, depleted of plasminogen, and iodinated using a chloramine T method (8). $^{125}$I-fibrin substrates were prepared in microtiter wells using the method of Klimetzek and Sorg (9). Plasminogen was purified from outdated plasma by lysine-Sepharose chromatography (10).

## RESULTS

**CTAP-III–induced PA secretion.** All fibrinolysis observed was plasminogen-dependent. PA activity was easily detected in both rheumatoid and non-rheumatoid cell lines; both intact cells and conditioned medium showed activity. PA activity of unstimulated cells varied from week to week and from cell line to cell line by as much as eightfold. Baseline enzyme activity did not appear to be influenced by the number of cell passages.

Addition of CTAP-III to cultures resulted in enhanced PA activity in 21 of 24 experiments in 8 of 9 cell lines assayed at different times over a 1-year period (Tables 1 and 2). Unstimulated fibroblasts cultured on $^{125}$I-fibrin solubilized 10.9 ± 8.7% of the substrate, and CTAP-III–stimulated cells solubilized 34.4 ± 17.9% ($P < 0.01$) (Table 1). Fibroblasts stimulated by conditioned medium from MNL cultures solubilized 43.9 ± 23.9% of the substrate (Table 1).

**Table 2.** Plasminogen activator in conditioned medium after stimulation of synovial fibroblasts by CTAP-III*

| Patient cell line (diagnosis)† | Stimulus | | |
|---|---|---|---|
| | None | CTAP-III (n) | MNL-CM (n) |
| CB (OA) | 8.6 | 35.8 (1) | 81.1 (1) |
| EG (RA) | 4.2 | 6.2 (1) | 47.1 (1) |
| NK (RA) | 5.2 | 52.1 (2) | 9.7 (2) |
| AB (OA) | 4.9 | 24.6 (3) | 7.1 (2) |
| AD (CPPD) | 87.0 | 61.0 (1) | – |
| WS (RA) | 3.7 | 53.0 (2) | 17.0 (1) |
| SH (OA) | 4.4 | 9.7 (2) | 51.1 (1) |
| All | 11.8 ± 23.8 | 35.7 ± 29.9 | 28.7 ± 27.9 |

* Synovial fibroblasts were cultured in uncoated wells in the presence or absence of connective tissue activating peptide III (CTAP-III, 20 μg/ml) or mononuclear leukocyte–conditioned medium (MNL-CM, 1:10) for 48 hours. Then the stimulants were removed and culture in Dulbecco's modified Eagle's medium containing lactalbumin hydrolysate (DME-LH) continued for 24 hours. Plasminogen activator activity in 50-μl aliquots of the DME-LH was measured as described in Materials and Methods. Values are % $^{125}$I-fibrin solubilized, mean ± SD.
† OA = osteoarthritis; RA = rheumatoid arthritis; CPPD = calcium pyrophosphate deposition disease.

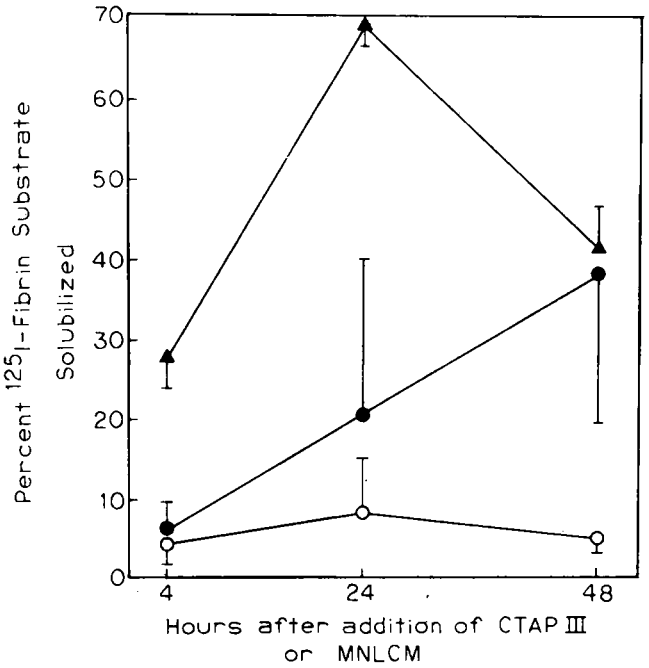


**Figure 1.** Induction of plasminogen activator (PA) secretion in synovial fibroblasts by connective tissue activating peptide III (CTAP-III) and mononuclear leukocyte-conditioned medium (MNL-CM). Mediators were added to cultures of synovial fibroblasts plated on $^{125}$I-fibrin, then PA activity was measured as described in Materials and Methods. CTAP-III enhanced PA activity 24–48 hours after addition to culture. Activation by MNL-CM was detected 4 hours after addition to culture. ○ = unstimulated; ● = CTAP-III; ▲ = MNL-CM.

Similar enhancement of PA secretion by CTAP-III and MNL-CM was observed when enzyme activity was measured in fibroblast-conditioned medium (Table 2). A cell line derived from a patient with pseudogout (patient AD) had extremely high basal activity and was not further stimulated by CTAP-III (Table 2). In 1 of 2 fibroblast lines derived from skin, CTAP-III induced PA activity (not shown). To determine whether a phagocytic stimulus could enhance enzyme induction by CTAP-III, latex particles were added during culture ($1–5 \times 10^2$ particles/cell); in 3 experiments, PA induction was not affected.

Neither CTAP-III nor MNL-CM altered fibroblast survival in culture, although MNL-CM altered cellular morphology, as described by Hamilton and Slywka (11). Addition of lipopolysaccharide ($10^{-11}$–$10^{-7}$ gm/ml) did not stimulate PA secretion, and preincubation of CTAP-III with the endotoxin-binding peptide polymixin B (12) did not prevent induction. These experiments suggest that PA induction was not caused by endotoxin contamination of the mediator preparation (13).

**Delayed induction of increased PA activity.** In 5 experiments using 2 cell lines, fibroblasts were cultured on $^{125}$I-fibrin with assay of enzyme activity 4–48 hours after stimulation. CTAP-III stimulated PA secretion after 24 hours in 1 experiment and after 48 hours in 4 experiments. Enhancement of PA secretion coincided with increases in glycolysis and glycosaminoglycan synthesis in stimulated cultures. This delayed induction differed from MNL-CM–induced enzyme secretion, which appeared within 4 hours (Figure 1).

**Effects of cycloheximide, actinomycin D, cytosine arabinoside, and dexamethasone on PA activity.** In 2 experiments, cycloheximide (1 μg/ml) reduced PA activity by more than 90%, indicating a requirement for continuous protein synthesis for PA secretion. Cytosine arabinoside ($10^{-4}M$), an inhibitor of DNA synthesis, had no effect on PA induction in 4 experiments.

In 6 experiments involving 4 cell lines, actinomycin D effects differed. With cell lines from patients with osteoarthritis (patient SH) and rheumatoid arthritis (patient WS), actinomycin D (0.3 μg/ml) abolished PA induction by both CTAP-III and MNL-CM. With 2

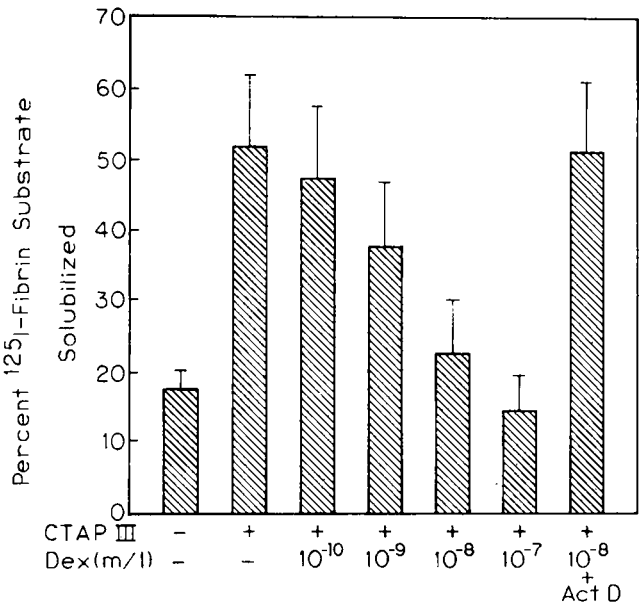


**Figure 2.** Effect of dexamethasone (Dex) on plasminogen activator (PA) induction by connective tissue activating peptide III (CTAP-III). The steroid was added at the same time as the peptide, as described in Materials and Methods, then PA activity was assayed 48 hours later. Actinomycin D (Act D) reversed the dexamethasone effect in 2 cell lines.

others, derived from patients with rheumatoid arthritis (patient EG) and psoriatic arthritis (patient GJ), the drug did not prevent PA induction by either stimulus. It is possible that these latter lines already contained RNA required for PA induction, perhaps because of a greater degree of endogenous activation.

Dexamethasone reduced PA induction by CTAP-III as well as by MNL-CM (Figure 2). In 2 cell lines (patients GJ and EG), actinomycin D reversed the dexamethasone effect, indicating that the drug was not toxic to the cells and, consistent with other observations (14), that corticosteroid inhibition of PA secretion required RNA synthesis.

## DISCUSSION

Plasminogen activator is a neutral protease implicated in the pathogenesis of chronic inflammation. PA itself may be a lymphocyte mitogen (15). In activating plasminogen, the enzyme generates degradative activity with a broad substrate specificity. In particular, within the articular space, plasmin can directly degrade proteoglycan (16) and activate the latent collagenase secreted by synovial cells (17). Plasmin can also induce secretion of collagenase (18). PA may, by initiating such protease activity, promote invasive properties of the synovial pannus, much as PA and plasminogen promote invasive properties of experimentally transformed fibroblasts (19).

Numerous immunologic and non-immunologic stimuli induce or enhance neutral protease secretion by inflammatory and synovial cells. Incubation of rabbit synovial fibroblasts with latex particles (20) or proteolytic enzymes (18) led to increased secretion of PA or collagenase. Interleukin-1, secreted by mononuclear phagocytes under immune stimulation, induced collagenase production by adherent synovial cells (21). Another monocyte factor, discovered and characterized by Hamilton et al (13), stimulated PA activity of human synovial fibroblasts.

The research reported here places CTAP-III, a naturally occurring and chemically characterized mediator, among those stimuli which enhance protease activity of synovial fibroblasts. Incubation of synovial cells regularly induced increased PA activity. Induction of PA was delayed, as are certain other CTAP-III–stimulated events, such as glycosaminoglycan secretion and accelerated glycolysis. Stimulation of enzyme secretion by MNL-CM occurred earlier and was usually of higher magnitude. Further characterization of the extent and regulation of protease induction by CTAP-III is under investigation.

Other properties of PA induction in fibroblasts by CTAP-III resemble those in other target cells by other stimuli. PA induction by CTAP-III was inhibited by submicromolar concentrations of dexamethasone. The drug affected MNL-CM–stimulated fibroblasts similarly (22). Dexamethasone likewise has reduced PA secretion in activated murine macrophages and in hepatoma cells (14,23). In the present work, inhibition of RNA synthesis reversed the steroid effect, again similar to dexamethasone inhibition of PA in diverse cell types (14,23). As observed by others (11,14,24,25), PA secretion was reduced by inhibition of protein synthesis, but not DNA synthesis.

It is postulated that CTAP-III–stimulated events are chronically and often inappropriately elicited "reparative" processes which contribute to the proliferative lesions of chronic inflammation (3). This report provides evidence that CTAP-III within inflammatory sites may also initiate degradative and invasive processes.

Platelets are not known to infiltrate inflammatory tissues, but platelet degranulation at sites of microvascular injury (26) could lead to diffusion of CTAP-III into synovium. Platelets have also been detected in synovial fluid (27,28) where immune complexes or lymphokines might stimulate degranulation (29,30). CTAP-III antigen levels are elevated in plasma in rheumatoid arthritis (31) and the mediator is present in rheumatoid synovium and synovial fluid (32,33). It is possible that CTAP-III stimulates, within inflamed synovium, the mitotic, metabolic, and proteolytic activity observed in vitro.

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