Vol. 96, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1980 Pages 498-505

STUDIES ON A LEUKOCYTE ELASTASE INHIBITOR PRESENT IN THE CULTURE MEDIUM OF INFLAMED SYNOVIAL TISSUE

Mary E. Englert, M. Jane Landes, Jay E. Birnbaum,
Arnold L. Oronsky and S. S. Kerwar[1]

Connective Tissue and Arthritis Research Unit
Metabolic Disease Research Section, Medical Research Division,
American Cyanamid Company, Lederle Laboratories, Pearl River, New York 10965

Received July 21,1980

Summary

The spent medium of cultured inflamed synovial tissue contains a potent inhibitor of leukocyte elastase. This leukocyte elastase inhibitor has no effect on leukocyte cathepsin G and pancreatic elastase is only marginally affected. The inhibitor is a glycoprotein, stable to heat, acid and reductive alkylation. Pretreatment of the inhibitor with either trypsin or chymotrypsin results in its inactivation.

Introduction

Studies from several laboratories (1-4) have suggested that the degradation of cartilage proteoglycans observed in inflammatory joint diseases is catalyzed, at least in part, by neutral proteases secreted by the polymorphonuclear leukocytes that are present in the synovial fluid. These leukocytes contain at least two neutral proteases, elastase and cathepsin G, which *in vitro*, can degrade the protein core of cartilage proteoglycans. Degradation of cartilage proteoglycans has also been observed upon intraarticular administration of these enzymes into joints of normal animals, suggesting that these enzymes can potentially contribute to the destruction of the cartilage matrix (5).

In inflammatory joint disease, studies by Barrett (6) indicate that at any time the synovial fluid contains at least 10^8 leukocytes. Since these cells survive approximately 3-4 hrs in the joint fluid, it has been suggested that approxi-

[1] To whom correspondence should be addressed.

0006-291X/80/170498-08$01.00/0

mately 8 mgs/day of neutral proteases can be potentially present in the inflamed synovial fluid. This large concentration of neutral proteases can very rapidly degrade the cartilage matrix. However, this does not occur because the synovial fluid contains α_2 macroglobulin and α_1 antitrypsin, known inhibitors of leukocyte neutral proteases (6).

The present studies indicate that the inflamed synovial tissue contains a potent inhibitor of leukocyte elastase but not of leukocyte cathepsin G. The inhibitor has been partially purified and its properties are described.

Materials and Methods

Inflamed synovial tissue from rabbits that had been immunized with bovine serum albumin was removed by surgery (7), coarsely minced and incubated in serum-free Minimal Essential Medium (MEM) containing glutamine and streptomycin/neomycin. After 48 hours, the medium was harvested and used as the source of the leukocyte protease inhibitor.

The inhibitor present in the spent medium was adsorbed on Concanavalin A Sepharose (0.9x7 cm) and eluted with 50 mM cacodylate buffer, pH 7.4 containing 5 mM $CaCl_2$ and 1 M α-methyl mannoside. This eluate after dialysis (50 mM Tris-Cl pH 8.0, 5 mM $CaCl_2$) was concentrated and fractionated on DEAE-cellulose (0.9x10 cm). After gradient elution with the above buffer containing 0.2 M NaCl, column fractions that contained the inhibitor were pooled and stored frozen.

SDS gel electrophoresis was conducted by the method of Weber and Osborne (8). Crude leukocyte neutral proteases were prepared from human leukocytes by the method of Barg *et al* (9). Pure human leukocyte elastase and partially purified leukocyte cathepsin G were prepared by the method of Baugh and Travis (10).

The assay system for protease activity using ^{35}S-labeled proteoglycans ($1.5x10^4$ cpm/ms dry beads) trapped in polyacrylamide beads has been described (9). Inhibitory activity in either the spent medium of the inflamed synovial cultures or in the partially purified inhibitor preparation was determined by adding aliquots to the assay system described (9). One unit of inhibitor represents the amount of protein required for 50 percent inhibition of enzyme activity and was determined by titrating the inhibitor with the enzyme in the above assays. Collagenase assays were conducted using [^{14}C]-labeled collagen as described (11). Protein concentrations were determined by the method of Lowry *et al* (12) using bovine serum albumin as the standard.

Results

The spent medium of cultured inflamed synovial tissue was examined for proteolytic activity using [^{35}S]-labeled proteoglycans. Results shown in Table I indicate that the spent medium was completely devoid of protease activity. Mixing

Table I

The Effect Of The Spent Medium Of Inflamed Synovial Tissue On Crude Human Leukocyte Neutral Proteases

	cpm[^{35}S] Released	Percent Activity
Synovial tissue spent medium (20 µg protein)	0	0
Crude leukocyte neutral proteases (8.6 µg protein)	3,961	100
Plus synovial tissue spent medium (20 µg protein)	2,572	65
(100 µg protein)	830	21

experiments of the spent medium with crude leukocyte neutral proteases indicated the presence of an inhibitor of proteolysis.

The inhibitor in the spent medium was purified by chromatography on Concanavalin A Sepharose and on DEAE-cellulose (Table II). SDS-polyacrylamide gel electrophoretic analysis (Figure 1) of the inhibitor (8) indicated that the material was not homogenous, one major and two faint bands were detected on staining with Coomassie Blue. To determine which of the enzymes in the crude leukocyte homogenate was susceptible to inhibition by the partially purified inhibitor, experiments were carried out with pure leukocyte elastase and partially purified cathepsin G. Using pure elastase, addition of the inhibitor to the assay system resulted in almost complete loss of enzymatic activity (Figure 2A). The mech-

Table II

Purification Of The Leukocyte Neutral Protease Inhibitor From The Spent Medium Of Inflamed Synovial Tissue

	Volume	mg Protein/ml	Total Protein (mg)	Specific Activity Units/mg	Total Units
Spent Medium	92	4.0	368	36	13,248
Con A Sepharose Pool	105	0.385	40.4	226	9,130
DEAE Cellulose Pool	42	0.440	18.5	376	6,974

1 unit of inhibitory activity represents the amount of protein required to inhibit crude leukocyte neutral protease activity (8.6 µg protein) by 50 percent. Other details of the assay are described in the text.

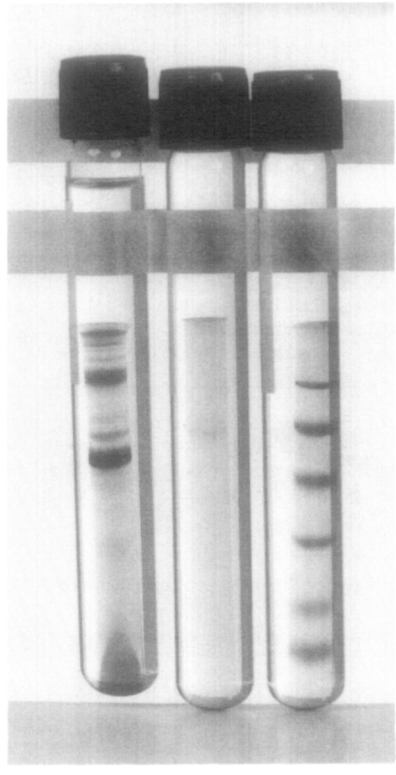

Figure 1 SDS gel electrophoretic analysis of Left crude spent medium of inflamed synovial tissue, Middle partially purified elastase inhibitor. Right Proteins of known molecular weight (Phosphorylase b M_r 94,000; Bovine serum albumin M_r 67,000; Ovalbumin M_r 43,000; Carbonic anhydrase M_r 30,000; Soy bean trypsin inhibitor M_r 20,000 and α Lactalbumin M_r 14,400).

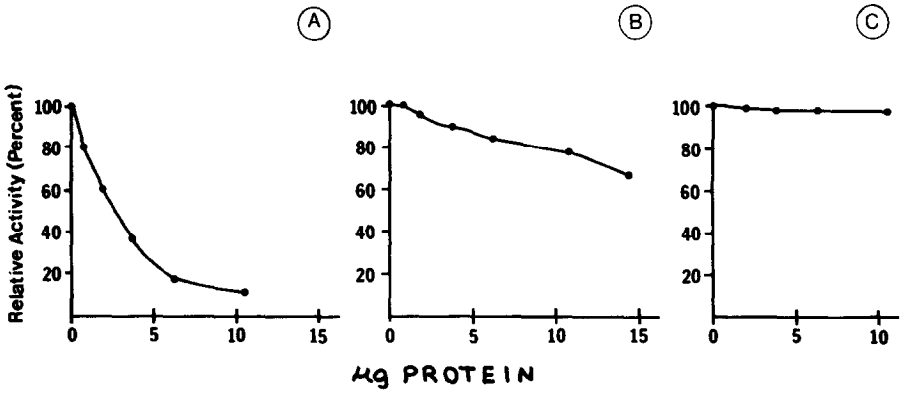

Figure 2 The effect of varying concentrations of the partially purified inhibitor on (A) pure leukocyte elastase (0.02 μg), (B) pure pancreatic elastase (0.2 μg) and (C) crude mammalian collagenase (10 μg).

Table III

The Effect Of Various Treatments Of The Inhibitor On Purified Leukocyte Elastase Activity

	Relative Activity (Percent)
0.03 µg Elastase	100
Plus native inhibitor (12.5 µg)	10
Plus heat treated inhibitor (60°, 15 min)	12
Plus acid treated inhibitor (pH 2.0 and neutralized)	25
Plus reduced alkylated inhibitor	22

Using the assay conditions described, 0.03 µg of leukocyte elastase releases approximately 4,000 cpm of [^{35}S] radioactivity from the labeled proteoglycans entrapped in polyacrylamide beads. Other details of the assay are described in the text.

anism of this inhibition is not known at the present time. The addition of the inhibitor to an assay system containing pancreatic elastase, indicated marginal inhibition (Figure 2B). The inhibitor had no effect on mammaliam collagenase activity (Figure 2C). In results not shown, the inhibitor had no effect on leukocyte cathepsin G activity.

Using pure human leukocyte elastase, the properties of the partially purified inhibitor were examined (Table III). The inhibitor was stable to heat and acid treatment. On reduction of the inhibitor with 0.1 M mercaptoethanol followed by alkylation with 0.2 M iodoacetamide (and subsequent dialysis) inhibitory activity was retained.

In results not shown, preincubation of the partially purified inhibitor (12.5 µg protein) with either trypsin (0.4 µg) or chymotrypsin (0.04 µg) for 15 minutes at 37°C resulted in the loss of inhibitory activity when assayed with pure elastase (0.02 µg) suggesting that the inhibitor is a protein.

When inflamed synovial tissue was incubated in MEM containing 10 µg/ml of cycloheximide, the amount of elastase inhibitor (units) in the medium was similar to that of control cultures not containing cycloheximide. These observations suggest that protein synthesis is not a requirement and that the inhibitor is pres-

ent in the inflamed tissue and released into the medium on incubation. Supporting this observation, inhibitory activity of leukocyte elastase could be detected in the homogenates of inflamed synovial tissue.

The possible relationship of the partially purified inhibitor to α_2 macroglobulin and α_1 antitrypsin, the two known leukocyte protease inhibitors, was examined. Using immunological techniques, the inhibitor did not cross react with either α_2 macroglobulin antisera or α_1 antitrypsin antisera (rabbit). The stability of the inhibitor to acid treatment confirms that the inhibitor is distinct from α_2 macroglobulin or α_1 antitrypsin. Both of these inhibitors are inactivated on acid treatment. Furthermore, the inhibitor does not effectively decrease pancreatic elastase activity indicating that it is not related to α_1 antitrypsin.

The relationship of the inflammatory state of the synovial tissue to the concentration of the inhibitor was examined. Synovial tissue with minimal inflammation (Grade 1) in tissue culture secretes neglible amounts of the elastase inhibitor. Highly inflamed synovial tissue (Grade 3 to 4) secretes significant amounts of the inhibitor (approximately 300 units/mg synovial tissue - wet weight). Inflamed synovial tissue contains dendritic cells, fibroblasts, giant cells, macrophages and lymphocytes and we believe that one of these cell types contains the elastase inhibitor.

Discussion

The present studies indicate that the spent medium of inflamed synovial tissue is completely devoid of any neutral protease activity. However, the medium contains a potent inhibitor of leukocyte elastase, an enzyme that has been viewed to be largely responsible for the degradation of proteoglycans of the articular cartilage in inflammatory joint diseases. Under conditions in which the leukocyte elastase is maximally inhibited, the partially purified inhibitor has only a marginal effect on pancreatic elastase and has no effect on cathepsin G, the other leukocyte enzyme that can degrade cartilage proteoglycans. These observations suggest some degree of specificity to the inhibitor.

The destruction of the cartilage connective tissue matrix, collagen and proteoglycans, involves collagenase, and, at least in part, leukocyte elastase, respectively. Collagenase inhibitors have been isolated from a variety of tissues (13) and it has been suggested that the rate of destruction of collagen in normal and diseased tissue may depend on the ratio of the concentrations of collagenase and its inhibitor. From the present studies, a similar type of control mechanism is also suggested for the degradation of proteoglycans. The degradation of proteoglycans by elastase may be regulated by at least three components, namely, α_2 macroglobulin, α_1 antitrypsin and the inhibitor secreted by the inflamed synovial tissue.

Acknowledgements. We thank Ms. V. Garti and Ms. J. Franklin for their assistance in the preparation of the manuscript. Trasylol (a trademark of Bayer) was a gift from Bayer, Wuppertal, W. Germany.

References

1. G. Feinstein and A. Janoff, Biochem. Biophys. Acta 403, 477 (1975).
2. H. Keiser. R. A. Greenwald, G. Feinstein and A. Janoff, J. Clin. Invest. 57, 625 (1976).
3. L. J. Ignarro, A. L. Oronsky and R. J. Perper, J. Clin. Immun. Immunopath. 2, 36 (1973).
4. J. E. Smolen and G. Weissman in Neutral Proteases of Human Polymorphonuclear Leukocytes (Ed. K. Havemann and A. Janoff) pp. 55-76, Urban and Schwarzenberg, Baltimore, Maryland (1978).
5. A. L. Oronsky and C. Buermann in Neutral Protease of Human Polymorphonuclear Leukocytes (Ed. K. Havemann and A. Janoff) pp. 361-372, Urban and Schwarzenberg Baltimore, Maryland (1978).
6. A. J. Barrett Agents and Actions 8, 11 (1978).
7. J. Panagides, J. Landes and A. Sloboda, Agents and Actions in press (1980).
8. K. Weber and M. Osborne, J. Biol. Chem. 244, 4406 (1969).
9. W. F. Barg, M. Englert, C. W. Buermann, A. L. Oronsky and S. S. Kerwar, Biochem. Pharmacol. 28, 2639 (1979).
10. R. J. Baugh and J. Travis, Biochemistry 15, 836 (1976).

11. J. C. Nolan, S. Ridge, A.L. Oronsky and S. S. Kerwar, Biochem. Biophys. Res. Commun. 83, 1183 (1978).

12. O. H. Lowry, N. J. Roseborogh, A. L. Farr and R. J. Randall, J. Biol. Chem. 193, 265 (1951).

13. C. A. Vater, C. L. Mainardi and E. D. Harris, J. Biol. Chem. 254, 3045 (1979).