# PROTEASES

## Potential Role in Health and Disease

Edited by

Walter H. Hörl

and

August Heidland

University of Würzburg
Würzburg, Federal Republic of Germany

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# PLASMA LEVELS OF INHIBITOR-BOUND LEUKOCYTIC ELASTASE IN RHEUMATOID ARTHRITIS PATIENTS

H. P. Schnebli[1], P. Christen[1], M. Jochum[2], R. K. Mallya[3] and M. B. Pepys[4]

[1] Research Department, Pharma Division, Ciba-Geigy, CH-4002 Basel, [2] Abt. Klinische Chemie, Chirurgische Klinik, Universität München, D-8000 Munich. [3] Dept. Rheumatology, Kings College Hospital, London, SE 5. [4] Immunological Medicine Unit, Dept. Medicine, Royal Postgraduate Medical School, London, W12, OH5

## INTRODUCTION

It is generally agreed that: (a) massive numbers of PMN leukocytes accumulate at sites of chronic inflammation (1), and in inflammatory synovial fluids (2), (b) these PMN leukocytes release elastase (3) as well as a number of other enzymes and inflammatory mediators (4) during phagocytosis or other mechanisms (5), (c) substantial quantities of elastase have been identified in the synovium (6,7) or, complexed to naturally occuring inhibitors in synovial fluids of RA patients (8-12) or penetrated into the articular cartilage (13,14).

Without entering the debate as to whether or not leukocytic elastase is responsible for tissue destruction in RA and other inflammatory diseases (for a review see Barrett, ref. 15), we have taken the findings listed above to suggest that leukocytic elastase released at sites of inflammation may lead to elevated plasma levels of this enzyme.

Indeed, elevated plasma levels of leukocyte elastase - $\alpha_1$PI complexes have previously been observed (16) and subsequently followed up in more detail (17) in patients with leukemia or septicaemia. Increased levels of elastase - $\alpha_2$M complexes were also found in septicaemic patients (18).

In the present study we measured the plasma levels of elastase - $\alpha_1$PI complexes by an immunological technique (ELISA) (Method A) and of $\alpha_2$M bound elastase with a synthetic chromogenic substrate (Method B) in RA patients (n=87) and healthy controls (n=24).

## PATIENTS AND METHODS

Twenty seven male and 60 female patients suffering from rheumatoid arthritis according to the criteria of the American Rheumatism Association were studied. Their disease activity was assessed by a new multivariate analysis comprising the following criteria: morning stiffness, pain scale, grip strength, articular index, haemoglobin concentration and erythrocyte sedimentation rate (19). On the basis of their global score in the analysis the patients were assigned to one of four grades of disease activity (MDAG 1-4).

Blood samples were drawn into EDTA-coated tubes and centrifuged immediately in order to remove cells; plasma was then stored at -20° until assayed.

Levels of immunreactive elastase in plasma were determined with a newly developed enzyme-linked immunoassay (ELISA) (12,20). This method (Method A) measures exclusively the elastase complexed to $\alpha_1$PI. Plasma samples were incubated in plastic tubes coated with antibodies against elastase. After washing with buffer, the surface fixed elastase-$\alpha_1$PI complexes were reacted with anti-$\alpha_1$PI antibodies labelled with alkaline phosphatase. Under the conditions used, the enzymatic activity of the alkaline phosphatase (assayed with p-nitrophenyl phosphate) was proportional to the concentration of elasatase-$\alpha_1$PI complex in the sample. (For details of this assay, see ref. 20).

Leukocyte elastase activity in plasma was measured by a modification (18) of the procedure described earlier (21). In this assay (Method B) the release of p-nitroaniline from Methoxy-Succinyl-L-Ala-L-Ala-L-Pro-L-Val-p-Nitroanilide (MeO-Suc-Ala-Ala-Pro-Val-pNA), a highly specific chromogenic substrate for leukocytic elastase (22), was determined photometrically; o-phenanthroline was included to inhibit an unrelated metal dependent elastase-like enzyme (18, 23). Briefly, 0.2 ml of plasma was diluted with 0.2 ml of buffer containing the substrate and the metal chelator; final concentrations: 0.1 M Tris-HCl, pH 7.5, 1 mM MeO-Suc-Ala-Ala-Pro-Val-pNA and 10 mM o-phenanthroline. The mixture was incubated at 37° for 1 to 6 hours and the reaction was stopped by the addition of 1 ml trichloroacetic acid (7.5 %). Precipitated protein was removed by centrifugation and the released nitroaniline was transformed to an azo-dye by the sequential addition of 25 µl each of sodium nitrite (1.8 %),

ammonium sulphamate (9 %) and N-1-naphtyl ethylene-diamine di-HCl (1.8 %) to the supernatant. The resulting color was measured at 550 nm. The corresponding enzyme activity is expressed in nmoles/ml/h of substrate hydrolyzed.

## RESULTS AND DISCUSSION

The present study was undertaken to determine whether the release of leukocyte elastase at sites of active inflammation is reflected in the plasma levels of this enzyme, and whether this may be diagnostically useful in RA. Two independent methods for the determination of HLE plasma levels were employed.

The design of the enzyme-linked immunoassay (20) ensures that only HLE bound to $\alpha_1$-proteinase inhibitor was measured (Method A). In our hands, the HLE levels in control plasma were compatible with the previously published values of 84 $\pm$ 25 ng/ml (20) and 60 ng/ml (median value) (24) using the same assay system.

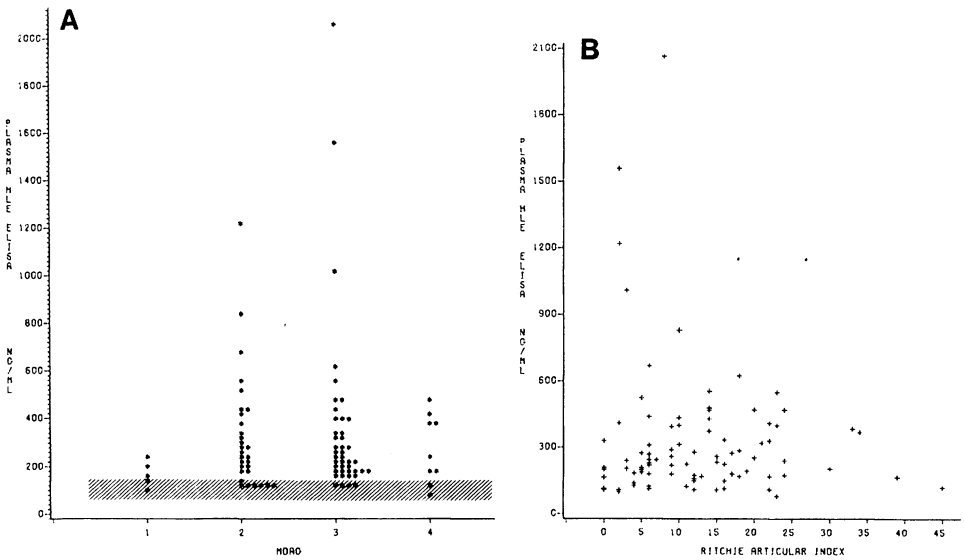


Fig. 1 HLE plasma levels measured immunologically as $\alpha_1$PI complex in 87 RA patients: comparison with MDAG (A), a weighted index of disease activity, or with the Ritchie articular index (B).

The majority of the plasma samples from RA patients had clearly elevated HLE-$\alpha_1$PI levels (Fig. 1A); 65 of 87 samples revealed values more than 2 standard deviations above the normal mean value. However, as can be seen in the same figure, the HLE concentration in plasma does not correlate with the "mean disease activity grade (MDAG)" (19), a weighted index based on 6 disease

parameters (see patients and methods) or with the Ritchie articular index (25) alone (Fig. 1 B). Interestingly, in the same patient collective, C-reactive protein (CRP) levels correlated well, both with the MDAG and the Ritchie index (26).

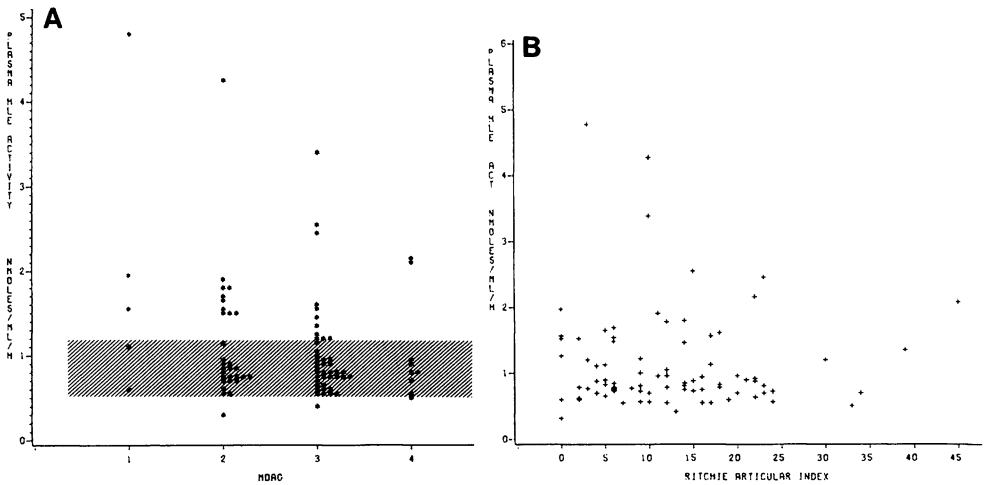


Fig. 2 HLE plasma levels measured as enzymic activity of $\alpha_2$M complexes in 87 RA patients: comparison with MDAG (A) or with the Ritchie articular index (B).

The measurement of elastase activity in plasma (Method B) is based on the fact that $\alpha_2$M bound proteinases retain hydrolytic activity towards low $M_r$ substrates, though they are unable to attack most large (protein) substrates. As shown previously with plasma from septicaemic patients (18), this elastase activity can be separated from the bulk of plasma proteins on Sephacryl S300. The apparent molecular weight (approx. 800 000 Da), the substrate specificity (MeO-Suc-Ala-Ala-Pro-Val-pNA) and the inhibition pattern (insensitive to EDTA and o-phenanthroline, sensitive to PMSF and the elastase specific inhibitor Eglin C) identifies this activity as leukocyte elastase-$\alpha_2$M complex. Compared with plasma from 24 healthy controls, HLE activity was elevated in a number of plasma samples from RA patients (20 out of 87 samples were elevated more than 2 standard deviations above the normal mean) (Fig. 2A).

However, again no correlation could be found between plasma HLE activity levels and disease activity (Fig. 2). Furthermore the HLE-$\alpha_1$PI levels (Method A) and the HLE-$\alpha_2$M levels (Method B) did not correlate ($R<0.1$, Fig. 3).

It must be stressed that the levels of elastase activity in plasma are very low, requiring a sensitive assay and prolonged reaction times (see methods). Normal plasma (n=24) hydrolyze approximately $0.9 \pm 0.3$ nmoles MeO-Suc-Ala-Ala-Pro-Val-pNA per

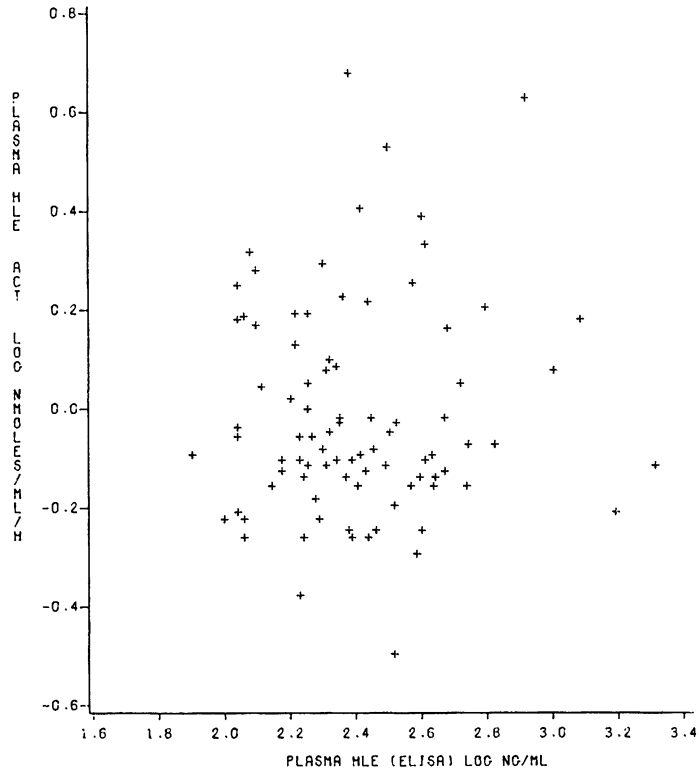


Fig. 3: HLE-$\alpha_1$PI levels and HLE-$\alpha_2$M activities in plasma of 87 RA patients (note logarithmic transformation).

ml per hour. Although an extrapolation from this value to the amount of enzyme actually present as $\alpha_2$M complex is not permissible, it can be estimated to be about 1-2 ng elastase/ml plasma. This is much less than the 84 ng elastase/ml bound to $\alpha_1$PI in normal plasma (see above). However, these values are quite compatible, when the known rates of complex formation and elimination are taken into account: the association rate constant of elastase/$\alpha_1$PI is 1.5 to 2 fold higher than that of elastase/$\alpha_2$M (27); together with the much higher plasma concentration of $\alpha_1$PI (approx. 10 times in molar terms) this accounts for a 15-20 fold higher rate of formation of the elastase/$\alpha_1$PI complex compared with the formation of the elastase/$\alpha_2$M complex in plasma. Indeed, it was found that 90 % of a small amount of radiolabelled elastase added to human serum was bound to $\alpha_1$PI (28). The half-time of elimination of elastase-$\alpha_2$M complexes in man is approx. 12 min, that of elastase -$\alpha_1$PI is approx. 60 min (29).

Using these numbers for a simple computer simulation experiment we calculated an influx of 47 ng/ml/h of elastase into plasma (of healthy controls) creating steady state levels of elastase bound to $\alpha_1$PI and to $\alpha_2$M of 90 ng/ml and 0.9 ng/ml, respectively, - surprisingly close to our estimated values of 84 and 1 to 2 ng/ml.

These calculations, although to be interpreted with caution, point to another important consideration. The flux of 47 ng of elastase per ml plasma per hour would indicate a total turnover of HLE of 2 to 3 mg per day, which is far below the estimated value of 300-400 mg elastase assumed to be released as a consequence of the normal life span of PMN leukocytes in healthy persons (28). It must be concluded, therefore, that only a small part of the elastase present in the neutrophil granules ever reaches the circulation. This might be one reason for the lack of correlation of plasma HLE levels and disease activity as observed here. Other reasons may be inflammation related alteration of the clearance rate (reticulo endothelial system) and altered rates of synthesis and consumption of proteinase inhibitors ($\alpha_1$PI is an acute phase reactant).

It is concluded that, although HLE is substantially elevated in the plasma of many RA patients, the plasma concentration of this enzyme by itself cannot serve as an indicator of disease activity. It is possible, however, that in longitudinal studies (within the same patients) changes in plasma HLE levels may be indicative of disease progression; indeed, changes in plasma levels of HLE have previously been shown to be indicative of disease progression in septicaemia (17).

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