Biochimica et Biophysica Acta, 429 (1976) 925—932

BBA 67777

THE INHIBITION OF HUMAN LEUCOCYTE ELASTASE AND CHYMOTRYPSIN-LIKE PROTEASE BY ELASTATINAL AND CHYMOSTATIN

G. FEINSTEIN *, C.J. MALEMUD and A. JANOFF **

Department of Pathology, State University of New York at Stony Brook, Stony Brook, N.Y. 11794 (U.S.A.)

(Received September 24th, 1975)

Summary

The ability of elastatinal and chymostatin, protease inhibitors of microbial origin, to inhibit human leucocyte proteases (EC 3.4.-) was studied. Elastatinal and chymostatin are capable of inhibiting the pancreatic enzymes elastase and chymotrypsin, respectively. It was found in these studies, with synthetic substrates, that elastatinal is a much weaker inhibitor of human leucocyte elastase than it is of porcine pancreatic elastase. Elastatinal caused no inhibition of the activity of human leucocyte chymotrypsin-like protease. Chymostatin was found to be a powerful inhibitor of human leucocyte chymotrypsin-like protease. Its affinity to the leucocyte protease was higher than its affinity to bovine pancreatic α-chymotrypsin. Chymostatin had a weak inhibitory effect on the activity of human leucocyte elastase.

Studies were also carried out on the ability of chymostatin to inhibit the release of $^{35}SO_4^{2-}$ from rabbit articular cartilage by human leucocyte chymotrypsin-like protease. Preincubation of the chymostatin with the protease before the latter was added to the $^{35}SO_4^{2-}$-labeled cartilage caused inhibition of proteolysis as measured by $^{35}SO_4^{2-}$ release. Preincubation of chymostatin with $^{35}SO_4^{2-}$-labeled cartilage prior to addition of the human chymotrypsin-like protease to the tissue also inhibited $^{35}SO_4^{2-}$ release. However, in the case of preincubation of cartilage with α_1-antitrypsin there was no such inhibition. It therefore appeared that chymostatin, unlike α_1-antitrypsin, was capable of penetrating the cartilage matrix and exerting its inhibitory effect upon the human leucocyte chymotrypsin-like protease that was subsequently added to the tissue.

Abbreviations: Boc-Ala-ONp, *N*-*t*-Butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester; Bz-Tyr-OEt, *N*-benzoyl-L-tyrosine ethyl ester; Ac-Phe-1-ONap, *N*-acetyl-L-phenylalanine-α-naphthyl ester; α_1-AT, human α_1-antitrypsin; Me_2SO, dimethyl sulfoxide.

* Permanent address: Department of Biochemistry, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel.

** To whom correspondence should be addressed.

Introduction

The role of leucocyte proteases (EC 3.4.-) in the pathogenesis of several human diseases has recently been reviewed [1—4]. Human leucocyte proteases appear to have a prime role in the inflammatory tissue injury which may contribute to pulmonary emphysema and certain joint diseases [1,5]. Human leucocyte elastase was purified in several laboratories from blood of either healthy donors [6—9] or from leukemic patients [10]. The elastase is a very basic protein, rich in basic amino acids, with a molecular weight of about 30 000 [9,10]. Human leucocyte chymotrypsin-like protease was likewise studied in several laboratories [11,12] and was purified from either healthy donors by us [13] or from leukemic patients by several groups [14—16]. The chymotrypsin-like protease appears to be an even more basic protein than either leucocyte elastase or lysozyme and it has a molecular weight of about 25 000 [13,15]. Both leucocyte proteases were found to be capable of degrading articular cartilage matrix [17—19] as well as purified cartilage proteoglycan [20]. It was reported that both of these leucocyte proteases can be inhibited by human serum α_1-antitrypsin inhibitor [13,16] which is the major endogenous protease inhibitor present in the circulation (mol. wt. 53 000). However, using immunohistochemical techniques, it was found that α_1-antitrypsin was incapable of penetrating into the cartilage matrix [19]. Thus, the efficacy of this endogenous antiprotease may be limited with respect to the protection of cartilage against the destructive action of leucocyte enzymes.

Recently, a large number of protease inhibitors were detected and purified from microbial cultures [21,22]. Compared to the classical protein inhibitors of proteases, these microbial inhibitors are relatively small molecules, with molecular weights of approx. 500. The microbial protease inhibitors, elastatinal [23,24] and chymostatin [25,26], were tested mostly for their ability to inhibit pancreatic enzymes.

The goals of the studies reported herein were to determine the inhibitory potency of the microbial inhibitors elastatinal and chymostatin against human leucocyte proteases and also to study the ability of the inhibitors to penetrate into cartilage matrix.

Experimental

Materials

Bovine α-chymotrypsin (lot CDS ICA), porcine elastase (lot ESFF 55B439) and human α_1-antitrypsin (lot HAAT 53E305) were obtained from Worthington Biochemical Corp., Freehold, N.J. *N*-Benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) was from Sigma Chemical Co., St. Louis, Mo. *N-t*-Butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester (Boc-Ala-ONp) was from Cyclo Chemical Corp., Los Angeles, Calif. $^{35}SO_4^{2-}$, as $Na_2{}^{35}SO_4$, was obtained from New England Nuclear, Inc., Boston, Mass. (specific activity, 773 Ci/M). New Zealand albino rabbits were from Hare Rabbits, Inc., Hewitt, N.J. Human blood, for preparation of leucocyte enzymes, was purchased from Inter-County Blood Services, Rockville Centre, N.Y, a division of the New York Blood Center. Human leucocyte elastase and chymotrypsin-like protease were purified in this lab-

oratory according to our previously published procedures [9,13]. Elastatinal and chymostatin were a generous gift from Dr. Takashi Sugimura, Director of the National Cancer Center Research Institute, Tokyo, Japan. All other materials were of reagent grade.

Methods

Enzymatic assays with synthetic substrates. All enzymatic assays were carried out at pH 7.5 in 0.10 M phosphate buffer at room temperature (20—22°C). A Gilford recording spectrophotometer (Model 2400-S) was used to determine enzymatic activities against various synthetic substrates. The hydrolysis of Bz-Tyr-OEt, in 5% dimethyl sulfoxide (Me_2SO), was followed at 256 nm according to Hummel [27] using an initial substrate concentration [S_0] of $5 \cdot 10^{-4}$ M. The hydrolysis of Boc-Ala-ONp, in 1% acetonitrile, was followed at 400 nm according to Visser and Blout [28] using an initial [S_0] = $5 \cdot 10^{-4}$ M.

Inhibition studies with synthetic substrates. Purified leucocyte enzymes or crude leucocyte granular extract were incubated for 10 min, at pH 7.5, with an inhibitor. The residual enzymatic activities of non-inhibited enzyme were determined after the addition of substrate.

Preparation of $^{35}SO_4^{2-}$-labeled rabbit articular cartilage. Rabbits were injected intravenously with $Na_2{}^{35}SO_4$ (450 μCi/kg) in physiological saline and killed 18 h later with pentobarbital. The proximal humeri and tibiae and the proximal and distal femora were removed and slices of articular cartilage were then dissected from the joints in the cold and subsequently stored at −80°C. Further details of these procedures were described earlier [17,19].

Assay of $^{35}SO_4^{2-}$ release. Radiosulphate-labeled cartilage was cut into pieces of about 1 mg (wet weight), each containing about 1500 cpm. The pieces were washed with ice-cold 0.05 M sodium phosphate buffer (pH 7.5) containing 0.3 M NaCl. Single pieces were placed in siliconized glass ampules to which 0.90 ml of warm (37°C) phosphate buffer was added. Then 0.10 ml buffer containing either 1 μg enzyme only or a preincubated mixture with an inhibitor was added, and the cartilage was incubated for 30 min at 37°C. At the end of the incubation period, the ampules were rapidly transferred to an ice bath, 0.5-ml aliquots of incubation medium were removed and mixed with 10 ml of 2.0% liquifluor in toluene: ethylene glycol monomethyl ether (1 : 1, v/v) (ACS). These samples were subsequently counted for radioactivity. The remainder of the incubation medium was immediately removed from all vessels, the cartilage briefly rinsed with ice-cold 0.1 M acetate buffer (pH 4.0), and the tissue then hydrolyzed at 110°C, overnight, in 8 M HCl. Aliquots of hydrolyzate were diluted with water and lyophilized to eliminate HCl. Lyophilates were redissolved in water, mixed with 10 ml of the scintillation liquid and counted. The fraction of radioactivity released into the medium and the fraction remaining in the cartilage at the end of each incubation interval were determined from the dpm values, and the released fraction was expressed as percent of total radioactivity initially present in the tissue sample. All samples were tested in quadruplicate.

Preincubation of $^{35}SO_4^{2-}$-labeled cartilage with inhibitors. The $^{35}SO_4^{2-}$-labeled cartilage pieces (about 1 mg wet weight) were washed and placed as before in siliconized 5-ml glass ampules. Then 0.1 ml of phosphate buffer containing inhibitor was added and the cartilage was incubated at 37°C. At the end of 1 h,

the cartilage pieces were removed, dried with tissue paper and placed in clean 5-ml siliconized glass ampules. Buffer and enzyme were added, as described above, and the incubation was carried on for 30 min at 37°C. In all cases, control samples without inhibitor or enzyme were run in parallel.

Protein determination. The Lowry et al. method [29] was used for protein determination using bovine serum albumin for calibration. The protein concentration of bovine α-chymotrypsin and porcine elastase were calculated from the absorbance at 280 nm. The following absorption coefficients ($A^{1\%}_{280\ nm}$) were used: chymotrypsin, 20.4; elastase, 20.2.

Results and Discussion

Inhibition of enzyme activities towards synthetic substrates

The effectiveness of elastatinal in inhibiting the esterolytic activities of pancreatic and leucocyte elastases upon Boc-Ala-ONp is shown in Fig. 1. Extrapolations to zero residual enzymatic activities show that whereas 0.25 μg of elastatinal would completely inhibit 1 μg porcine elastase, 37 μg of elastatinal would be required to inhibit 1 μg of human leucocyte elastase. Since the molecular weighs of the two enzymes are close to each other, it appears that the affinity of elastatinal for the porcine elastase is much stronger than its affinity for the human leucocyte elastase. It was calculated from these studies that the K_i of elastatinal with porcine elastase and with leucocyte elastase are about $2 \cdot 10^{-7}$ and $5 \cdot 10^{-5}$ M, respectively, (assuming equimolar binding).

In contrast to the foregoing results, it appears that chymostatin has stronger affinities for both pancreatic and leucocyte chymotrypsins (Fig. 2). Extrapolations to zero residual enzymatic activities show that the inhibition of either 1 μg of human leucocyte chymotrypsin-like protease or of bovine α-chymotrypsin would require about 0.035 and 0.075 μg chymostatin, respectively. It was estimated that the K_i of chymostatin with both enzymes is about $1 \cdot 10^{-8}$ — $3 \cdot 10^{-8}$ M (assuming equimolar binding).

Kunimoto et al. [30] studied the inhibition of pepsin by pepstatin, another inhibitor of microbial origin, and found equimolar binding with a K_i of about $1 \cdot 10^{-10}$ M. It seems that chymostatin and especially elastatinal are less effec-

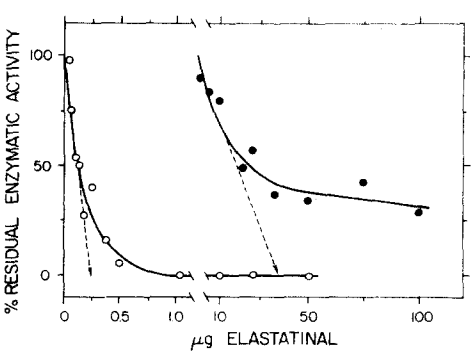

Fig. 1. The inhibition of elastases by elastatinal. The residual enzymatic activities in hydrolyzing Boc-Ala-ONp of porcine elastase (1 μg, ○——○) and of human leucocyte elastase (1 μg, ●——●) were determined after 10 min incubation with elastatinal at room temperature and at pH 7.5.

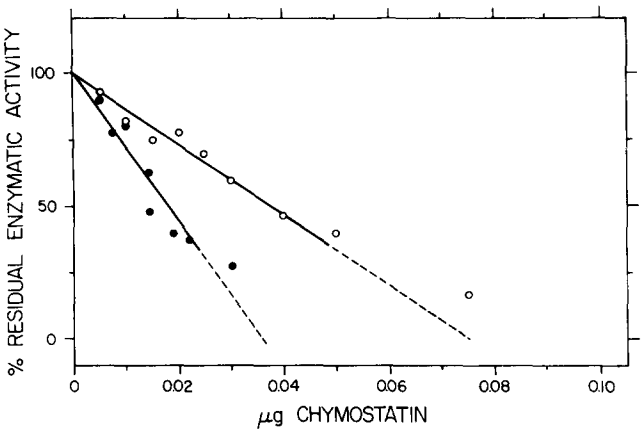

Fig. 2. The inhibition of chymotrypsins by chymostatin. The residual enzymatic activities in hydrolyzing Bz-Tyr-OEt of bovine α-chymotrypsin (1 μg, ○——○) and of human leucocyte chymotrypsin-like protease (1 μg, ●——●) were determined after 10 min incubation with chymostatin at room temperature and at pH 7.5.

tive in inhibiting human leucocyte proteases. It was also found in these studies (Fig. 3) that chymostatin has a weak inhibitory effect upon human leucocyte elastase activity. This is not surprising in view of our earlier report [9] indicating that leucocyte elastase has weak affinity towards the typical chymotrypsin substrate, *N*-acetyl-L-phenylalanine-α-naphthyl ester (Ac-Phe-1-ONap). On the other hand, even 200 μg of elastatinal had no effect on 1 μg of leucocyte on chymotrypsin-like enzyme activity on the latter's synthetic substrate. The different affinities of chymostatin to the leucocyte chymotrypsin-like protease compared to that of elastatinal to leucocyte elastase are shown in Fig. 3.

Inhibition studies with $^{35}SO_4^{2-}$ cartilage

It was reported earlier that leucocyte enzymes were capable of releasing $^{35}SO_4^{2-}$-labeled digestion fragments from $^{35}SO_4^{2-}$ cartilage [17,19,31]. Those studies used either purified enzymes [19] or mixtures of enzymes [17,31]. The

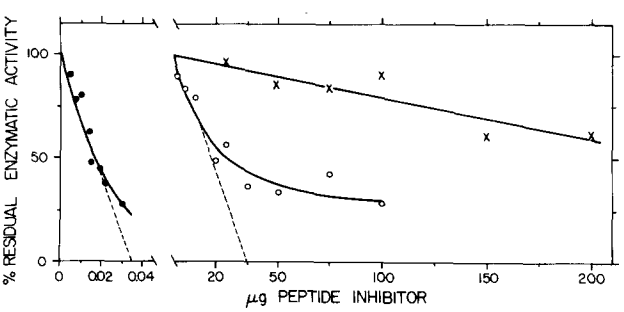

Fig. 3. The inhibition of human leucocyte enzymes by elastatinal and chymostatin. The residual enzymatic activities of leucocyte enzymes (1 μg) were determined after 10 min incubation with inhibitors at room temperature and at pH 7.5. Chymotrypsin-like protease and chymostatin, substrate was Bz-Tyr-OEt, ●——●; elastase and elastatinal, substrate was Boc-Ala-ONp, ○——○; elastase and chymostatin, substrate was Boc-Ala-ONp, X——X.

two leucocyte proteases were also reported to be capable of hydrolyzing isolated cartilage proteoglycan [20]. It seemed of interest to test the effect of the microbial inhibitors in this system. Pepstatin was reported to be an effective inhibitor of cartilage proteoglycan degradation by cathepsin D [32] as well as of cartilage autolysis [32]. Since our studies had already indicated the relative ineffectiveness of elastatinal against the leucocyte enzymes we directed our efforts towards studying chymostatin inhibition of leucocyte chymotrypsin-like protease-mediated $^{35}SO_4^{2-}$ release. Preincubation of chymostatin with leucocyte chymotrypsin-like protease resulted in 75% inhibition of $^{35}SO_4^{2-}$ release from cartilage (Table I). Similar results had been obtained earlier when human α_1-antitrypsin (α_1-AT) was preincubated with either leucocyte elastase or chymotrypsin-like protease [19,20]. However, when the pieces of cartilage were preincubated with α_1-AT (Table I) and then leucocyte enzyme was added to the pretreated pieces of cartilage, very little inhibition was observed. Thus, it appeared that α_1-AT, the major endogenous circulatory inhibitor, had not penetrated into the cartilage in sufficient amount to inhibit the leucocyte protease. This observation is consistent with our previous report [19] that α_1-AT could not be detected inside cartilage by immunohistochemical procedures after the cartilage was preincubated with serum. On the other hand, our present studies show that when cartilage was preincubated with chymostatin, subsequent release of $^{35}SO_4^{2-}$ by leucocyte chymotrypsin-like protease was mostly inhibited (86%, Table I). It appeared that chymostatin, perhaps because of its relatively small size compared to α_1-AT, was able to penetrate into the cartilage matrix. Thus, when the leucocyte protease was added at the second step, its degradative activity against the cartilage proteoglycan was inhibited by the chymostatin that had preceded it into the tissue.

The possible involvement of leucocyte neutral proteases in inflammatory diseases was the subject of several recent reviews and symposia [1—4]. These proteases can be inhibited in vitro by the major circulatory protein inhibitors,

TABLE I

THE EFFECT OF INHIBITORS ON THE RELEASE OF $^{35}SO_4^{2-}$ FROM $^{35}SO_4^{2-}$ CARTILAGE BY HUMAN LEUCOCYTE CHYMOTRYPSIN-LIKE PROTEASE

Treatment	Release (%)	Inhibition (%)
Enzyme *	100 **	—
Enzyme * + 20 μg chymostatin	25	75
Enzyme ***	100 **	—
Enzyme *** + cartilage pre-incubated with 100 μg α_1-AT	93	7
Enzyme *** + cartilage pre-incubated with 100 μg chymostatin	14	86

* 1 μg enzyme with or without inhibitor was pre-incubated for 10 min and then added to a piece of cartilage followed by 30 min additional incubation.

** Enzyme, without inhibitor, usually released under these experimental conditions 20—30% of total tissue radioactivity. These values for percent of $^{35}SO_4^{2-}$ released have been normalized to 100 for convenient comparison. All data were corrected for background $^{35}SO_4^{2-}$ release during incubation from cartilage alone. This rarely exceeded 3% of total tissue radioactivity.

*** Pieces of cartilage alone or with inhibitors were pre-incubated in buffer for 1 h at 37°C. Thereafter, only the cartilage was transferred to another ampule, fresh buffer and 1 μg enzyme were added and the usual 30 min incubation at 37°C was then carried on.

α_1-antitrypsin and α_2-macroglobulin. However, as suggested earlier [5], if leucocyte proteases are released by inflammatory cells which are either in direct contact with or in close proximity to the target tissue, there would be little opportunity for the endogenous protein inhibitors to act. This could be the case even though the protein inhibitors might be present in sufficient quantities in the extracellular fluid. As a result, tissues close to the inflammatory site would be damaged, and the presence of a large extracellular inhibitory potential would not prevent this damage. On the other hand, application of smaller-sized inhibitors such as chymostatin, which is able to penetrate into cartilage, may more effectively prevent tissue degradation by leucocyte proteases. Such agents may therefore prove to have therapeutic value in combatting selected inflammatory diseases.

Acknowledgements

We would like to thank the New York Blood Center for their cooperation in supplying human blood for the collection of leucocytes. We also thank Dr. Takashi Sugimura, Director of the National Cancer Center Research Institute, Tokyo, Japan, and Dr. Taijiro Matsushima of the University of Tokyo, Japan, for kindly providing us with elastatinal and chymostatin. We thank Rosemarie Dearing for her technical assistance throughout the experiments. This work was supported by N.I.H. Grant HL-14262 and by a Phebe Cornell Maresi Center Grant of the New York State Arthritis Foundation.

References

1 Janoff, A. (1972) Ann. Rev. Med. 23, 177—190
2 Mittman, C. (1972) Pulmonary Emphysema and Proteolysis, Academic Press, London
3 Perper, R.J. (1975) Mechanism of Tissue Injury with Reference to Rheumatoid Arthritis, Ann. N.Y. Acad. Sci. 256, 1—450
4 Reich, E., Rifkin, D. and Shaw, E. (1975) Cold Spring Harbor Symposium on Proteases and Biological Control, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
5 Janoff, A. (1975) Ann. N.Y. Acad. Sci. 256, 402—408
6 Janoff, A. (1973) Lab. Invest. 28, 458—464
7 Baugh, R. and Travis, J. (1975) Fed. Proc. 34, 484
8 Taylor, J.C. and Crawford, I.P. (1975) Arch. Biochem. Biophys. 169, 91—101
9 Feinstein, G. and Janoff, A. (1975) Biochim. Biophys. Acta 403, 493—505
10 Ohlsson, K. and Olsson, I. (1974) Eur. J. Biochem. 42, 519—527
11 Schmidt, W. and Havemann, K. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1077—1082
12 Gerber, C.A., Carson, J.H. and Hadorn, B. (1974) Biochim. Biophys. Acta 364, 103—112
13 Feinstein, G. and Janoff, A. (1975) Biochim. Biophys. Acta 403, 477—492
14 Olsson, I. and Venge, P. (1974) Blood 44, 235—246
15 Odeberg, H., Olsson, I. and Venge, P. (1975) Lab. Invest. 32, 86—90
16 Rindler-Ludwig, R. and Braunsteiner, H. (1975) Biochim. Biophys. Acta 379, 606—617
17 Malemud, C.J. and Janoff, A. (1975) Arthritis Rheum. 18, 361—368
18 Janoff, A., Blondin, J., Sandhaus, R.A., Mosser, A. and Malemud, C.J. (1975) Cold Spring Harbor Symposium on Proteases and Biological Control (Reich, E., Rifkin, D. and Shaw, E., eds.), Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
19 Janoff, A., Feinstein, G., Malemud, C.J. and Elias, J.M. (1976) J. Clin. Invest., in the press
20 Keiser, H.D., Greenwald, R.A., Feinstein, G. and Janoff, A. (1976) J. Clin. Invest., in the press
21 Umezawa, H. (1972) Enzyme Inhibitors of Microbial Origin, University of Tokyo Press
22 Wingender, W. (1974) Proteinase Inhibitors, Bayer Symposium V (Fritz, H., Tschesche, H., Greene L.J. and Truscheit, E., eds.), pp. 548—559, Springer-Verlag, Berlin, Heidelberg, New York
23 Umezawa, H., Aoyagi, T., Okura, A., Morishima, H., Takeuchi, T. and Okami, Y. (1973) J. Antibiot. 26, 787—789

24 Okura, A., Morishima, M., Takita, T., Aoyagi, T., Takeuchi, T. and Umezawa, H. (1975) J. Antibiot. 28, 337—339
25 Umezawa, H., Aoyagi, T., Morishima, H., Kunimoto, S., Matsuzaki, M., Hamada, M. and Takeuchi, T. (1970) J. Antibiot. 23, 425—427
26 Tatsuta, K., Mikami, N., Fujimoto, K., Umezawa, S., Umezawa, H. and Aoyagi, T., (1973) J. Antibiot. 26, 625—646
27 Hummel, B.C.W. (1959) Can. J. Biochem. Physiol. 37, 1393—1399
28 Visser, L. and Blout, E.R. (1972) Biochim. Biophys. Acta 268, 257—260
29 Lowry, O.H., Rosebrough, N.J., Farr A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
30 Kunimoto, S., Aoyagi, T., Mishizawa, R., Komai, T., Takeuchi, T. and Umezawa, H. (1974) J. Antibiot. 27, 413—418
31 Ignarro, L.A., Oronsky, A. and Perper, R.J. (1973) Clin. Immunol. Immunopathol. 2, 36—51
32 Woessner, J.F. (1972). Biochem. Biophys. Res. Commun. 47, 965—970
33 Dingle, J.T., Barrett, A.J. and Poole, A.P. (1972) Biochem. J. 127, 443—444