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ELAFIN IS A POTENT INHIBITOR OF PROTEINASE 3

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Elafin, a human skin derived inhibitor of human leukocyte elastase, was tested for inhibitory activity against proteinase 3, an elastin degrading proteinase of neutrophils. The inhibitory activity of elafin was compared with antileukoprotease and eglin C. Elafin proved to be a potent inhibitor of elastin-FITC degradation showing an IC 50 of 9.5×10^{-9} M. Potency was found to be more than 100-fold higher as compared with antileukoprotease and eglin C.

Proteinase 3 was first described as the third serine proteinase of human neutrophils (1) beside human leukocyte elastase (EC 3.4.21.37) and cathepsin G. The lack of specific synthetic substrates may have caused the late discovery of relevant functions of this elastinolytic proteinase in man. Recent studies have shown that proteinase 3 is capable of causing lung emphysema-like tissue destruction in animals after instillation into the airways with the same potency as human leukocyte elastase (2). In addition, proteinase 3 was shown to be the target antigen of anti-neutrophil cytoplasm autoantibodies specific to Wegener's granulomatosis (3,4,5). Further understanding of the physiological and pathophysiological role of this proteinase requires more information about its regulation in man. Whilst searching for physiological inhibitors of proteinase 3 we looked for the potency of human peptide inhibitors for elastinolytic enzymes to inhibit proteinase 3, such as the recently discovered peptide elafin (6) as well as antileukoprotease (7), in comparison with the medical leech derived recombinant eglin C (8).

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METHODS

Preparation of elafin: Elafin was purified according to Wiedow et al. (6) from human horny layers. In brief, an acidified extract of scales, derived from patients suffering from psoriasis, was cleared by centrifugation and filtration (5μm) and diafiltrated against 0.1 M Na-acetate, pH 6.0. Separation from serine proteases was achieved by cation exchange chromatography (TSK CM 3 SW-HPLC) using a gradient to 1 M sodiumchloride in the buffer mentioned above. Fractions containing the human leukocyte elastase inhibitor were further purified by reversed phase C8 HPLC using a gradient from 0.1 % aqueous trifluoracetic acid (TFA) to 0.1 % TFA in acetonitrile. Relevant fractions were further purified on a poly-sulfoethyl-aspartamide column (Poly LC-HPLC). The column was equilibrated with 5 mM KH_2PO_4, pH 3.1 + 25 % acetonitrile and elution was achieved by increasing the KCl content from 0 to 0.5 M. The inhibitor was finally purified by reversed phase C18-HPLC to homogeneity. Purity was found to be more than 95% based on complete absence of contaminants in SDS-PAGE, N-terminal amino acid sequence analysis, and titration of active molecules with active site titrated human leukocyte elastase (Elastin Products Corporation, MO, USA; active site titration of the enzyme was performed with recombinant eglin C).

Preparation of antileukoprotease: Antileukoprotease was prepared from human horny layers using the same extraction procedure as for elafin. Peaks in the above-mentioned cation exchange chromatography (TSK CM 3 SW-HPLC) showing inhibitory activity against human leukocyte elastase and cathepsin G were purified separately on RP_8-HPLC using a gradient from 80% of 0.1% aqueous TFA and 20% of 0.1% TFA in acetonitrile to 0.1% TFA in acetonitrile. Fractions inhibiting human leukocyte elastase and cathepsin G were combined, lyophilized and solubilized in 1 ml 0.1 % aqueous TFA and subjected to RP18 chromatography using a gradient from 80% of 0.1% aqueous TFA and 20% of 0.1% TFA in acetonitrile to 0.1% TFA in acetonitrile. Active fractions were combined, lyophilized , solubilized in 0.1% TFA and applied to a CN-Baker column. The column was developed using a gradient from 90% 0.1% aqueous TFA and 10% n-propanol to 100% n-propanol. The major human leukocyte elastase and cathepsin G inhibitory peak was finally purified by RP_{18}-HPLC using the same gradient as mentioned above. The human leukocyte elastase and cathepsin G inhibitory activity appeared to be pure as it eluted as a single symmetric peak in analytical RP_{18}-HPLC, analytical TSK 2000 size exclusion-HPLC and gave a single line on SDS-PAGE under reducing conditions. The N-terminal sequence analysis revealed a sequence SGKSFKAGV?PPKKSAQ?LRYKKPE?QSDWQ?PGKKR matching the sequence reported for antileukoprotease (7). Cystein residues were not identified since the sample was not derivatised prior to sequence analysis. Identity in the C-terminus was not established.

Preparation of proteinase 3: Proteinase 3 was purified from degranulation supernatants of human polymorhonuclear leukocytes (10) using the method of Kao et al. (2). Absence of human leukocyte elastase and cathepsin G was proven by the lack of activity of the preparation towards specific substrates. For human leukocyte elastase we used methoxy-succinyl-ala-ala-pro-val-p-nitroanilide, succinyl-ala-ala-ala-p-nitroanilide, and succinyl-ala-ala-val-p-nitroanilide, for cathepsin G the substrate succinyl-ala-ala-pro-phe-p-nitroanilide (all substrates from Sigma) according to the method of Nakajima et al. (9). Purity was further proven by the absence of contaminants in SDS-

PAGE with subsequent silver staining (3) and by immunoblotting using sheep antisera against human leukocyte elastase and cathepsin G (Serotec, Oxford, UK).

Determination of proteinase 3 activity: The elastinolytic activity was determined by hydrolysis of bovine ligamentum nuchae elastin-FITC (Elastin Products Corporation, MO, USA). Proteinase 3 (0.3 µg) was preincubated with 0 - 1 µg inhibitor in 100 µl 0.1 M carbonate buffer, 0.01% Brij 35, pH 8.2 for 30 min. at 37°C, prior to the addition of 1 mg Elastin-FITC in 100 µl 0.1 M carbonate buffer, 0.01% Brij 35, pH 8.2. The mixture was incubated at 37°C with gentle agitation at 37°C for 4 hrs and was subsequently centrifuged at 3000 x g. Supernatants were diluted 1:20 and the fluorescence of solubilized elastin-FITC was determined by a Perkin Elmer LS 50 microtiterplate reader at Ex.489 nm, Em.513 nm. Fluorescence signals were corrected by enzyme-free controls and inhibition was expressed in % of inhibitor-free controls. Recombinant eglin C was kindly provided by Dr. Schnebly, Basel.

RESULTS

Elafin was found to be a potent inhibitor of proteinase 3 activity with an IC_{50} of 9.5×10^{-9} M (Fig. 1). Antileukoprotease and recombinant eglin C were also able to inhibit proteinase 3 activity, but they were more than 100-fold less active than elafin based on the molar ratio. The inhibitory activity was apparently not affected by the preincubation period of the enzyme with elafin since 5 min. preincubation, no preincubation and addition of the inhibitor to the enzyme adsorbed to elastin gave approximately the same results (Fig. 2). The proteinase 3 inhibiting effect of all three inhibitors could be confirmed

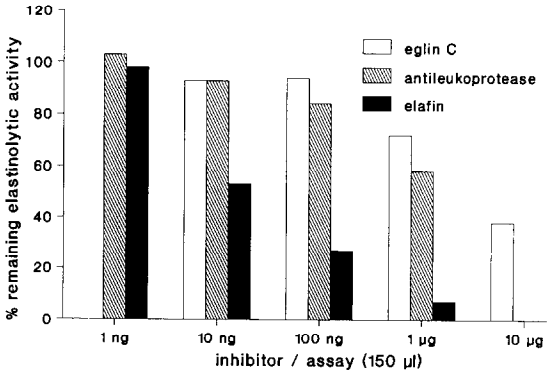

Fig.1. Inhibition of elastin-FITC hydrolysis by proteinase 3. Proteinase 3 (0.3 µg) was incubated with increasing amounts of elafin (MW 7017), antileukoprotease (MW 11726) and recombinant eglin C (MW 8100) for 30 min. prior to incubation with 1 mg elastin-FITC for 4 hrs at 37°C. Hydrolysis was followed by fluorometric determination (Ex. 489 nm, Em. 513 nm) of FITC in a 3000 x g supernatant.

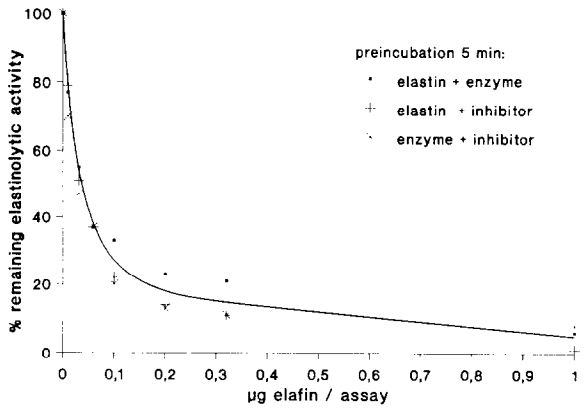

Fig.2. Influence of 5 min. preincubation on elafin mediated inhibition of proteinase 3. Elafin was preincubated either with proteinase 3 or elastin-FITC or added to proteinase 3 preincubated with elastin-FITC.

further using the synthetic sustrate α-naphtylacetate (data not shown) according to the method of Kao et al. (2)

DISCUSSION

Proteinase 3 is the third elastin degrading serine protease, beside human leukocyte elastase and porcine pancreatic elastase (EC 3.4.21.36), that is effectively inhibited by elafin. Since elafin appears to be a physiological regulator of human leukocyte elastase mediated tissue proteolysis in skin and probably in other organs as well, it is of interest that this inhibitor also inhibits proteinase 3. Both enzymes, human leukocyte elastase and proteinase 3, are constituents of the azurophilic granules of neutrophils and both are able to induce emphysema-like tissue destruction in animals after instillation of the enzyme into the airways (2). Since elafin is able to inhibit either enzyme after preadsorption to the substrate (data for human leukocyte elastase not shown), it seems to be likely that this inhibitor could have therapeutic value in lung diseases with elastase and proteinase 3 mediated tissue destruction. Furthermore, proteinase 3 has been shown to be the target antigen of anti-neutrophil cytoplasm autoantibodies specific to Wegner's granulomatosis (3,4) by a preliminary N-terminal amino acid sequence of the purified antigen (3) corrected by DNA-sequencing (5). Since there is no answer to the question whether the occurrence of autoantibodies against proteinase 3 or the occurrence of proteinase 3 itself plays the major role in the pathophysiology of this disease,

further investigations into regulation, elimination and a probable influence of the therapeutical inhibition of this proteinase are required. The discovery of a potent human inhibitor of this proteinase may be a step forward to achieving these aims.

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REFERENCES

1. Baggiolini, M., Bretz, U., Dewald, B., and Feigenson, M.E. (1978) Agents Actions 8, 3-10.
2. Kao, R.C., Wehner, N.C., Skubitz, K.M., Gray, B.H., and Hoidal, J.R. (1988) J. Clin. Invest. 82, 1963-1973.
3. Lüdemann, J., Utecht, B., and Gross, W.L. (1990) J. Exp. Med. 171, 357-362.
4. Lüdemann, J., Csernok, E., Ulmer, M., Lemke, H., Utecht, B., Rautmann, A., and Gross, W.L. (1990) Neth. J. Med. 36, 157-162.
5. Jenne, D.E., Tschopp, J., Lüdemann, J., Utecht, B., and Gross, W.L. (1990) Nature 346, 520.
6. Wiedow, O., Schröder, J.M., Gregory, H., Young, J.A., and Christophers, E. (1990) J. Biol. Chem. 256, 14791-14795.
7. Seemüller, U., Arnhold, M., Fritz, H., Wiedenmann, K., Machleidt, W., Heinzel, R., Appelhans, H., Gassen, H.-G., and Lottspeich, F. (1986) FEBS Lett. 199, 43-48.
8. Seemüller, U., Meier, M., Ohlsson, K., Müller, H.P., and Fritz, H. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1105-1117.
9. Nakajima, K., Powers, J.C., Ashe, B.M., and Zimmerman, M. (1979) J. Biol. Chem. 254, 4027-4032.
10. Lüdemann, J., Utecht, B., and Gross, W.L. (1988) J. Immunol. Methods 114, 167-174.