CONNECTIVE TISSUE ACTIVATION

XXXV. Detection of Connective Tissue Activating Peptide–III Isoforms in Synovium from Osteoarthritis and Rheumatoid Arthritis Patients: Patterns of Interaction with Other Synovial Cytokines in Cell Culture

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Objective. To determine whether extracts of un-incubated osteoarthritis (OA) and rheumatoid arthritis (RA) synovial tissue contain connective tissue activating peptide–III (CTAP-III) isoforms and prostaglandin E_2 (PGE_2), and whether such extracts have growth-promoting activity, and to determine whether binary combinations of CTAP-III with other cytokines reported to be present in synovial tissue lead to synergistic, additive, or inhibitory effects on growth.

Methods. Acid–ethanol extracts of human synovium were examined for growth-promoting activity by measuring formation of ^{14}C-glycosaminoglycan (^{14}C-GAG) and ^3H-DNA in synovial cell cultures; PGE_2 was measured by enzyme immunoassay, and CTAP-III isoforms were identified by Western blotting of extracted proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Growth-promoting activity of CTAP-III and other cytokines was tested in synovial cultures treated with the agonists singly and in binary combination, by measuring changes in synthesis of ^{14}C-GAG and ^3H-DNA.

Results. Platelet-derived CTAP-III and a cleavage isoform with the electrophoretic mobility of CTAP-III–des 1-15/neutrophil-activating peptide–2 (NAP-2)

and PGE_2 were found in biologically active extracts of synovial samples from patients with RA and OA. Five growth factors (recombinant epidermal growth factor [rEGF], recombinant interleukin-1β [rIL-1β], basic fibroblast growth factor [bFGF], PGE_1, and PGE_2) in binary combination with CTAP-III showed synergism in stimulating GAG synthesis; two (recombinant platelet-derived growth factor type BB [rPDGF-BB] and recombinant transforming growth factor β [rTGFβ]) had an additive effect. In combination with CTAP-III, rEGF and rPDGF-BB had a synergistic effect in promoting DNA synthesis, rTGFβ and rbFGF had an additive effect, and rIL-1β, PGE_1, and PGE_2 were antagonistic.

Conclusions. The results suggest that, in addition to endogenous factors, CTAP-III and other platelet-derived cytokines may play roles in regulating synovial cell metabolism in RA and OA, and that combinations of growth factors may be more significant than single agents in amplification or suppression of important cell functions.

Cytokines that may participate in driving the proliferative and destructive activity of rheumatoid synovitis are derived from (a) synovial fibroblastic (type B) or macrophage-like (type A) cells, endothelial cells, and lymphoid cells resident in the hyperplastic synovial membrane itself, and/or (b) components from the circulation that are targeted to the joints (1–5). Earlier experiments indicated that extracts of both normal and rheumatoid human synovial cells grown in cell culture had substantial growth factor activity when tested against normal synovial cell cultures; rheumatoid synovial cells had ~4 times the growth-promoting activity found in normal cells (1,2). In the last 2 decades many components of synovial tissue

with biologic activity have been resolved into a variety of well-defined growth factors; many are thought to be synthesized by cell types found in intact synovial tissue (6–8). Growth factors of endogenous origin reported in rheumatoid arthritis (RA) synovium include interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNFα), macrophage colony-stimulating factor, granulocyte–macrophage colony-stimulating factor, interferon-γ, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), connective tissue activating peptide–V (CTAP-V), and possibly, transforming growth factor β (TGFβ).

Since earlier evidence showed that both leukocyte and platelet extracts have potent cytokine activities when added to synovial cell cultures, it is important to consider their potential effect in the diseased synovium (3,4). In active RA, large numbers of polymorphonuclear cells are continuously targeted to joints; most apparently exit the synovial membrane and ultimately make their way into the synovial fluid. If platelets are similarly targeted to inflamed joints, significant quantities of platelet-derived cytokines may be delivered to the inflamed human synovial membrane. Well-described platelet-derived cytokines include PDGF (9), CTAP-III (10,11), TGFβ (12), an epidermal growth factor (EGF)–like protein (13), and a platelet-derived endothelial cell growth factor (14).

Evidence suggesting a link between platelets and articular inflammation includes the association between thrombocytosis and decreased platelet survival time in patients with severe RA (15–21). Direct evidence indicating significant platelet traffic through inflamed synovial membrane includes detection of extravascular platelets and/or platelet antigens in RA and other inflammatory synovial membranes and joint fluid samples (22–26). An important observation was the demonstration that [111]indium-labeled platelets can localize to inflamed rheumatoid joints (15).

In the present study, we have shown that extracts of unincubated RA and osteoarthritis (OA) human synovial tissue contain growth-promoting activities. Agents in these extracts that may contribute to these activities were shown to include prostaglandin E_2 (PGE$_2$), CTAP-III, and a cleavage isoform(s) with the electrophoretic mobility of CTAP-III–des 1-15/neutrophil-activating peptide–2 (NAP-2). Further, utilizing many of the cytokines thought to be present in inflammatory synovium, we measured the effects of several binary combinations of factors on growth-related activities in synovial cell cultures.

MATERIALS AND METHODS

Extraction of human synovial tissue. Synovial tissue removed during arthrotomy or joint replacement surgery was fixed for morphologic studies, and the remainder was stored at $-80°C$ for extraction studies. Aliquots of each sample were weighed and dried to a constant weight over a desiccant at 37°C for several days; water content was determined by the difference. Weighed aliquots (0.9–1.1 gm) of frozen tissue were also homogenized with a Virtis 45 Micro Homogenizer (The Virtis Co., Gardiner, NY) for ~10 minutes at 4°C in 2 ml or 3 ml of an extraction vehicle. Extraction vehicles were either phosphate buffered saline (PBS), pH 7.0, or acid–ethanol (95% ethanol–5% 1.25N HCl). After homogenization, extraction was continued for 48 hours at 4°C with slow stirring. Extracts were centrifuged at 20,000g for 10 minutes, and the supernatant fluid was dialyzed against Dulbecco's phosphate-buffered saline (D-PBS) and filter sterilized prior to testing for biologic activity. Samples of supernatant fluid to be examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were dialyzed against water, lyophilized, and reconstituted in a smaller volume for study.

Tissue prostaglandin measurements. OA and RA synovial tissue was homogenized in acid–ethanol as noted above and analyzed for PGE$_2$ content (27). In a solid-phase extraction, samples were adsorbed onto prewetted C-18 cartridges, the cartridges washed with 20% methanol, and the eicosanoids eluted with 80% methanol. Eluates were dried under an airstream and reconstituted with assay buffer. Extraction recoveries, typically 70–80%, were monitored with a radioactive thromboxane tracer, and assay results corrected accordingly. An ACE enzyme immunoassay (EIA) kit (Cayman, Ann Arbor, MI) was used, with acetylcholinesterase-linked PGE$_2$ as tracer, rabbit anti-PGE$_2$ as the first antibody, and a mouse anti-rabbit monoclonal as the second antibody. The range of detection for the assay is 2.6–250 pg/ml.

Isolation of CTAP-III. CTAP-III was isolated from human platelets by acid–ethanol extraction, acetone precipitation, gel filtration, heparin affinity chromatography, and immunoaffinity chromatography as reported previously (11,28). CTAP-III in human synovial tissue was extracted with acid–ethanol and dialyzed against PBS for subsequent testing.

Protein measurement and development of antiserum. Protein was measured by a colorimetric method (29) and/or by ultraviolet absorption (30). Antiserum to platelet-derived 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 0.15M NaCl in Freund's complete adjuvant. Booster injections with antigens in incomplete Freund's adjuvant were given 6 weeks and 12 weeks later. Animals were bled at 4 weeks and then at biweekly intervals after the initial immunization. Measurement of CTAP-III by radial immunodiffusion utilized filtered, heat-inactivated rabbit anti-human CTAP-III.

Analytic polyacrylamide gel electrophoresis. CTAP-III and its isoforms were analyzed by SDS-PAGE in 8M urea–8% total acrylamide (28,31). Proteins separated by SDS-PAGE were detected by both silver and Coomassie

brilliant blue R-250 staining (28,32). Proteins were separated by SDS-PAGE and 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 (28,31). Alternatively, Western blots of proteins following electrophoretic separation were performed by 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 an Immunoblot assay kit (Bio-Rad, Richmond, CA) by staining for horseradish peroxidase or for alkaline phosphatase using the amplification method.

Amino acid sequence determination. Aqueous protein solutions were applied to a glass-fiber filter pretreated with 2.0 mg of Biobrene (Applied Biosystems, Foster City, CA) and subjected to NH_2-terminal sequencing using an Applied Biosystems 470A Gas Phase Protein Sequencer interfaced with an Applied Biosystems 120A Analyzer (33).

Cell culture methods. Cultures of normal human and rheumatoid synovial cells were developed from explants obtained at arthrotomy (34,35). Monolayer cultures were grown in T-75 flasks in CMRL 1066 medium (Gibco, Grand Island, NY) supplemented with 5% human serum, 10% neonatal calf serum, 5% fetal calf serum (FCS), sodium bicarbonate, L-glutamine, 0.02M HEPES buffer, penicillin, streptomycin, and gentamicin. Cell strains were studied at passages 2–5 after removal from explants with trypsin.

Measurement of growth by isotope incorporation studies. Measurement of ^{14}C-glucosamine incorporation into ^{14}C-glycosaminoglycan (^{14}C-GAG), primarily ^{14}C–hyaluronic acid (^{14}C-HA), was accomplished by plating cells in 96-well microtiter plates at a density of 10^4 cells/well in 200μl of semisynthetic medium (Leibovitz medium, L-15; Hazelton Biologics, Lenexa, KS) containing 1% FCS, L-glutamine, penicillin, gentamicin, streptomycin, sodium carbonate, and 0.02M HEPES buffer, pH 7.6 (28,36). Test materials and control vehicles were in contact with the 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 (28,36).

To measure ^3H-thymidine incorporation into synovial cell DNA, cells were plated at 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 (28,37). After a preliminary 20-hour incubation, test samples or vehicles were added (5–15 μl/well) and incubation continued for 24 hours; ^3H-methylthymidine (1.5 μCi/15 μl ESM/well) was then added, and incubation was resumed for 24 hours. Medium was aspirated, discarded, and cell sheets were washed twice, each separately, with PBS, pH 7.0, 5% trichloroacetic acid, and absolute methanol. After air drying at 35°C, the cells were lysed for 1 hour at 37°C with 50 μl of 0.3N sodium hydroxide. Fifty microliters of cell lysate was pipetted into a counting vial containing Ecolume cocktail, and radioactivity was measured in a scintillation counter.

Cytokine interaction studies. Native CTAP-III was prepared in our laboratory. Recombinant cytokines were obtained from the following sources: PDGF from PDGF Inc.

(Boston, MA), rTGFβ from Biosource International (Westlake Village, CA), bFGF from Synergen, (Boulder, CO), rIL-1β from Intergen (Purchase, NY), rEGF from Collaborative Research, (Bedford, MA), PGE_1 from Upjohn Pharmaceutical (Kalamazoo, MI), and PGE_2 from Warner Lambert Research Institute (Morris Plains, NJ).

The interactive effects of cytokines in combinations of two were tested in bioassays employing nonrheumatoid human synovial cells. Dose–response curves were set up to measure the magnitude of single growth factor effects on ^{14}C-GAG synthesis and ^3H-DNA formation and to define saturation levels for these experimental conditions. The interaction of two cytokines (A and B) was then tested by adding an intermediate amount of a second agent (B) at the low and high ends of the dose–response curve for the first agent (A). Growth factors were added to well cultures at room temperature, the second agent following the first within a minute. Within the framework of the same experiment, the reverse study was also done; that is, a dose–response curve of cytokine B was supplemented at its high and low ends by cytokine A and the metabolic consequences were measured. In the graphic presentation of the cytokine data, each data point is the mean of values obtained from at least 4 microtiter wells. Standard error bars are incorporated into all figures; in many figures the error bar is so small, it is hidden within the data point symbol.

RESULTS

Composition and biologic activity of synovial tissue extracts. Approximately 85% of the wet weight of human synovium is water (83.5 ± 3.9%, mean ± 1

Table 1. Characteristics of synovial tissue extracts*

Synovial tissue sample no. (diagnosis)	Degree of inflammation	Extraction procedure	Protein concentration, μg/ml†	Biologic activity, incremental cpm‡	
				^{14}C-GAG	^3H-DNA
1998 (RA)	4	PBS	568.0	2,128	961
1997 (RA)	2	PBS	415.0	2,173	2,236
1998 (RA)	4	Acid–ethanol	59.8	711	27,229
1997 (RA)	2	Acid–ethanol	40.0	689	14,504
1999 (OA)	0–1	Acid–ethanol	25.6	427	35,305

* Tissue was obtained from 2 patients with rheumatoid arthritis (RA) and 1 with osteoarthritis (OA). The degree of inflammation was estimated from stained sections: 0 = no hyperplasia or cellular infiltration, and 4 = marked synovial hyperplasia, cellular infiltration, and capillary growth. GAG = glycosaminoglycan; PBS = phosphate buffered saline.
† Concentrations shown reflect the amount used in culture wells to assess growth-related activity.
‡ Incremental cpm = total cpm per 100 μl medium (GAG) or total cpm per 10^4 cells (DNA) in experimental wells minus control values from vehicle-treated wells.

SD of 5 samples). Seven to fifteen percent of the dry residue was saline extractable, and 1–2% was extractable with acid–ethanol. Both the relatively protein-rich saline extracts and the acid–ethanol extracts stimulated DNA and GAG synthesis by synovial cells in culture; clearly, the specific activity in relation to total protein is greater for the acid–ethanol extracts (Table 1) (calculations not shown). SDS-PAGE of the extracts showed that most of the extracted proteins were large, with mobilities similar to that of albumin. Radial immunodiffusion measurements identified albumin as 76% of the protein extracted from RA tissue with acid–ethanol, while 30% of the protein extracted by this procedure from OA tissue was albumin. PGE_2 extracted by acid–ethanol was measured by EIA, and the results are presented in Table 2. There was marked variability among samples: Both low concentrations and relatively high concentrations of PGE_2 were seen in samples from both inflammatory disease synovium and degenerative joint disease synovium.

Detection of CTAP-III isoforms in extracts of human synovium. Acid–ethanol extracts of OA and RA synovium were shown, by SDS-PAGE and Western blot utilizing anti–rCTAP-III antisera, to contain CTAP-III and its small isoforms (Figure 1). The samples from RA patients each showed a band with the mobility of CTAP-III and a second, smaller band with the mobility of CTAP-III–des 1-15/NAP-2. A sample from a patient with juvenile rheumatoid arthritis showed 1 band at 9.0 kd and 1 at 6.2 kd (results not shown). Only the 6.2-kd form of CTAP-III was recovered from the extract of normal-appearing synovium from 1 patient with OA. Lanes 2 and 5 in Figure 1 also reveal higher molecular weight isoforms (15–16 kd) which are recognized by the anti–rCTAP-III antibody;
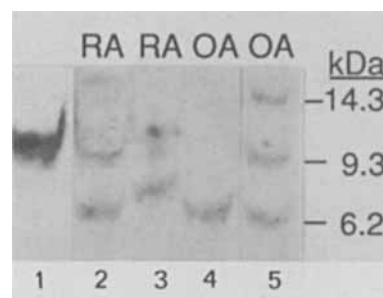

Figure 1. Western blot of synovial cell extracts from patients with rheumatoid arthritis (RA) or osteoarthritis (OA), stained with alkaline phosphatase to demonstrate connective tissue activating peptide–III (CTAP-III) and its cleavage isoforms. Lane 2, Sample 1997; lane 3, sample 1998; lane 4, sample 1999; lane 5, sample 2009 (see Tables 1 and 2). Higher molecular weight isoforms were also detected with patient samples. Lane 1 shows the mobility of CTAP-III purified from blood bank platelet packs outdated for clinical use (~6 days post–blood donation). After imunoaffinity purification, the preparation was shown by amino-terminal sequencing to be the CTAP-III parental form. In spite of prolonged exposure to plasma components, 6.2-kd proteolytic cleavage forms are not seen.

these probably represent disulfide-linked dimers and are of unknown biologic significance.

It is important to judge whether the processed forms of CTAP-III are derived from the extravascular domain of the synovial membrane or whether they might be easily found within the plasma compartment. Indirect evidence bearing on this issue derives from examination of the CTAP-III isoforms discharged by platelets into the platelet-rich plasma of platelet packs from a blood bank (lane 1, Figure 1). It is clear that CTAP-III in the plasma environment remains mostly in the 9.3-kd form for several days, in contrast to the CTAP-III isoforms in synovial extracts, which exhibit a marked degree of processing to a smaller form(s).

Growth effects of synovial cytokines. In preliminary studies (results not shown), recombinant PDGF type AA (rPDGF-AA) and EGF were weak stimulators of synovial cell growth, measured as increases in ^{14}C-GAG and ^3H-DNA. In contrast, CTAP-III, rPDGF-BB, and rTGFβ markedly stimulated these growth parameters. Recombinant IL-Iβ behaved differently in that it was a potent stimulant of ^{14}C-GAG formation but inhibited ^3H-DNA synthesis, under conditions where PGE_2 synthesis was not blocked. It was of interest that, at saturating concentrations of the individual agents, the most potent agonists induced similar levels of growth stimulation. CTAP-III is found in serum at concentrations of 10–40 $\mu g/ml$; it was active at concentrations well below this range in these

Table 2. Prostaglandin E_2 (PGE_2) in extracts of human synovial tissue*

Sample no. (diagnosis)	Degree of inflammation†	PGE$_2$	
		ng/gm tissue	ng/ml tissue water
1998 (RA)	4	2.7	3.2
1999 (OA)	1	0.8	1.0
2008 (JRA)	4	70.9	85.0
2009 (OA)	2	25.3	30.3
2010 (RA)	4	0.25	0.29

* PGE$_2$ samples were obtained in acid–ethanol extracts. RA = rheumatoid arthritis; OA = osteoarthritis; JRA = juvenile rheumatoid arthritis.
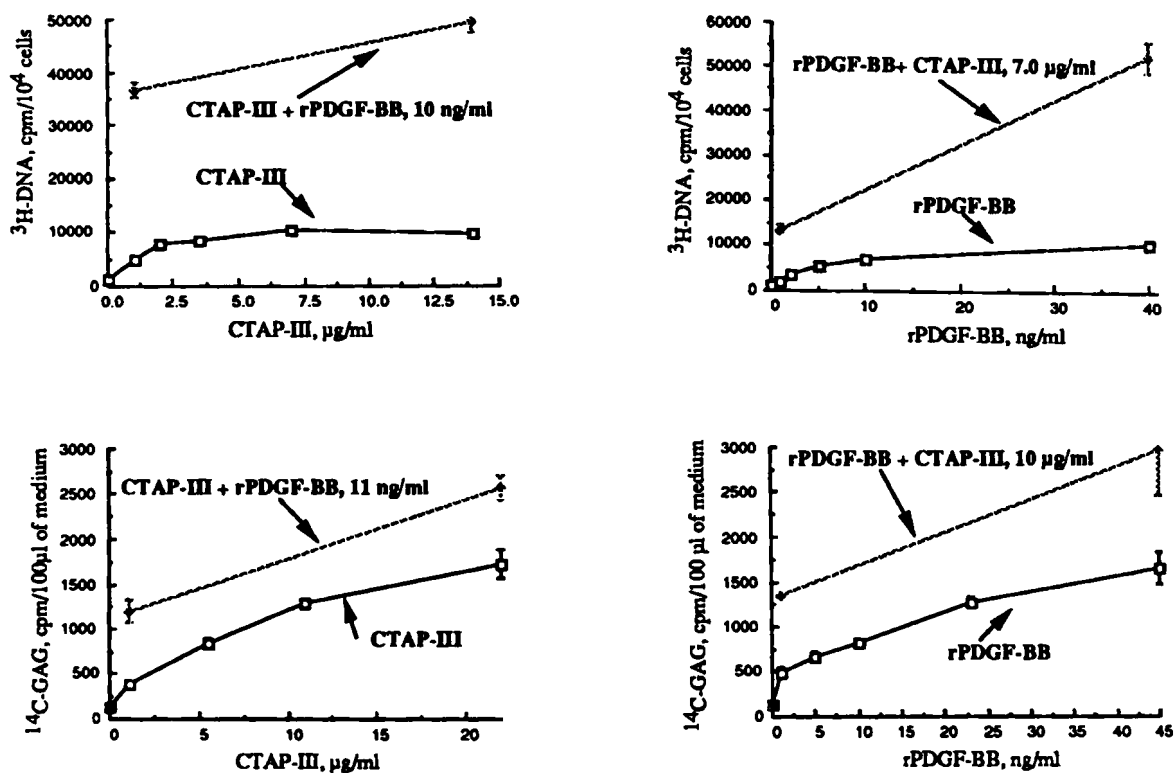† See Table 1.

Figure 2. Growth effects of connective tissue activating peptide–III (CTAP-III) in combination with recombinant platelet-derived growth factor type BB (rPDGF-BB) in synovial cell cultures. The combination of CTAP-III and rPDGF-BB had a synergistic effect on promotion of DNA synthesis; the effect on stimulation of glycosaminoglycan (GAG) formation was additive. Values are the mean and SEM.

assays. The other agents were active in the low ng/ml and high pg/ml range. Little data are available concerning plasma or tissue levels of these agonists.

Interactions of synovial cytokines in binary combinations. Although the actual number of cytokines that play a significant functional role in the synovial membrane is unknown, there is evidence for the presence of 10–12 separate agonists, including the 6 studied in the present investigation. It is not unreasonable to expect that resident synovial cells are confronted with many or most of the possible cytokines in variable combinations. The number of combinations of n different growth factors, taken r at a time, may be expressed as:

$$C(n,r) = n!/r! (n - r)!$$

Thus, the number of combinations of 10 growth factors, taken 2 at a time, is 45; if taken 3 at a time there are 120 combinations, taken 4 at a time the number would be 210, etc. These combinations of cytokines might have effects that are antagonistic, additive, or

synergistic; it is also possible that the metabolic effects of combinations are not very different from those of single agents. Several combinations of two agonists were examined in the present investigation.

Measurements of the growth effects of CTAP-III in combination with other agonists are shown in Figures 2–7. CTAP-III and rPDGF-BB were mutually synergistic in stimulating ^{3}H-DNA synthesis in synovial cell cultures (Figure 2). The combination of CTAP-III and rPDGF-BB increased DNA synthesis to levels 2–3 times the sum of their individual values. Both agents stimulated ^{14}C-GAG synthesis to a similar degree; in combination, the effects appeared to be fully additive. Recombinant EGF alone had less growth-stimulating activity in the GAG and DNA assays than did CTAP-III (Figure 3). Treatment with a combination of rEGF and CTAP-III caused a moderately synergistic interaction in stimulating ^{14}C-GAG synthesis; while the increase was severalfold higher than the maximum value with rEGF alone, it represented only a 33% increase over the maximum with CTAP-III
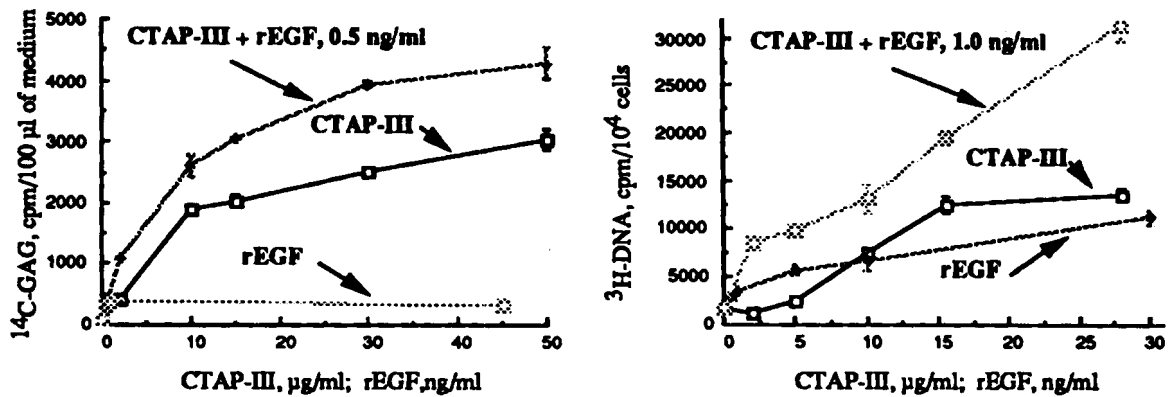
Figure 3. Growth effects of CTAP-III in combination with recombinant epidermal growth factor (rEGF) in synovial cell cultures. The combination of CTAP-III and rEGF had a clearly synergistic effect on promotion of both GAG and DNA synthesis. Values are the mean and SEM. See Figure 2 for other definitions.

alone. These 2 agonists in combination also stimulated DNA formation in a synergistic manner; the maximum level of DNA synthesis was more than doubled by addition of a low concentration of rEGF, which had little activity alone.

Recombinant bFGF at saturating concentra-

tions was less potent than CTAP-III in stimulating DNA synthesis; however, the agonists had similar activity in promoting GAG formation (Figure 4). Synovial cell cultures exposed to CTAP-III and bFGF in combination showed a simple additive effect in the DNA assay and a modest synergistic effect with re-
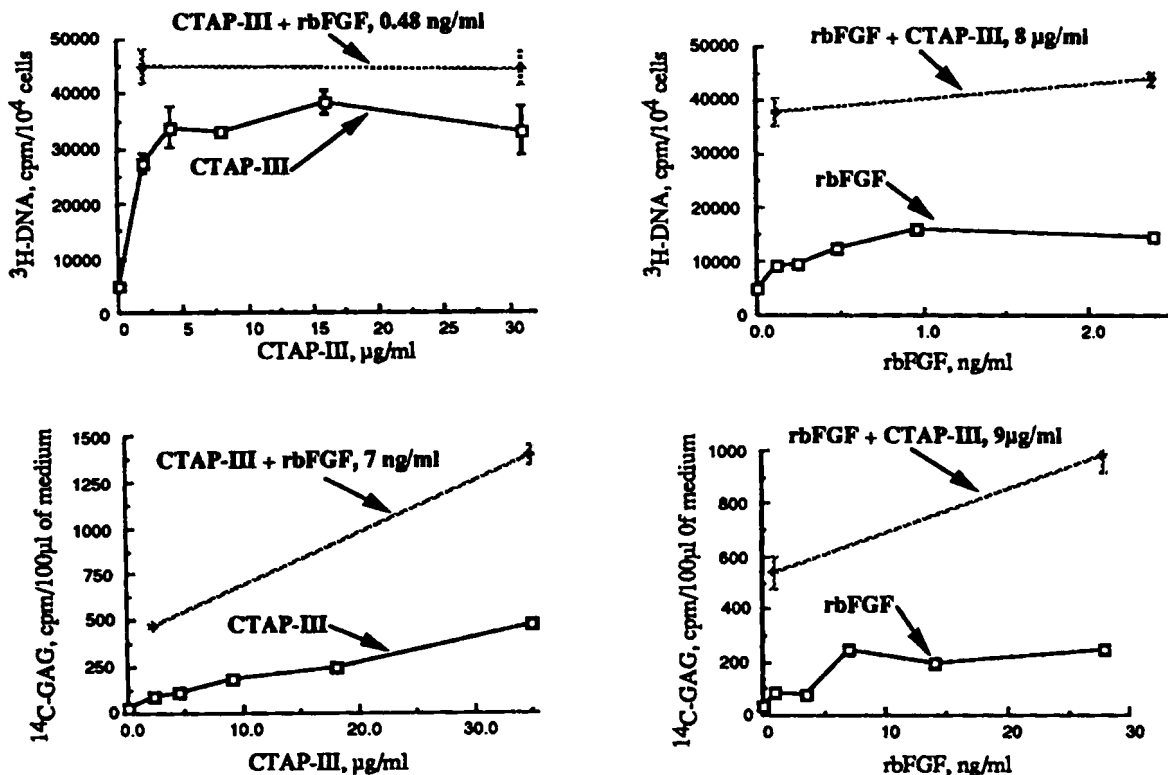

Figure 4. Growth effects of CTAP-III in combination with recombinant basic fibroblast growth factor (rbFGF) in synovial cell cultures. The combination of CTAP-III and rbFGF had an additive effect on promotion of DNA synthesis and a synergistic effect on stimulation of GAG formation. Values are the mean and SEM. See Figure 2 for other definitions.
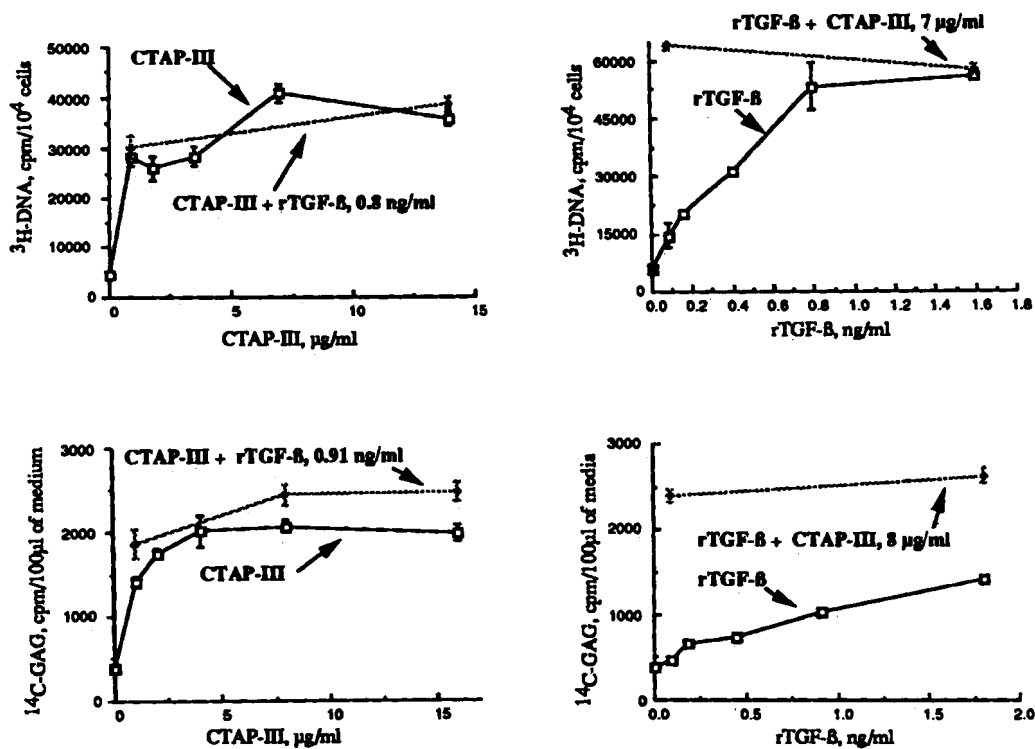
Figure 5. Growth effects of CTAP-III in combination with recombinant transforming growth factor β (rTGF-β) in synovial cell cultures. The combination of CTAP-III and rTGF-β had a partly additive effect on stimulation of GAG formation and a weakly additive effect on promotion of DNA synthesis. Values are the mean and SEM. See Figure 2 for other definitions.

spect to GAG synthesis. Recombinant TGFβ and CTAP-III at maximum stimulatory concentrations induced similar increments in DNA synthesis; rTGFβ, however, was less effective in stimulating GAG synthesis (Figure 5). The combination of rTGFβ and CTAP-III was incompletely additive in promoting GAG and DNA formation; in the case of DNA, the additive effect was often detectable only with very low concentrations of the agonists. Recombinant IL-1β caused a decrease in DNA synthesis, and when combined with CTAP-III, it had an antagonistic effect on the mitogenic action of the platelet-derived agent (Figure 6). Recombinant IL-1β was a potent inducer of synovial ^{14}C-GAG synthesis, and in combination with CTAP-III, it had a moderately synergistic effect on promotion of GAG formation.

Interactions of PGE$_1$ and PGE$_2$ with CTAP-III in synovial cell culture. PGE$_1$ and PGE$_2$ showed a similar, modest ability to stimulate GAG synthesis and depress DNA formation in basal synovial cell cultures (Figure 7). Each prostaglandin had a markedly synergistic effect with CTAP-III in promoting GAG formation,

and each partially reversed CTAP-III stimulation of DNA synthesis. In these experiments, PGE$_2$ had a greater specific activity than did PGE$_1$, especially with respect to reducing CTAP-III–stimulated DNA synthesis. Of interest, CTAP-III stimulation of DNA synthesis was not completely suppressed by PGE$_1$ and PGE$_2$ even at higher concentrations.

DISCUSSION

We have demonstrated growth factor activity in extracts of unincubated synovial membranes from patients with osteoarthritis and rheumatoid arthritis: DNA synthesis was stimulated by as much as 240% and GAG formation by up to 680% in human synovial cultures exposed to such extracts. Prostaglandins, measured as PGE$_2$, were found in concentrations ranging from 0.3 to 85.0 ng/ml tissue water, determinations that should be regarded as minimal values. Tissue PGE$_2$ concentrations had no obvious relation to degree of inflammation, as judged by light microscopy of tissue sections. It was, however, of interest that the
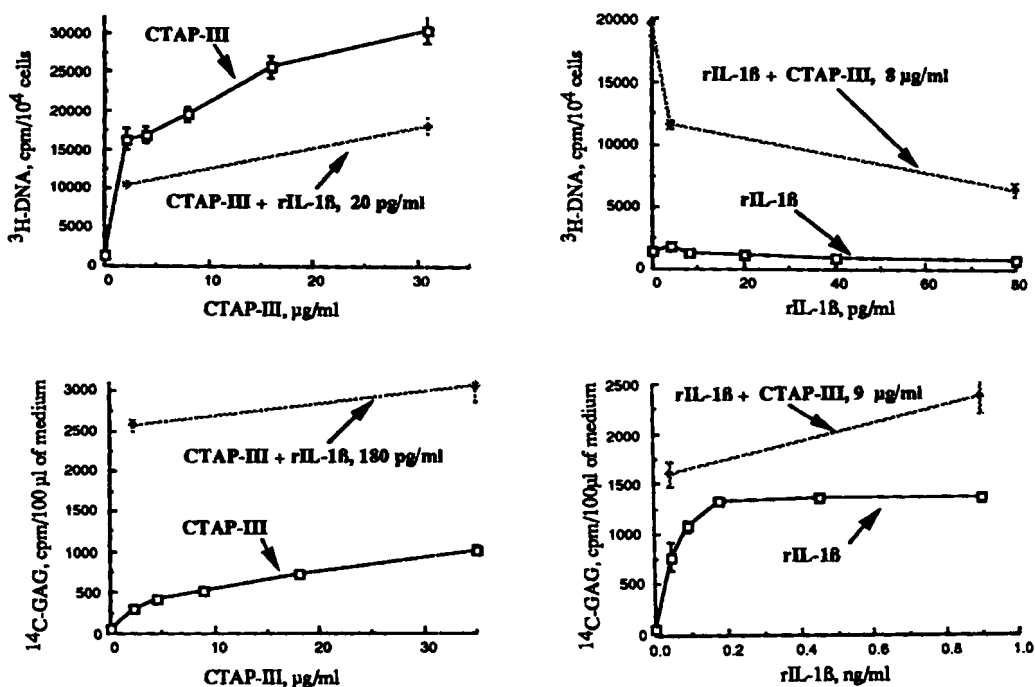
Figure 6. Growth effects of CTAP-III in combination with recombinant interleukin-1β (rIL-1β) in synovial cell cultures. Recombinant IL-1β had an antagonistic effect on CTAP-III promotion of DNA synthesis; the combination of CTAP-III and rIL-1β had a synergistic effect on stimulation of GAG formation. Values are the mean and SEM. See Figure 2 for other definitions.

concentrations detected were clearly in a range that modifies growth activity in vitro, by acting either alone or in combination with defined protein growth factors.

Detection of CTAP-III in acid–ethanol extracts of human synovial tissue was accomplished by Western blotting using monospecific polyvalent rabbit antiserum raised against recombinant CTAP-III. This antiserum is specific in its recognition of native CTAP-III and its des 1-13, 1-14, and 1-15/NAP-2 cleavage isoforms. In spite of the marked homology CTAP-III shares with platelet factor 4, NAP-1/IL-8, and melanoma growth stimulation activator (MGSA), anti-rCTAP-III antiserum does not recognize these proteins. The finding that CTAP-III was present in
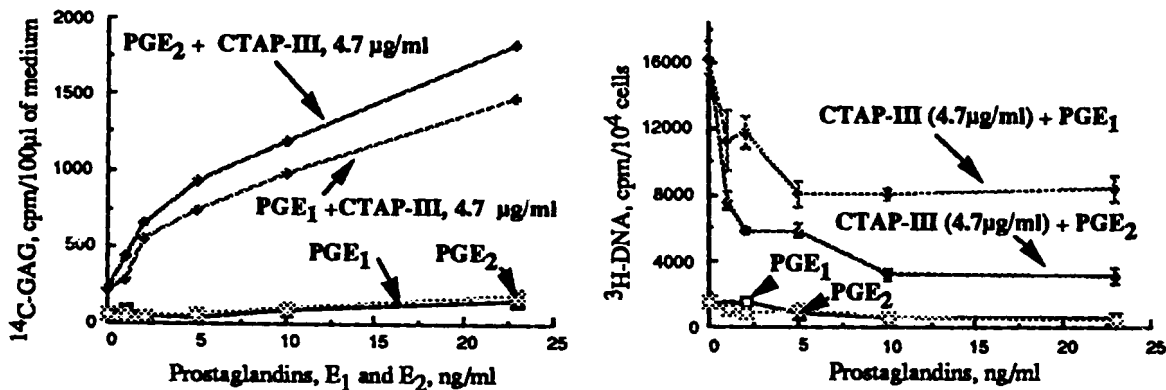

Figure 7. Growth effects of CTAP-III in combination with prostaglandin E_1 (PGE$_1$) and PGE$_2$ in synovial cell cultures. The combination of CTAP-III with either PGE$_1$ or PGE$_2$ had a synergistic effect on stimulation of GAG formation; both PGE$_1$ and PGE$_2$ partially antagonized the mitogenic effect of the platelet agent. Values are the mean and SEM. See Figure 2 for other definitions.

synovial extracts was of interest for several reasons. It was detected as easily in OA tissue as in RA tissue; in both cases, a major component was in a low molecular weight isoform with the electrophoretic mobility of CTAP-III–des 1-15/NAP-2.

Detection of a large proportion of the CTAP-III antigen as a cleavage isoform suggests that the agonist was recovered from the extravascular space and that it had been separated from the synovial circulation for an extended period of time prior to surgery. This interpretation is based on our experience that CTAP-III extracted from fresh platelets with acid–ethanol, from fresh platelet-releasate, and from platelet-releasate that is several days old (Figure 1) shows little or no evidence of proteolyic cleavage. It therefore seems likely that the CTAP-III antigens demonstrated in Figure 1 did not originate from platelets that were merely trapped in vessels at the time of surgery, but derive instead from earlier platelet α-granule releasate from platelet thrombi in the microvasculature or in the extravascular matrix.

The mobility of the cleavage isoform on SDS–PAGE is compatible with the presence of des 1-15/NAP-2, but the far less common (and also biologically active) des 1-14 and 1-13 forms cannot be excluded until microsequencing has been accomplished (11,38). It is also pertinent to note that the presence of CTAP-III isoforms implies the delivery of at least 4 other platelet-derived growth factors to the synovial membrane, all with the potential for influencing inflammation in that tissue. Findings of a recent study provided evidence that, in the rat, platelets mediate glomerular cell proliferation in immune complex nephritis induced by antimesangial cell antibodies (39), suggesting a central role for platelets in the inflammatory process.

Cleavage of CTAP-III to the des 1-15/NAP-2 isoform not only may increase the anabolic activity of the agonist, it also confers neutrophil-activating and chemotactic activity to the agonist. Both native and chemically synthesized NAP-2 have been shown to induce cytosolic free calcium, elastase release, and chemotaxis in human neutrophils (40,41). Other data indicate that CTAP-III is not an attractant; chemotactic activity appears to result from amino terminal cleavage to the CTAP-III des 1-13 and des 1-15/NAP-2 isoforms, which are able to interact with the NAP-1/IL-8 receptor on human neutrophils (42). Other findings suggest that both CTAP-III–des 1-15/NAP-2 and gro/MGSA interact with two classes of receptors for NAP-1/IL-8 (43). The pathologic significance of these results is highlighted by recent data showing that intra-

Table 3. Synovial growth effects induced by CTAP-III in binary combination with other cytokines

Combination*	Effect on growth measurements	
	^{3}H-DNA	^{14}C-GAG
CTAP-III + rEGF	Synergistic	Synergistic
CTAP-III + rPDGF-BB	Synergistic	Additive
CTAP-III + rTGFβ	Partly additive	Partly additive
CTAP-III + rIL-1β	Antagonistic	Synergistic
CTAP-III + rbFGF	Additive	Synergistic
CTAP-III + PGE$_1$	Antagonistic	Synergistic
CTAP-III + PGE$_2$	Antagonistic	Synergistic

* CTAP-III = connective tissue activating peptide–III; see Figures 2–7 for other definitions.

dermal injection of CTAP-III–des 1-15/NAP-2 into rabbit skin promotes neutrophil accumulation and plasma leakage when administered along with vasodilating substances (44).

Evidence supporting the presence of multiple cytokines in abnormal human synovium is persuasive; however, data are scarce concerning tissue cytokine concentrations or how these agonists act in the aggregate to influence synovial cell behavior. Since the net effects of a "cytokine network" may reflect the consequences of multiple cytokine–cellular interactions, we have attempted to enhance understanding of the behavior of synovial cells when they are confronted with more than one cytokine at a time.

The measurements of growth-related activity of selected cytokines in binary combinations are summarized in Table 3. It is clear that synergistic interactions were more common when cytokine combinations were evaluated in the ^{14}C-GAG assay; only the combinations of rPDGF-BB and rEGF with CTAP-III were synergistic in stimulating DNA synthesis. EGF is the best known member of a large family of cytokines that includes TGFα, platelet-derived EGF-like protein, and amphiregulin, all able to bind to EGF receptors and presumably to evoke characteristic EGF responses (45). Other widely distributed proteins with EGF-like domains, not yet known to bind to EGF receptors, include tissue-type plasminogen activator, clotting factors IX and X, low density lipoprotein receptor, and proteoglycan core protein.

The ability of PGE$_1$ and PGE$_2$ to antagonize the action of CTAP-III on DNA synthesis was marked, but not complete. Others have shown that rIL-1β and rTNFα in combination had a marked synergistic effect on both prostaglandin E and collagenase production, but only additive effects on GAG (hyaluronic acid) formation (46). PDGF-BB has also been shown to act

synergistically with IL-1α in promoting production of metalloproteinases in rabbit articular chondrocyte cultures (47). These data indicate that PDGF-BB and IL-1α each up-regulate cell receptors for the other agonist. The possibility that this particular binary combination may exist in rheumatoid synovium is supported by immunologic evidence for the presence of PDGF-B–like peptide in these tissues (48).

The present results and earlier findings indicate that the difference between additive and synergistic effects is influenced by the concentrations of the agonists that are combined. The data suggest that, in addition to endogenous factors, CTAP-III and other platelet-derived cytokines may have roles in the regulation of synovial cell metabolism in RA and OA. It is likely that particular combinations of growth factors may be more important than single factors in the amplification or suppression of important cell functions.

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