CONNECTIVE TISSUE ACTIVATION

XX. Stimulation of Prostaglandin Secretion by Mediators from Lymphocytes (CTAP-I) and Platelets (CTAP-III)

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Connective tissue activating peptides from lymphocytes (CTAP-I) and platelets (CTAP-III) are known to stimulate glycosaminoglycan synthesis, glycolysis, and mitogenesis in connective tissue cell cultures. Direct evidence suggested that increased accumulation of cyclic AMP was involved in the action of these peptide agonists, and increased prostaglandin E synthesis was postulated on the basis of indirect evidence. In the present experiments, CTAP-I and -III were incubated with human and murine cells in culture, and prostaglandin E was measured by radioimmunoassay using antibody directed primarily to prostaglandin E2. Both CTAP-I and -III markedly stimulated the elaboration of prostaglandin E into culture medium, the earliest evidence of increased synthesis occurring at 4 hours with maximal concentrations found at 24 hours. Substantial residual stimulation persisted at least through 48 hours. Indomethacin (13.0 μg/ml) obliterated basal and incremental synthesis of prostaglandin in the presence of mediators. Cycloheximide (8.7 μg/ml) did not affect the stimulation of prostaglandin synthesis by CTAP-I and -III. Three nonrheumatoid and 3 rheumatoid synovial cell strains showed similar basal levels of prostaglandin E and similar responses to CTAP-I. A murine fibroblast cell strain (3T3) showed increased prostaglandin E synthesis on exposure to CTAP-I, and the KB tumor cell strain was markedly stimulated by CTAP-III. These studies confirm the increased synthesis of E series prostaglandins postulated to occur in human connective tissue cells on exposure to CTAP-I and -III, and clarify the mechanism of action of these agonists on "activated" target cells. The importance of elevated extracellular concentrations of prostaglandins is uncertain, although they may act directly on sensitive cell types as well as potentiate the actions of CTAP-I and -III on neighboring cells.

Recent data suggest that substances released from leukocytes and platelets during the acute phase of the inflammatory response may be instrumental in activating connective tissue and initiating repair activities in that cellular system (2–5). Products (connective tissue activating peptides [CTAP]) derived from platelets during the coagulation sequence (CTAP-III), from lymphoid cells (CTAP-I), and from exudative leukocytes (CTAP-PMN) are all capable of stimulating connective tissue cells. CTAP-I (lymphocytes) stimulates energy metabolism and glycosaminoglycan synthesis, whereas CTAP-III (platelets) possesses the additional ability to stimulate DNA synthesis. The lymphocyte and platelet materials have been isolated and characterized, the amino acid composition of both is known, and the sequence of the platelet factor has been reported (4,6). One of the major differences is the essential sulfhydryl

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Table 1. Basal and CTAP stimulated PGE2 synthesis in normal and rheumatoid synovial cultures

	PGE pg/1.5 × 10^5 cells/24 hrs*		
	Control	CTAP-I	CTAP-III
Normal cell strains			
CF-C	76 ± 36	236 ± 97†	184 ± 47†
CF-G	115 ± 5	5490 ± 853‡	240 ± 98§
CB-E	50 ± 18	703 ± 53¶	552 ± 238#
WP-F	13 ± 7	166 ± 59**	762 ± 181¶
Rheumatoid cell strains			
CS-F	477 ± 101	4773 ± 654‡	1178 ± 128¶
OD-H	13 ± 3	372 ± 129††	1590 ± 540††
HD-E	113 ± 42	815 ± 14‡	333 ± 114#

* Data are the mean ± 1 SD of triplicate measurements in a single experiment. Similar data were obtained when cell strains CF, WP, and CS were studied in additional experiments.
† $P < 0.025$.
‡ $P < 0.001$.
§ $P < 0.20$.
¶ $P < 0.005$.
** $P < 0.02$.
†† $P < 0.01$.

component of CTAP-I as opposed to two essential disulfide bridges in the CTAP-III platelet factor.

An early proposal for the mechanism of action of the connective tissue activating peptides suggested that they interacted with the cell membrane at a complex receptor site (7). Stimulation of glycolysis and hyaluronate synthesis was shown to depend on both RNA and protein synthesis, and this process appeared to require the participation of a coupled prostaglandin-cyclic AMP system (7,8). This latter component of the mechanism of activation was thought to be important in making energy and carbon available for the glycosaminoglycan synthetic processes. Indirect evidence suggested a role for the participation of prostaglandins in the mechanism of synovial cell activation by CTAP. For instance, connective tissue activation was blocked by inhibiting prostaglandin synthesis with indomethacin and acetylsalicylic acid, or by antagonizing the action of prostaglandins with agents such as polyphloretin phosphate or 7-oxa-13-prostynoic acid (8). Further, we demonstrated earlier that the actions of connective tissue activating peptides were markedly potentiated by low concentrations of prostaglandins, which in and of themselves had no measureable activity. Lastly, direct measurements of cellular cyclic AMP content showed this mediator to be substantially stimulated by the addition of prostaglandins of the E and F series to synovial cultures (8).

In the present experiments, we provide direct evidence that synovial cell cultures release prostaglandin E (PGE) upon exposure to either CTAP-I or CTAP-III.

MATERIALS AND METHODS

Cell culture methods. Human synovial membrane specimens were obtained at arthrotomy or amputation, and a portion of each was fixed for histology. The remainder of the samples was divided into 1 mm^3 explants from which primary cell strains were developed, as previously reported (9). Human dermal cultures were established in a similar manner. Rheumatoid synovial specimens were from patients fulfilling the American Rheumatism Association criteria (10). Guinea pig lung fibroblast cultures were established following enzymatic disaggregation of the tissue (11). Human and mammalian cell strains and lines were recovered from frozen storage for use in these studies as needed. Routine culture medium for human connective tissue cells consisted of 80% synthetic medium 1066, 10% fetal calf serum, and 10% heat inactivated human serum supplemented with L-glutamine, penicillin, streptomycin, and 0.02*M* Hepes buffer. Human serum was inactivated at 56°C for 1 hour. Cells were counted with an electronic cell counter (Model B, Coulter Electronics, Hialeah, FL).

Sources of CTAP. CTAP-I was isolated from human spleen and from a continuous human lymphocyte line as reported earlier (2). CTAP-III was isolated from outdated human platelets by procedures published recently (4).

Isotope incorporation studies. Incorporation of ^{14}C-glucosamine into glycosaminoglycans (GAG) was carried out as described previously (12). In brief, fibroblasts were plated in well cultures at 20 × 10^3 cells per well and nourished by 200 μl of medium containing 0.5 μCi/ml of D-[U-^{14}C]-glucosamine HCl. After 40 hours of incubation, the medium was removed, and the isotope content of macromolecular species in the medium was estimated by scintillation counting after separation from unincorporated isotope as reported previously (12), or alternatively, by selectively precipitating the GAG on filter paper with cetylpyridinium chloride and washing away unincorporated isotope, a method to be reported in detail elsewhere. Characterization of glycosaminoglycan mixtures was accomplished by incubation of radiolabeled glycosaminoglycans with specific carbohydrases before cetylpyridinium chloride precipitation, washing, and measurement of radioactivity. Incubation conditions have been defined (13), and the specificity of the fungal hyaluronidase (14) and chondroitinase (15) has been established.

Radioimmunoassay (RIA) of PGE2 was carried out by standard methods with antisera from Miles-Yeda Ltd. (16). This antiserum showed less than 0.01% cross-reactivity with F-series prostaglandins, but showed 60% cross-reactivity with PGE1. Data were calculated in PGE2 equivalents from a PGE2 standard curve ranging from 7.8 to 500 pg/ml with a detection limit of approximately 900 fg/assay tube. Serum-free Eagle's synthetic medium (ESM) was used for the cell culture experiments. This medium was shown not to contain any materials that interfere in the RIA for PGE2. One-half milliliter of medium was used to support 1.5 × 10^5 cells in each culture well during these experiments. Cells were planted at zero time in serum containing medium and allowed to attach and spread; the following day the medium was changed to serum-free ESM and various additives as indicated, and in-

Table 2. Basal and CTAP stimulated PGE2 synthesis in nonsynovial cell cultures

Cell strain	PGE pg/1.5 × 10^5 cells/24 hrs		
	Control	CTAP-I	CTAP-III
CF-S-F human skin fibroblast	271 ± 83	1162 ± 64*	-
KB human epidermoid cancer	1165 ± 122	1212 ± 73†	4487 ± 1404‡
Guinea pig lung fibroblast	743 ± 4	1428 ± 527§	-
3T3 murine fibroblast	757 ± 120	1670 ± 352‡	236 ± 23¶

* $P < 0.001$.
† Not significant.
‡ $P < 0.02$.
§ CTAP-I-like material prepared from guinea pig spleen. $P < 0.20$.
¶ $P < 0.005$.

cubation was carried out for 24 hours. Purified prostaglandins used in the culture experiments were made available through the courtesy of Dr. John E. Pike, the Upjohn Company, Kalamazoo, Michigan.

RESULTS

Three normal synovial cell strains synthesized measureable amounts of prostaglandin E in the course of 24 hours (Table 1). These concentrations were substantially increased by the addition of either CTAP-I or CTAP-III. Further, three rheumatoid synovial cell strains exhibited a similar increase in secretion of PGE on exposure to these mediators. The first rheumatoid cell culture that was studied had a very high basal rate of prostaglandin synthesis and proved to be very responsive to both CTAP preparations. However, such high basal synthetic rates did not prove to be a consistent finding in all rheumatoid cultures (Table 1).

Cell strains other than synovial cultures were also capable of releasing PGE in response to the CTAPs (Table 2). A human skin fibroblast strain synthesized four-fold more prostaglandin on exposure to CTAP-I, and a human epidermoid carcinoma cell strain proved to be quite sensitive to CTAP-III but failed to respond to the lymphocyte peptide. Both a primary lung fibroblast strain and an established line of murine fibroblasts were responsive to CTAP-I.

In two experiments, the increasing concentrations of prostaglandins induced by CTAP-I or CTAP-III were plotted against time (Figure 1). Both peptides stimulated increased PGE2 secretion detectable by 8 hours; maximal values were observed by 24 hours (strain CB, Figure 1). An experiment with another primary synovial cell strain suggested that the elevated concentrations of PGE in the media may decay relatively slowly (strain WP, Figure 1). A dose response curve for CTAP-III (Figure 2) indicated that the secretion of PGE into the culture medium is a function of CTAP-III concentration. Increases in CTAP-I concentrations also lead to increased PGE secretion (data not shown).

Comparative effects of prostaglandins on net ^{14}C-GAG formation. Previous studies clearly showed that PGE1, PGE2, PGF1α, and PGF2α stimulated GAG (hyaluronic acid) formation by human synovial cells in vitro (8). In the present study, we compared CTAP-I, CTAP-III, bovine insulin, and several prostaglandins for their capacity to stimulate ^{14}C-glucosamine incorporation into hyaluronate secreted into the culture medium. In both experiments recorded in Table 3, CTAP-I, CTAP-III, and PGE2 showed a striking ability to

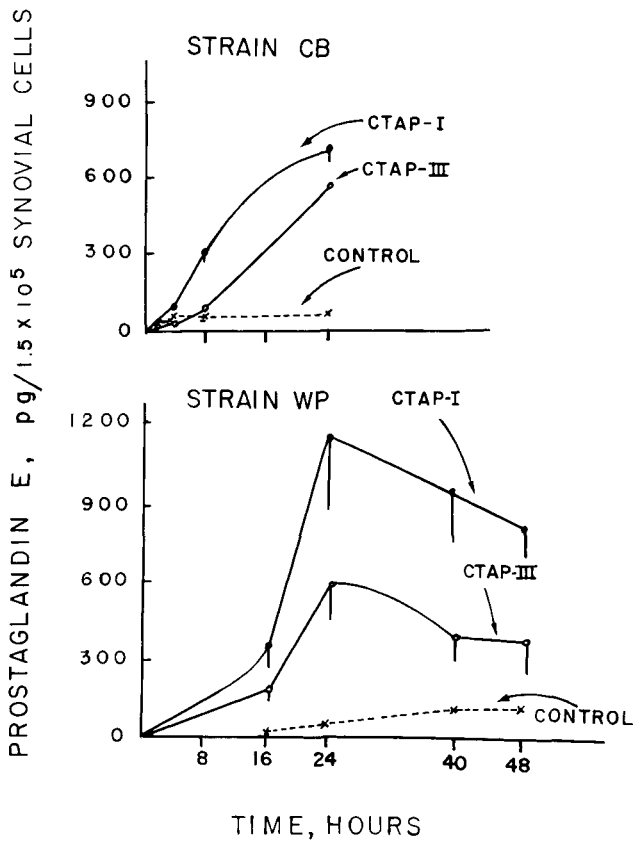

Figure 1. Stimulation of PGE synthesis by CTAP-I (15.6 μg/ml) and -III (35 μg/ml) as a function of time.

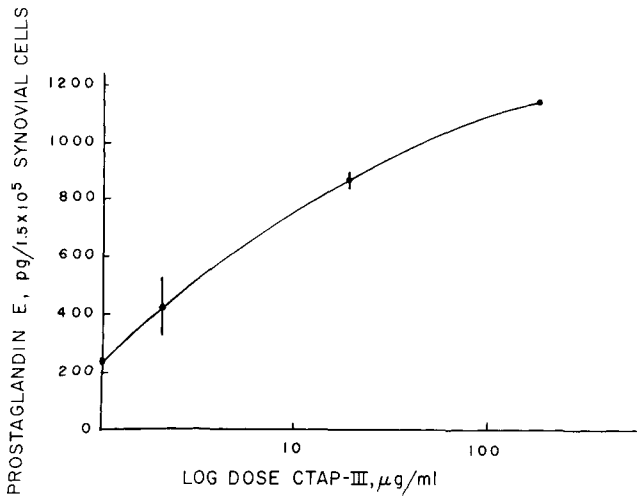

Figure 2. CTAP-III stimulation of PGE synthesis, a dose-response study. Values plotted are mean ± SD. Incubation time was 24 hours.

stimulate hyaluronate formation, and on a "specific activity" basis (Table 3, last column), PGE2 was the most potent prostaglandin tested. Several other prostaglandins also induced hyaluronate formation in the concentration tested, albeit to a lesser extent for this particular cell type (Table 3).

Effect of drugs on PGE formation. The effects of selected drugs on CTAP-stimulated PGE2 synthesis are recorded in Figure 3. Both CTAP-I and CTAP-III stimulated PGE synthesis markedly over control levels. Cycloheximide failed to block the effect of either CTAP on prostaglandin synthesis and secretion (upper panel, Figure 3). Conversely, indomethacin markedly reduced the stimulatory effect of both CTAP-I and CTAP-III on PGE synthesis in cultivated synovial cells. In a similar experiment, PGE synthesis was suppressed by acetylsalicylic acid as well as by indomethacin (lower panel, Figure 3). It was interesting in this experiment that hydrocortisone totally suppressed the stimulatory effect on PGE synthesis of the lymphocyte peptide, but was somewhat less suppressive in the case of CTAP-III.

DISCUSSION

Regulating synovial cell synthesis and secretion of prostaglandins may have pathophysiologic importance in view of reports documenting their tissue distribution and studies of agents able to stimulate or inhibit their formation. Explant cultures of rheumatoid synovial membrane have been shown to synthesize and secrete primarily PGE2 (17). Interestingly, colchicine stimulated PGE2 synthesis in this system. The relevance of these observations to serious clinical aspects of synovitis was indicated in parallel studies suggesting that PGE2 from rheumatoid synovium induced bone resorption as measured by calcium release (18). Immunofluorescent studies with antisera to PGE localized prostaglandin E in the synovial intimal cells of rheumatoid synovial tissue and showed that the intensity of PGE staining in synovial cells was positively correlated with the degree of lymphocyte infiltration (19). This latter finding is consistent with the possibility of a significant role for CTAP-I in rheumatoid synovitis, since this lymphocyte-derived mediator was shown here to markedly stimulate PGE formation by synovial cells.

Synthesis and secretion of PGE by human synovial and skin cells in monolayer cultures are known

Table 3. Comparison of substances stimulating ^{14}C-glucosamine incorporation by human synovial cells

Additive	Concentration of additive, μg/ml	Incremental ^{14}C-hyaluronate in media* CPM/2 × 10^4 cells	CPM/2 × 10^4 cells /nmole of additive	*P*
Experiment #1				
CTAP-I	50.5	8278	9016	<0.01
CTAP-III	77.7	2297	1375	<0.01
Insulin†	5.0	405	2430	<0.01
PGE1	1.0	968	1379	<0.01
PGE2	1.0	1085	1546	<0.01
PGF1α	1.0	107	152	<0.05
PGF2α	1.0	321	457	<0.01
6-keto-PGF2α	1.0	39	56	NS
PGD2	1.0	172	245	<0.10
PGI2	1.0	441	628	<0.01
Experiment #2				
CTAP-I	50.5	7640	8321	<0.01
CTAP-III	15.5	979	2929	<0.01
Insulin	25.0	789	947	<0.01
Serum, human‡	714.0	1006	479	<0.01
PGE1	1.0	857	1221	<0.01
PGE2	1.0	1554	2214	<0.01
PGI2	1.0	454	647	<0.01
6-keto-PGF2α	1.0	391	557	<0.10

* Cultures consisted of 20,000 cells in 200 μl media. Incremental ^{14}C-HA (over saline controls) in media was measured by scintillation counting after removal of free ^{14}C-glucosamine. In both experiments, the mean incremental ^{14}C-HA/20,000 cells was derived from four replicate well cultures for each substance. Control ^{14}C-HA measurements (saline vehicle) were 221 ± 63 CPM/20,000 cells in Experiment #1 and 533 ± 172 CPM/20,000 cells in Experiment #2. *P* values for the significance of differences between quadruplicate test wells and saline control wells are shown.

† Bovine insulin.

‡ Molecular weight of albumin used for calculation in last column.

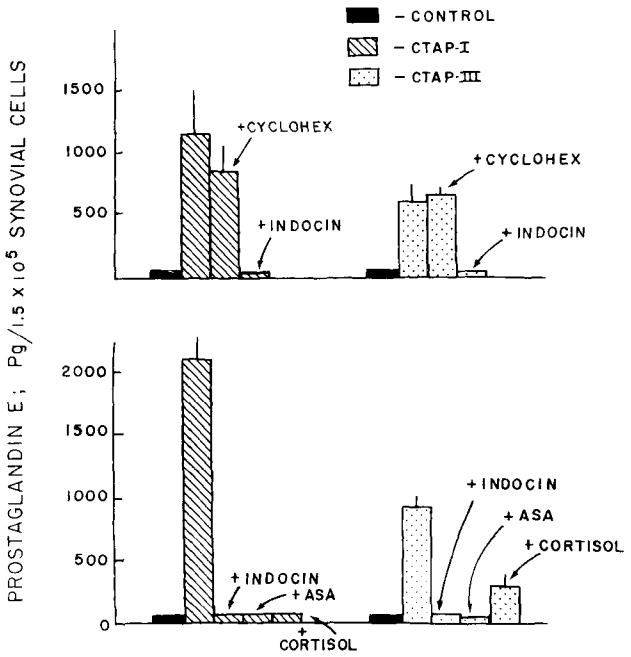

Figure 3. Effect of drugs on CTAP-stimulated PGE synthesis. Drug concentrations were: cycloheximide, 8.7 μg/ml; indomethacin, 15.0 μg/ml; acetylsalicylic acid, 300 μg/ml; cortisol, 1.0 μg/ml.

to be regulatable (20). Both human interferon and the interferon inducer, poly(I) · poly(C), markedly stimulated PGE formation and secretion, effects totally reversed by cortisol, 2.0 μg/ml.

The present data indicate that both CTAP-I and CTAP-III stimulated synthesis of PGE in cultures of human connective tissue cells. It is likely that the RIA for PGE is actually measuring PGE2 rather than PGE1, in view of other data linking PGE2 to the synovium (17,18). Responsive cell types included both normal and rheumatoid synovial cells and normal dermal fibroblasts. Agents known to interfere with PGE2 formation, including indomethacin, acetylsalicylic acid, and hydrocortisone, blocked most of the CTAP-induced prostaglandin synthesis, while cycloheximide was ineffective in this regard. The findings with acetylsalicylic acid, indomethacin, and cycloheximide are consistent with the belief that synovial cells contain an intact, functional cyclooxygenase, and that the protein synthesis required for activation does not include this prostaglandin synthetase system. Hydrocortisone inhibition of PGE synthesis by human synovial cells probably is related to blockade of arachidonate release, in view of recent evidence showing that dexamethasone blocked a lipase specific for arachidonate in rabbit synovial cells (21).

The data are consistent with our proposal that CTAP-induced connective tissue activation is mediated at least in part by endogenous prostaglandin synthesis (8). It is not clear whether CTAP-induced PGE synthesis influences connective tissue activation in a particular stimulated cell by re-uptake of newly secreted PGE, or whether the newly formed PGE is partitioned in some fashion, with a portion staying within the cell of origin. Exogenous consequences of secreted PGE were suggested by the experiments showing calcium release in a target tissue caused by PGE2 secreted by rheumatoid synovium (18). In a similar manner, our earlier data are compatible with an amplification mechanism whereby high concentrations of PGE2 secreted by one cell might directly stimulate GAG synthesis in a nearby cell; at a more distant site (at a lower concentration), it might serve to potentiate a threshold concentration of a CTAP mediator.

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