Connective Tissue Activation

I. The Nature, Specificity, Measurement and Distribution of Connective Tissue Activating Peptide

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A polypeptide which induces hypermetabolism in normal synovial fibroblasts in tissue culture is described. The metabolic changes induced included a marked increase in hyaluronate formation, lactate formation and glucose uptake; formation of soluble and fibrous collagen was depressed. An assay method is presented which permits quantitative comparisons of samples for connective tissue activating peptide (CTAP) activity. The CTAP appeared to be widely distributed in human tissues, the amount of activity being a function of cell density. A role for CTAP in rheumatoid inflammation is proposed.

Human synovial connective tissue cells can be activated in vitro by live leukocytes, extracts of human lymphocytes, polymorphonuclear leukocytes, thrombocytes, and extracts of embryonic human kidney cells and fibroblasts grown in tissue culture (1, 2). The term "activation" was used to focus attention on the increased fibroblast metabolic activity induced by such materials, including marked stimulation of hyaluronic acid formation, glucose uptake and lactate formation. It is clear that both allogeneic and syngeneic activator materials are effective in stimulating human synovial cells, and that a single exposure to such materials has effects that linger for weeks (2). Since this type of metabolic hyperactivity is known to be characteristic of rheumatoid synovitis (3, 4), it is suggested that activator materials may play a *central role in initiating and regulating the progression of the inflammatory process from the exudative to the reparative (proliferative) phase* (2).

The present report provides information concerning the chemical nature of connective tissue activators and demonstrates the existence of these materials in several human tissue types. A striking difference in the responsiveness of synovial and dermal fibroblasts to activator substances suggests that there is a degree of heterogeneity in the elements of the connective tissue acti-

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vating process, dependent on the anatomic location of the reactive connective tissue cells. A combined tissue culture-biochemical assay procedure is proposed which permits quantitative measurement and comparison of activator activity in biologic samples. Finally, the potential role of connective tissue activating peptide in regulating the transition from acute to chronic inflammation in the synovial membrane is discussed.

MATERIALS AND METHODS

Techniques for generating primary synovial and skin cell fibroblast cultures have been described earlier (5–7). Newer modifications of the author's procedures, including the methodology for frozen storage of fibroblasts using DMSO have been reported recently (8). Where the author has deviated from the usual culture procedures, as in the assay technique for measurement of connective tissue activating peptide, these alterations of methodology are treated in the body of the text. The sources of synovial tissue included amputations, arthrotomies, and punch biopsies, and the samples of other human tissues were obtained at postmortem examination.

Protein was measured by the method of Oyama and Eagle (9) and DNA was measured through its deoxyribose content by the method of Burton (10) after the isolation of the desoxyribonucleoprotein (11). Glucose was measured by a glucose oxidase method (12) *, and lactic acid was measured by the method of Barker and Summerson (13). Hyaluronic acid was measured by isolating the polymer (14) and determining its uronic acid moiety by a colorimetric procedure (15). Acid phosphatase was measured in cellular extracts and culture media by measuring hydrolysis of a p-nitrophenylphosphate substrate, and β-glucuronidase was measured by virtue of its capacity to hydrolyze phenolphthalein β-glucuronide (16, 17). Cells examined for acid phosphatase and β-glucuronidase activity were suspended at a concentration of 3.0×10^6 cells/1.0 ml of pH 7.0, 0.05M phosphate buffer, including 0.15M NaCl. This suspension was frozen and thawed three times and then centrifuged at 17,300 × g for 10 min at 4°C. The supernatant fluid was used for enzymatic assays.

In the section dealing with the development of methodology for assaying connective tissue activating peptide through its ability to stimulate formation of hyaluronic acid, a simplified procedure was used to isolate and measure hyaluronate. In order to obviate the need for proteolysis before isolating the hyaluronate polymer, the assays were carried out in serum-free Eagles' synthetic medium. In the serum-free assay experiments, 1.7 ml of media was pipetted into a 30 ml Corex centrifuge tube. Cetylpyridinium chloride (5% CPC in 0.2M Na_2SO_4) was added, 0.2 ml/sample, followed by 15 ml of distilled water to reduce the ion concentration in the mixture to below the critical salt concentration of CPC-HA. The preparations were incubated for 1 hr at 37°C to promote optimal flocculation of the CPC-HA complex. The CPC-HA complex was sedimented by centrifugation at 17,300 × g for 10 min at room temperature. The supernatant fluids were decanted, the precipitates in the Corex tubes were washed with approximately 25 ml of distilled water and again centrifuged at 17,300 × g for 5 min at room temperature. The wash was decanted and 20 ml of methanol containing 10% sodium acetate was added to each tube to facilitate conversion of the CPC-HA complex to sodium hyaluronate. This conversion requires approximately 1 hr or less at 37°C. The sodium hyaluronate is sedimented by centrifugation at room temperature at 17,300 × g for 10 min and the supernatant fluid is decanted. One and two-tenths milliliters of a phosphate buffer, 0.05M, pH 7.0, containing 0.15M NaCl and testicular hyaluronidase†, 0.33 mg/ml, were added to each tube. To assure complete solution of the sodium hyaluronate, the mixture was allowed to stand for 1 hr at 37°C with mixing on a vortex at 15-min intervals. Uronic acid is measured in 1.0 ml of this solution using the method of Bitter and Muir (15).

Connective tissue activating peptide was extracted from solid tissues by homogenizing the tissue in a Waring blender or a Virtis homogenizer using 1.0 g of solid tissue/10 ml of extraction buffer. The extraction buffer consisted of 0.05M phosphate buffer, pH 7.0 containing 0.15M NaCl supplemented by 0.1% β-mercaptoethanol and 0.001M EDTA (ethylenedinitrilo) tetraacetic acid, disodium salt.

*Glucostat, Worthington Biochemical Corporation, Freehold, New Jersey.

†Worthington Biochemical Corporation, Freehold, New Jersey.

RESULTS

Chemical Nature of Connective Tissue Activating Substance(s)

Rheumatoid cell strain, DW, 10^7 cells/ml, was extracted for 2 hr in distilled water after three freeze-thaw cycles. The 23,500 × *g* supernatant fluid (crude extract) from this extraction proved to be a potent stimulator of connective tissue metabolism (Table 1). Disruption by sonic disintegration* was equally effective in breaking the cells. Isotonic saline buffered (0.05M phosphate) at pH 7.0, as well as 0.25M sucrose, were also satisfactory extraction vehicles. As given in Table 1, the crude DW water extract lost no activity after 6 hr incubation at 37°C in 0.05M Tris buffer, pH 7.8, but virtually all biological activity was destroyed where the proteolytic enzyme pronase† (200 μg/ml) was included in the incubation mixture. In the controls where appropriate mixtures of buffers and pronase were added, no alteration of culture performance was detected. Making an active extract 10% with respect to trichloroacetic acid destroyed all biological activity, as did heating at 80°C for 30 min.

Dialysis of a crude *activator* preparation against water for 16 hr at 6°C did not detract from the activity of the material, and the concentrated dialysis bath revealed

*Branson Sonifer, model S-75, Branson Instruments Inc, Great Neck, Long Island. Sonication was carried out for 30 sec at Power 6 with the sample in an ice bath.

†Pronase, a proteolytic enzyme derived from *Streptomyces griseus* was obtained from California Corporation for Biochemical Research, Los Angeles, California.

Table 1. Destruction of Activator by a Proteolytic Enzyme*

Culture additives	Final fibroblast count × 10^{-6}	HA/flask† (μg)	HA synthesis rate (μμg/cell/day)	Glucose uptake (μM/10^6 cells/day)	Lactate output (μM/10^6 cells/day)
Saljne buffer + Tris buffer	4.01 (3.78–4.27)	114 (112–116)	28.3 (27.2– 29.7)	1.95 (1.84–2.06)	6.35 (6.19– 6.51)
Saline buffer + Tris buffer + Pronase	4.25 (4.11–4.39)	110 (108–112)	25.9 (25.5– 26.3)	2.75 (2.58–2.92)	5.07 (4.90– 5.24)
Tris buffer‡ + Activator	4.25 (3.73–4.00)	467 (448–480)	121.8 (111.9–128.9)	7.55 (7.33–7.76)	16.73 (15.52–17.94)
Activator incubated with Tris buffer 37° C for 6 hr.	3.99 (3.79–4.12)	488 (466–511)	122.6 (114.9–128.8)	7.79 (7.48–8.10)	13.41 (13.39–13.42)
Activator + Tris buffer + Pronase, 6 hr incubation	4.36 (4.21–4.44)	149 (144–153)	34.4 (32.6– 36.1)	2.78 (2.21–3.34)	4.60 (3.66– 5.55)

* The values recorded are the means from 3 flasks, and the figures in parentheses represent the ranges of the observations.

† HA is an abbreviation for hyaluronic acid.

‡ The activator used in this experiment was derived from rheumatoid synovial cell strain DW.

no evidence, of dialyzable activity (Table 2). When subjected to centrifugation* at 265,000 × *g* for 1 hr, the biological activity was found in the supernatant fluid while the pellet was essentially inactive (Table 2). Ammonium sulfate precipitated biological active material over a range from 35 to 90% saturation at pH 7.0, and was not helpful in recovering a purified active protein in good yield. Chromatography of activator preparations on Sephadex G-25† and Bio-Gel P-6‡ columns equilibrated with 0.15M NaCl, 0.05M PO_4, pH 7.0 buffer demonstrated that the activator protein was eluted with the excluded volume, suggesting that the molecular weight exceeded 4000, in agreement with the dialysis experiments. Similar chromatographic studies with Sephadex G-50 and Bio-Gel P-10 columns revealed retardation of the active principal, indicating a molecular weight of less than 10,000 and providing a useful tool for fractionating the polypeptide activator from crude mixtures.

The semipurified activator peptide preparations tended to lose activity with repeated manipulation, even in frozen storage. Evidence was sought that a labile SH group was required for the biologic activity of the polypeptide connective tissue activator, and the results are given in Table 3. In an alkylation experiment, a semipurified activator (41 μg protein) was reacted with 10.0 μg of iodoacetamide at pH 7.4 (PO_4 buffer) for 1 hr at room temperature in a 1.2 ml volume. The alkylated activator, and an aliquot of untreated activator, were dialyzed overnight against water in the dark to remove unbound iodoacetamide

*Beckman L2-65 Ultracentrifuge.

†Pharmacia Fine Chemicals, Inc, 800 Centennial Ave, Piscataway, New Market, New Jersey.

‡California Biochemical Corporation, Los Angeles, California.

Table 2. The Effect of Dialysis and Ultracentrifugation on Crude Activator Extracts

Culture additive	Final fibroblast count × 10^{-6}	Hyaluronate/flask (μg)	HA synthesis rate (μμg/cell/day)
None	5.26	198	12.6
	(4.94–5.43)	(188–208)	(12.2–12.8)
Undialyzed Activator*	6.33	357	18.9
	(6.18–6.62)	(350–368)	(17.8–19.8)
Dialyzed Activator	5.77	321	18.6
	(5.50–6.20)	(316–326)	(17.3–19.8)
Concentrated dialysis bath	5.23	189	12.0
	(4.94–5.40)	(182–194)	(11.7–12.3)
None	3.35	175	17.4
	(3.24–3.44)	(164–180)	(15.9–18.5)
Activator† extract	3.23	521	53.8
	(3.05–3.40)	(490–552)	(50.4–56.8)
265,000 × *g* Supernatant fluid	3.27	495	50.6
	(3.13–3.52)	(490–500)	(47.4–52.9)
265,000 × *g* Pellet	3.66	201	18.3
	(3.31–3.91)	(194–206)	(17.2–19.5)

* The activator used in the dialysis experiment was derived from normal synovial strain WaK.

† The activator used in this ultracentrifuge experiment was extracted from rheumatoid synovial cell strain DW.

Table 3. Evidence for Important Sulfhydryl Functions in the Connective Tissue Activating Polypeptide

Culture additive	HA synthesis rate (μg HA/mg cell protein/24 hr)
Buffer	6.5
Activator peptide*	26.8
Alkylated activator peptide	8.0
Buffer	17.9
Activator† + PCMB‡	12.6
Activator + PCMB + Dithiothreitol	21.7

* A chromatographic fraction of the HE_{p-2} cell strain taken from retarded volume of a P-10 column.

† A semi-purified fraction from HE_{p-2}.

‡ PCMB = *p*-chloromercuribenzoate. (See text for explanation of manipulative procedures related to activator preparations.)

and iodide. When 0.1 ml of each of these preparations (3.4 μg protein) was added to a normal synovial monolayer, the unmodified activator stimulated a threefold increase in the HA synthesis rate, while the alkylated protein was without effect. A similar experiment demonstrated that an effective activator could be incubated at room temperature for 6 hr with 0.01M dithiothreitol at pH 7.0 with no loss of activity, suggesting that exposed disulfide bonds are not critical to the biologic function of activator protein. To support the evidence from the iodoacetamide experiments suggesting that a sulfhydryl function was vital, the author attempted to block the biologic activity by forming a mercaptide with *p*-chloromercuribenzoate. A semipurified activator preparation (60 μg of protein) was reacted with 50 μg of p-chloromercuribenzoate at 25°C in 0.05M Tris buffer, pH 7.0. After 2 hr, the sample was split and one-half was exposed to 0.1M dithiothreitol, while the control received the vehicle during a second 2-hr incubation. Both preparations were dialyzed overnight against water at 6°C and then assayed for activity. As given in Table 3, the activator blocked by PCMB did not have the capacity to stimulate HA synthesis, whereas the preparation subsequently treated with dithiothreitol, in an attempt to remove the mercurial compound, did actually show a modest restoration of activity. On the basis of these experiments suggesting that a reactive sulfhydryl group in the activator molecule was vital to the expression of its biologic activity, the extraction procedures were modified by including 0.1% β-mercaptoethanol in the extracting vehicle. This lead to a marked increase in the amount of activator activity which could be recovered from tissue homogenates, as much as a 100-fold increase in some instances, and provided indirect confirmation of the importance of the sulfhydryl function in the activator polypeptide.

The Behavior of Activated Synovial Connective Tissue Cells

Since the earlier reports demonstrating the stimulation of hyaluronic acid and glucose metabolism induced by activator in cultivated synovial cells, the effects of connective tissue activation on collagen metabolism have been examined. A representative experiment, given in Table 4, indicates that cultures exposed to an effective activator form much less soluble and fibrous collagen in the face of increased hyaluronate formation and glucose metabolism. As pointed out earlier (19), towards the end of a 9-day culture-period, the proportion of glucose uptake which can be accounted for as lactate tends to fall, suggesting a shift in the energy-producing pattern of the cell at the time when collagen

Table 4. Depression of Net Collagen Formation During Activation*

Parameter measured	Cultures: Control	Cultures: Activated
Final fibroblast count, $\times 10^{-6}$	10.49	6.75
HA/flask, sum of last two medium changes (μg)	924.0	1896.0
Soluble collagen/flask, sum of last two medium changes (μg)	315.0	121.0
Fibrous collagen/flask (μg)	270.9	106.0
HA synthesis rate† ($\mu\mu$g/cell/day)	21.7	45.0
Fibrous collagen synthesis rate ($\mu\mu$g/cell/9 days)	25.8	15.7
Glucose uptake (μM/10^6 cells/day)†	1.79	3.72
Lactate output (μM/10^6 cells/day)†	1.38	5.16

* This experiment was carried out in 9-day cultures whose media was supplemented at each of 4 changes with 200 μg ascorbate/ml of medium. The rationale for using this system for collagen study, and detailed presentation of the method of calculation are in recent reports (8,18). Activated cultures received the polypeptide during the final two medium changes.

† Computed for the fourth (final) medium change.

formation becomes a dominant activity. It is usually also true that the 7–9 day-interval reveals leveling off or deceleration of the hyaluronic acid synthesis rate. In a sense, it appears that the activator forces the connective tissue cell to continue its metabolic emphasis on hyaluronate formation sustained by a high rate of glycolysis, and in the process delays the shift to conditions favorable to collagen formation—an activity known to dominate the latter portion of simple inflammation as well as being a terminal activity of the model culture system.

In view of the interest in lysosomal enzymes as possible mediators of tissue injury in aggressive inflammatory reactions, evidence was sought concerning the effect of connective tissue activation on two typical lysosomal enzymes, acid phosphatase and β-glucuronidase. As given in Table 5, 6 separate experiments with two nonrheumatoid cell strains provided an opportunity to make 10 comparisons. In general, activated cells had more of a given lysosomal enzyme than did their controls. In 2 of 10 comparisons, the controls were higher, in 5 of 10 the increase in enzyme extracted from activated cells was minor, and in only 3 of 10 comparisons was the amount of enzyme from activated cells more than double the control values. In view of the variability of the enzyme measurements within a single cell strain, it is unwise to attach great significance to the relatively minor apparent increases in lysosomal enzymes induced by exposure to a single dose of activator. Assay of the culture media provided no evidence indicating significant release of

Table 5. The Effect of Activation on Lysosomal Enzymes*

Cell source	Acid phosphatase, units/10^6 cells: Control	Acid phosphatase, units/10^6 cells: Activated	β-glucuronidase, units/10^6 cells: Control	β-glucuronidase, units/10^6 cells: Activated
RN-E†	0.23	1.50	0.60	2.27
RN-F	0.76	0.90	3.88	3.62
RN-G	0.84	1.21	—	—
CaW-D	1.39	1.21	—	—
CaW-F	.66	.88	1.57	3.41
CaW-G	0.65	1.09	2.21	2.38

* In each of six separate experiments the cells were exposed to an activator for 2 days before harvesting cells and media for counting and chemical analysis. In each experiment, activation of the cells was confirmed by evidence of marked stimulation of HA formation in the activated flasks.

† Denotes the passage status of a cell strain; E represents the fifth passage since separation from the donor explants.

these two enzymes into the extracellular milieu of either control or activated flasks.

Specificity of the Synovial Fibroblast Response to Activator Materials

In a preliminary experiment, skin fibroblasts (JJ-S) were exposed to an activator, prepared from cultivated rheumatoid synovial cells, in a plan to examine the effect of the peptide on sulfated mucopolysaccharide formation. Surprisingly, the hyaluronate formation by these skin cells was virtually unaffected. Further, the sulfated mucopolysaccharide fraction isolated from the cellular mat (by proteolytic digestion and cetylpyridinium chloride fractionation (20)), was actually decreased 50% below that of the control flasks. This refractoriness of skin fibroblasts to activator of synovial origin proved to be a reproducible phenomenon. As shown in Table 6, dermal and synovial fibroblast strains were established from the same individual. On exposure to a modest amount of activator, derived from rheumatoid synovial cells, the target synovial cells responded with a modest increase in hyaluronate and glucose metabolism. Skin fibroblasts from the same patient were *not* stimulated by the synovial activator. To further examine the capacity of skin fibroblasts to respond to activator materials, activator substance from human spleen, HE_{p-2} tissue culture cells, and from two strains of dermal fibroblasts were prepared. From Table 7, it is apparent that skin fibroblasts JJ-skin and CaW-skin showed increased formation of HA *only* in *response* to *activator from dermal fibroblasts,* although all of the activators were effective against synovial strain HH. It was interesting that the dermal fibroblasts did show increased glucose uptake and lactate formation with virtually all types of activator while responding only to dermal activator with respect to increased HA formation. The JJ-skin strain responded equally to syngeneic and allogeneic skin cell activator, but the CaW-skin strain was less responsive to allogeneic activator. It is noteworthy that in these experiments the magnitude of stimulation of HA synthesis by skin cell strains, when it did occur, was modest by comparison to synovial strains exposed to the same amount of the same activator preparations.

Assay of Connective Tissue Activating Peptide (CTAP)

To assay connective tissue activating peptide the following procedure was

Table 6. Failure of Skin Cells to Respond to Synovial Activator

Target fibroblast strain	Activator source	HA synthesis rate ($\mu\mu$g/cell/day)	Glucose uptake (μM/10^6 cells/day)	Lactate output (μM/10^6 cells/day)
CaW-Synovium	Buffer	8.9 (7.9–10.1)	1.6 (1.4 –1.7)	1.3 (1.3– 1.3)
CaW-Synovium	DW Cells*	19.5 (16.5–23.0)	2.0 (1.98–2.01)	3.1 (3.1– 3.1)
CaW-Skin	Buffer	31.0 (27.6–32.7)	6.6 (6.5 –6.8)	9.8 (8.6–10.9)
CaW-Skin	DW Cells	24.9 (22.1–29.0)	5.5 (5.3 –5.8)	8.3 (7.9– 8.7)

* DW activator material from cultivated rheumatoid synovial cells was added in 50 μg amounts (equivalent to 5×10^6 cells) to the 10 ml of media in each of the three experimental flasks.

Table 7. Selective Response of Skin Fibroblasts to Activator from Dermal Fibroblasts

Target fibroblast strain	Activator source	HA synthesis rate ($\mu\mu$g/cell/day)	Glucose† uptake (μM/mg protein/day)	Lactate† output (μM/mg protein/day)
HH*-Synovium	Buffer	17.8	3.2	7.7
HH-Synovium	Spleen	46.3	7.8	14.5
HH-Synovium	CaW-Skin	70.2	8.4	16.4
HH-Synovium	JJ-Skin	68.0	7.3	15.4
JJ-Skin	Buffer	12.8	1.9	5.6
JJ-Skin	Spleen	14.0	4.8	10.9
JJ-Skin	JJ-Skin	27.6	4.8	11.0
CaW-Skin	Buffer	25.2	8.6	13.8
CaW-Skin	HE_{p-2}	18.7	9.8	20.0
CaW-Skin	JJ-Skin	27.6	6.2	17.2
CaW-Skin	CaW-Skin	32.5	8.7	21.8
JJ-Skin	Buffer	10.1	5.0	7.1
JJ-Skin	HE_{p-2}	11.1	7.6	14.0
JJ-Skin	JJ-Skin	27.6	4.8	11.9
JJ-Skin	CaW-Skin	30.8	6.9	14.4

* The letters designating the fibroblast strains refer to the initials of the donors.
† Measurements are in terms of μM/mg of cell protein/24 hr.

adopted: normal target synovial cells were trypsin dispersed and planted out before noon to provide 4–6 hr for attachment and spreading. Inocula consisted of 1.0×10^6 cells/T-15 flask, nourished by 2.0 ml of standard serum-containing media. After attachment of the synovial fibroblasts to the flask surface, the standard media was replaced by Eagles' synthetic medium buffered to pH 7.4 with 0.02M HEPES* buffer and supplemented as usual with penicillin, streptomycin and L-glutamine. Omission of serum simplifies the subsequent analysis of the medium for hyaluronate and prevents cell multiplication. Experimental flasks received 0.1 to 0.3 ml of the test sample, and duplicate control flasks received the same volume of the appropriate vehicle. Incubation was carried out at 35–37°C. The progressive increment in hyaluronate formed by activated cultures is illustrated in Fig 1. The amount of hyaluronate formed by a culture increased steadily with incubation time for at least 48 hr, at which point glucose in the medium was not yet exhausted. To define the relationship between concentration of activator peptide and culture response in terms of hyaluronate synthesis, a series of increasing concentrations of activator peptide were tested, terminating the assay at 40-hr incubation. As shown in Fig 2, the early portion of the dose-response curve is essentially zero order and then becomes curvilinear with no further increase in hyaluronate formation at the higher concentrations of activator. A unit of activator activity is defined as that amount which induces a culture to form 10 μg of hy-

*N-2-Hydroxyethylpiperazine-N-2-Ethanasulfonic acid, from Calbiochem, Los Angeles, California.

aluronate/mg of cell protein/24 hr more than the nonactivated control cultures. Such measurements must be made with dilute preparations of activator whose activity will fall on the linear portion of the dose response curve. One of the major difficulties with this system lies in intrinsic differences among different target cell strains (indicator strains). Dose-response curves generated by different cell strans in response to a single activator are usually similar, but seldom identical. Measurements made on a group of unknown samples will fall into the same rank order when measured against different indicator cell strains, but the actual units of activity of a given preparation measured against two different indicator cell strains may vary by as much as 50%. It is therefore important to attempt to use one cell strain routinely as an indicator strain, or if more than one strain is to be used, to assure oneself of the equivalent performance of the multiple cell strains. In any case, when a quantitative comparison between two or more samples is required, accuracy would be improved by assaying them simultaneously against the same indicator cell strain. The following relation may be used to calculate the concentration of activator in test samples:

$$\text{units of activator/ml} = \frac{A_2 - A_1}{10 \times V}$$

in which A_2 = hyaluronic acid synthesis rate in the stimulated culture corrected to μg hyaluronate/mg cell protein/24 hr; A_1 is this measurement in the unstimulated control flasks; and V = the volume of activator material added to the standard test system in milliliters.

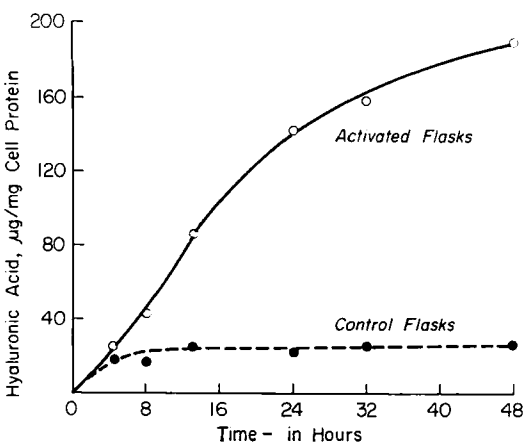

Fig 1. Control flasks showed only a minor increment in HA as a function of time, while activated flasks demonstrated more than a sixfold increase over the controls by 48 hr. Each point is the mean of duplicate flasks.

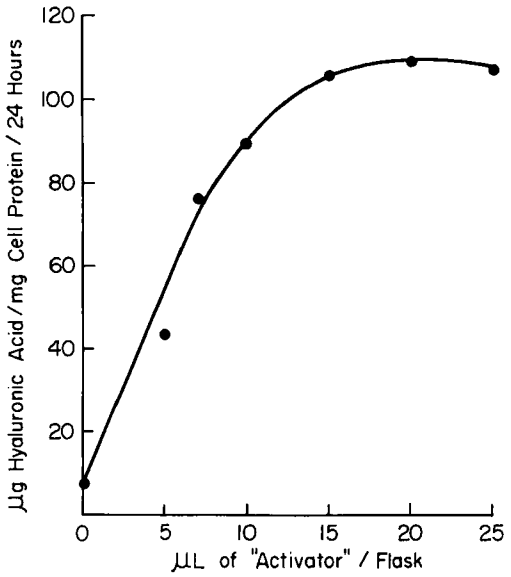

Fig 2. A normal synovial strain responded to increasing amounts of activator from a rheumatoid synovial strain, DW, by producing increasing amounts of HA until a maximal rate was achieved.

Distribution of Activator Peptide in Human Tissues

Quantitative data concerning the concentration of activator peptide in several

Table 8. Connective Tissue Activating Peptide (CTAP) in Human Tissues

Patient	Diagnosis	Fibroblast assay strain	CTAP, Units/100 μg DNA					
			Spleen	Liver	Lymph node	Kidney	Heart	Lung
OD	Carcinoma of breast, ascites and acute passive congestion of lungs	CaW-H	6.4	5.7	—	6.5	7.4	—
FL	Cirrhosis, brain infarct	HH-G	12.5	6.9	2.4	—	—	—
Jur	Pneumonia	HH-G	7.2	—	—	—	—	1.6
CB	Gunshot wound	HH-G	8.6	7.2	—	—	—	—
EFr	Chronic renal failure, Hemodialysis	HH-H	2.7	1.7	13.1	—	—	—
EP	Congenital heart disease, acute passive congestion of spleen	HH-H	12.0	3.5	—	7.5	—	—
JC	Diffuse interstitial pulmonary fibrosis, Prednisone	HH-F	5.3	2.3	1.9	—	—	—
MW*	Felty's syndrome	HH-G	8.6	—	—	—	—	—
KB*	Felty's syndrome	HH-G	6.5	—	—	—	—	—
MW	Felty's syndrome	FS-E	14.4	—	—	—	—	—
KB	Felty's syndrome	FS-E	10.6	—	—	—	—	—
Mean			8.6	4.6	5.8	7.0	7.4	1.6

* Made available through the courtesy of Dr. John L. Decker, Chief of the Arthritis and Rheumatism Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

human tissues is given in Table 8. Since it was clear that the activator peptide was a function of the number of cells extracted, the concentration of activator has been related to the amount of DNA found in the tissues. Examined in this way, there appeared to be no startling differences among the tissues examined with respect to the concentration of activator peptide. The variation in determinations of CTAP resulting from measurement with different indicator cell strains may be appreciated from Table 8 where data from patients MW and KB are recorded. It is noteworthy that the ratio of the measurements, MW / KB was virtually constant, being 1.32 in the first measurement and 1.36 in the second instance. Since the determinations in Table 8 may be influenced by pathologic processes and drug therapy, no conclusions about normal tissue levels of CTAP should be drawn from this random sample. It was interesting that the highest splenic values were found in circumstances associated with congestion or hyperplasia of the organ, while the lower values were associated with glucocorticoid therapy (JC) and a protracted debilitating illness (EFr). In patient Jur normal lung exhibited 1.6 units of CTAP/100 μg DNA, while acutely inflamed lung had 2.5 units of CTAP/100 μg DNA.

As shown in Table 9, similar concentrations were found in both normal and rheumatoid synovial tissue. The mean value for activator peptide extracted from rheuma-

Table 9. Connective Tissue Activating Peptide (CTAP) in Non-Rheumatoid and Rheumatoid Synovium

Patient	Diagnosis*	Treatment	DNA (μg/g tissue)	CTAP (Units/g tissue)	CTAP (Units/100 μg DNA)
CaW	Normal	0	188	33.1	17.6
HH	Normal	0	332	14.5	4.4
FS	Normal	0	381	14.7	3.9
JaK	TA	0	366	22.4	6.1
JKa	TA	0	189	13.0	6.9
WA	DJD	Oral steroids	189	0.0	0.0
CF	Ank. Spon.	Indomethacin	592	11.0	1.9
TH†	? JRA	Physical therapy	343	19.0	5.5
Mean			323	16.0	5.8
WH	RA	IA steroids Hydroxy-chloroquin	482	27.3	5.7
EF	RA	IA steroids	422	26.9	6.4
JJ	RA	IA steroids	618	3.4	0.6
EP	RA	IA Steroids, Indometha-cin Methotrexate	757	0.0	0.0
IK	RA	Oral steroids	617	32.0	5.3
HD	RA	Oral, IA steroids	569	24.5	4.3
MaH	RA	Phenylbutazone, Hydroxychloroquin	1019	19.7	1.9
Mean			641	19.1	3.5

* TA = Traumatic arthritis, DJD = degenerative joint disease, JRA = juvenile rheumatoid arthritis, and RA = rheumatoid arthritis, IA = intra-articular.

† TH is a 15-year-old boy with monarticular arthritis of uncertain etiology, trauma and JRA appearing most likely.

toid synovium was somewhat less than that from controls, a finding that may be related to the modality of treatment. All of the very low measurements of CTAP in synovial membrane were associated with oral and/or intraarticular glucocorticoid therapy, including a patient (WA) treated with oral steroids for degenerative joint disease.

In addition to the quantitative measurements presented in tabular form, activator peptide was demonstrated in human skeletal muscle and in established tissue culture cell strains such as HE_{p-2}, Chang liver, and J-111 malignant monocytes. The BHK hamster kidney fibroblast, the LM mouse fibroblast and a transformed human synovial cell strain were essentially without activity.

DISCUSSION

Current data indicate that the connective tissue activating substance is a water and saline soluble polypeptide with a molecular weight between 4000 and 10,000. It possesses one or more labile sulfhydryl groups per molecule which are essential to those biologic actions which we have studied. In disrupted cell preparations, the connective tissue activating peptide (CTAP) is found in the nonparticulate fraction after centrifugation at 265,000 × *g*. Extraction of CTAP activity from human

tissues in the absence of a thiol (dithiothreitol or β-mercaptoethanol) is difficult, and yields low levels of evanescent activity. The rapid disappearance of CTAP from the disordered environment of a tissue homogenate probably depends to a significant degree on the lability of sulfhydryl functions which readily undergo oxidation, alkylation, participate in disulfide interchange reactions, and form mercaptides. It seems unlikely that CTAP activity would persist for long in an extracellular site following injury and cell disruption, or that it could diffuse very far in an active form. It is not surprising then, that the CTAP was not detected in serum or cell-free joint fluid.

The peculiar sensitivity of fibroblasts from synovial membrane to the action of CTAP from diverse sources is unexplained. It is intriguing to recall that synovitis is frequently associated with granulomatous or proliferative processes in other organ systems, including sarcoidosis, ulcerative colitis and psoriasis, clinical entities wherein the articular manifestations often subside with control of the primary disease. As shown in this report, dermal fibroblasts are less sensitive and apparently have specific requirements best found in extracts of dermal cells themselves, adding yet another dimension to the array of differences known to exist between fibroblasts of synovial and dermal origin (7, 21). A survey of the activation process in fibroblasts from multiple organs and tissues is an important area for further study, and in this connection it is interesting that CTAP from rheumatoid synovial strain DW induced moderate hyperfunction in fibroblasts derived from human retroorbital tissue (22).

The assay procedure for measurement of CTAP is relatively rapid, reproducible and permits quantitative comparisons of biologic samples. The 40-hr incubation of the test sample with an indicator culture may be planned to terminate at 8–9 AM, which allows adequate time for measurment of cell protein and medium HA within 48 hr of initiation of the assay. All of the manipulations of hyaluronic acid in the serum-free procedure, except the final colorimetry, may be carried out in the Corex centrifuge tube which initially received the medium sample.

It is clear from the tissue survey that CTAP is widely distributed, and the absolute amount found in a given tissue is significantly related to cell density. When CTAP from a given set of cells is permitted to act on synovial fibroblasts, the latter cells are promptly induced to exhibit hypermetabolism characterized by a 3–40-fold increase in hyaluronic acid formation, marked stimulation of glucose uptake, lactate formation and hydrogen ion liberation. Concurrent with this burst of hypermetabolism, formation of soluble and fibrous collagen is depressed.

If one views simple inflammation as an integrated sequential process designed to repair an injury, it is reasonable to postulate signal mechanisms to turn on the anabolic (proliferative or reparative) phase of inflammation and to expect the origin of this signal mechanism to lie in the antecedent (exudative) phase. Such a role for CTAP (activator) in synovial inflammation is shown diagrammatically in Fig 3. The activator peptide is visualized as arising from necrotic tissue cells and from leukocytes of the exudative stage, and inducing increased energy metabolism and mucopolysaccharide (HA) formation in the synovial connective tissue cells. Clearly, *all* of the events of the reparative phase are *not* stimulated by CTAP. It would not be unreasonable to speculate concerning the existence of a family of activators working in sequence to complete the anabolic

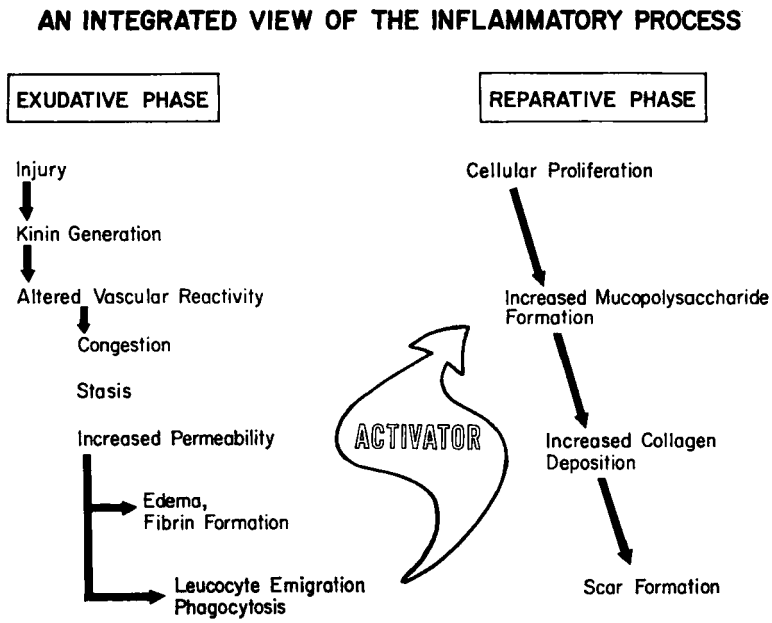

Fig 3. An abbreviated schematic summary of the exudative and reparative phases of the inflammatory process, showing an activator substance regulating the transition from one to the other.

process. Of particular interest in the context of rheumatoid inflammation is an analysis of the consequences of prolonged, excessive stimulation by CTAP. One would expect chronic overproduction of hyaluronate and increased synovial energy metabolism, as seen in garden variety rheumatoid synovitis. In addition, the tendency of CTAP to freeze metabolic activities of synovial cells at the level of mucopolysaccharide formation, and to depress collagen formation, might well delay the completion of the reparative process. Chronic hyperstimulation of rheumatoid synovial tissue may be a consequence of CTAP derived from polymorphonuclear cells of the synovial fluid, lymphoid cells of the stromal infiltrate, and possibly an augmented content of CTAP in the surface synovial cells themselves (23). Future measurements of CTAP in synovial tissue would be most useful if the confusion incident to drug effects could be relieved, and if the superficial and deep layers were examined separately. It is possible that turn over studies rather than single time point measurements will be required to adequately assess the role of activator in rheumatoid synovitis.

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