The binding of tissue prokallikrein to isolated human neutrophils

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Abstract

We have investigated the binding of human tissue kallikrein and the corresponding proenzyme to isolated human lymphocytes and neutrophils, respectively, by the means of steady-state binding assays. It was demonstrated that only prokallikrein bound to washed neutrophils in a time-dependent, specific and saturable manner, whereas in vitro binding of activated kallikrein to neutrophils or lymphocytes could be excluded. Our data provide strong evidence for the specific interaction of prokallikrein with intact human neutrophils and indicate a neutrophil surface binding site for the tissue kallikrein precursor which could be involved in kallikrein endocytosis and selective prokallikrein-to-kallikrein conversion.

Key words: Kallikrein; Neutrophil; Neutrophil cell binding; Proenzyme

1. Introduction

Human tissue kallikreins (EC 3.4.21.35) comprise a subfamily of trypsin-like glycoproteins of closely related structure which share common biological features, especially the highly selective kininogenase activity. Limited proteolysis of their endogenous substrates high and low molecular weight kininogen (HMWK and LMWK) liberates the vasoactive decapeptide kallidin (Lys-bradykinin), which is involved in the regulation of local blood flow and vascular permeability [1]. The hitherto characterized tissue kallikreins, called urinary, pancreatic and salivary-gland kallikrein, are expressed by a common gene, which encodes a pre-pro-enzyme with a 17-amino acid signal peptide, that is subsequently split off during protein translocation within the cells. The originating enzymatically inactive prokallikrein carries an N-terminal activation peptide of seven amino acids in length with a sequence motif, which is identical in all kallikrein species examined so far [2]. Cleavage of the propart occurred by limited proteolysis of the precursor, which could be achieved in vitro by plasma kallikrein, plasmin, thermolysin and trypsin to yield mature kallikrein [3]. However, these proteinases possess no in vivo significance for the proenzyme conversion, leaving the physiological activation process yet unidentified.

The active proteinase and varying proportions of prokallikrein together are present in a variety of tissues like kidney, pancreas and salivary glands, but also found in their secretions and different body fluids, namely plasma, synovial and bronchoalveolar lavage fluid [4]. Recently, the localisation of tissue kallikrein in cytoplasmic granules of circulating neutrophils has been demonstrated in normal human blood and bone marrow by immunocytochemical techniques, whereas the enzyme was not detectable in eosinophils, lymphocytes, macrophages, mega-karyocytes and platelets [5]. The functional importance of this discovery is illustrated by the specific attachment of both HMWK and LMWK [6,7], as well as plasma prekallikrein [8], on the exterior surface of human neutrophils, displaying a spatial relationship of substrates and corresponding proteinases involved in kinin liberation.

Whereas the presence of tissue kallikrein in neutrophils has been reported, the source of this kininogenase remains unclear. Contradictory discussion arouses whether the neutrophil tissue kallikrein was due to in situ synthesis of a kallikrein precursor [4] or to the capture of mature kallikrein by endocytosis from plasma after cell binding [9]. To prove the latter hypothesis, we have studied the ability of human salivary-gland kallikrein, that is in the zymogen or activated form, to interact with isolated human neutrophils. In addition, the identification of such binding sites is a prerequisite which provides new insights into the prokallikrein conversion and function of tissue kallikrein in neutrophils during physiological and pathological events.

2. Materials and methods

2.1. Materials

Human recombinant salivary-gland prokallikrein (M_r = 35,000) was purified from the previously described pVLKA56-based baculovirus expression system [2]. Trypsin activation of the proenzyme [3] and separation of the activation peptide by gel filtration on a Superose 12 HR 10/30 column (Pharmacia) using 10 mM sodium phosphate, 100 mM NaCl, pH 7.2 with a flow rate of 0.3 ml/min yielded mature salivary-gland kallikrein (M_r = 33,000). Human urinary kallikrein (M_r = 41,000) was a kind gift from R. Geiger (München).

2.2. Isolation of human neutrophils and lymphocytes

Separation of blood cells occured by density centrifugation on Neutrophil Isolation Medium (NIM; Paesel + Lorei) using a modified version of the manufacturer's recommendations. Briefly, whole blood of healthy donors was collected by venipuncture into 1/49 volume 10% EDTA and gently layered in 7 ml-fractions over 4 ml NIM followed

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by centrifugation at 400 × g for 30 min at 20°C. The lymphocyte-containing plasma of the upper band and the neutrophil-enriched fraction of the lower band were aspirated separately and diluted with equal volumes Krebs-Ringer buffer (121 mM NaCl, 4.9 mM KCl, 16.5 mM Na₂HPO₄, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, pH 7.4) to remove NIM-traces by sedimentation at 450 × g for 10 min. Cell pellets were washed twice with RPMI1640 medium supplemented with 20% fetal calf serum, resuspended in the same medium and used within 6 h after preparation.

This procedure yielded approximately 6×10^6 cells/ml whole blood, for neutrophils and lymphocytes respectively, as counted in a Thoma chamber. The cell viability was over 97% assessed by the Trypan blue exclusion test.

2.3. Kallikrein binding studies

Before the binding studies, neutrophils and lymphocytes were washed twice with Krebs-Ringer buffer containing 0.05% Brij 35 and resuspended in the same buffer at a final concentration of 3×10^7 cells/ml. Incubation of the cells occurred at 37°C with increasing amounts of different tissue kallikrein samples in a total assay volume of 300 μl. The reaction was terminated at the indicated periods by centrifugation for 10 min at 5000 rpm in a microfuge, and the supernatant was analyzed for unbound kallikrein species by an enzymatic assay with the fluorogenic peptide substrate H-prolyl-phenylalanyl-arginine-7-amido-4-methylcoumarin (H-Pro-Phe-Arg- AMC; Bachem) according to [10] by means of a Perkin Elmer LS 50 spectrofluorimeter ($\lambda_{exc} = 380$ nm; $\lambda_{em} = 460$ nm). The amidolytic kallikrein activity was measured directly or after trypsin activation of the proenzyme. Total binding was calculated as the difference between the kallikrein activity of cell-free reaction mixtures and the corresponding binding assays. The results are represented in arbitrary units (AU) where one unit is defined as that amount of enzyme which releases 2 pmol AMC/s under the described conditions.

3. Results and discussion

The preparation of human salivary-gland prokallikrein expressed in recombinant baculovirus-infected insect cells together with in vitro proenzyme conversion has enabled our laboratory to investigate the physiological function of the tissue kallikrein precursor which is not interfered with by trace amounts of proteinases in prokallikrein fractions from natural sources [12]. The recombinant material displayed full biological activity and recognition of all three glycosylation sites [2] which might be essential for protein–cell interaction.

Cell-binding experiments with different components of the kallikrein–kinin system have been performed in situ

by immunolocalization [5,7–9] or in vitro by binding assays with radiolabeled ligands [6,11]. This study introduces an enzymatic assay based upon the selective tissue kallikrein substrate H-Pro-Phe-Arg-AMC [10] to monitor the amidolytic activity of unbound kallikrein using sensitive fluorescence measurements. To assess misrepresentations of fluorescence intensity which might occur by the kallikrein content of freshly isolated cells, washed neutrophils alone at a concentration of 3×10^7 cells/ml were incubated under assay conditions, and the amidolytic kallikrein activity in the supernatant was measured directly or after trypsination to cover prokallikrein. Values amounted to 0.02 AU and to 0.04 AU in the latter case (data not shown) which were subtracted as background from kallikrein activity in cell binding experiments. Cell viability was not affected by the assay conditions as can be proved, thus excluding the release of kallikrein from cell lysis.

Further investigations were conducted to ascertain whether the external membrane of neutrophils contains unoccupied binding sites for pro- or mature kallikrein. Increasing concentrations of recombinant pro-kallikrein, recombinant kallikrein and urinary kallikrein, respectively, were added to washed human neutrophils and bound kallikrein species quantified by differential amidolytic assays (Fig. 1). To avoid nonspecific protein–protein or protein–cell interactions, all solutions were supplemented with the nonionic detergent Brij 35 at a concentration of 0.05%. As the amount of added prokallikrein increased, the level of binding increased until saturation was observed at ~25 nM prokallikrein with approximately 5 pmol prokallikrein bound per 10^7 neutrophils, which corresponds to about 3×10^5 molecules of prokallikrein bound per cell. In contrast to prokallikrein binding, no association of recombinant mature kallikrein to neutrophils was detected, whereas the final extent of binding of urinary kallikrein was reduced 40–50%. The discrepancy in the binding behaviour of the activated tissue kallikrein in recombinant or native form is due to the proenzyme content of kallikrein isolated
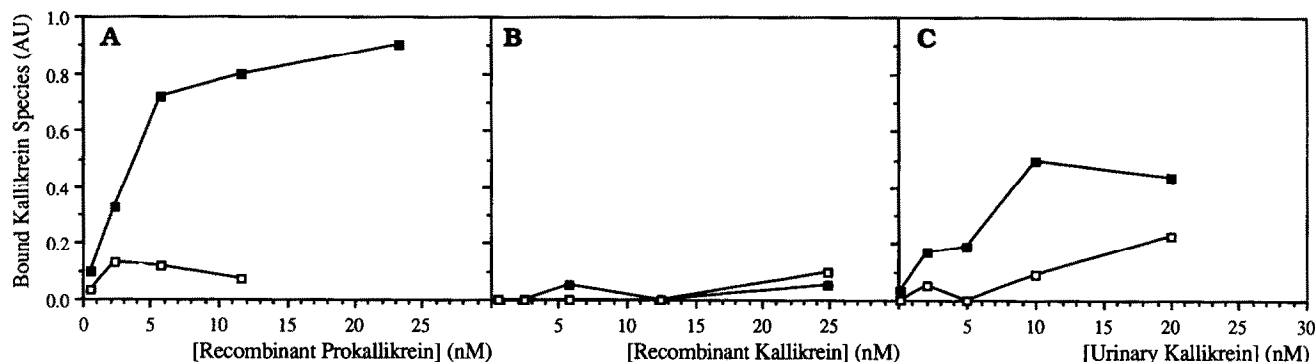

Fig. 1. Concentration-dependent binding of different human tissue kallikrein species to human white blood cells. Neutrophils (■) and lymphocytes (□) at a concentration of 3×10^7 cells/ml each were incubated with various concentrations of (A) recombinant salivary-gland prokallikrein, (B) recombinant salivary-gland kallikrein and (C) native urinary kallikrein for 1 h, respectively. The plotted data represent total binding.
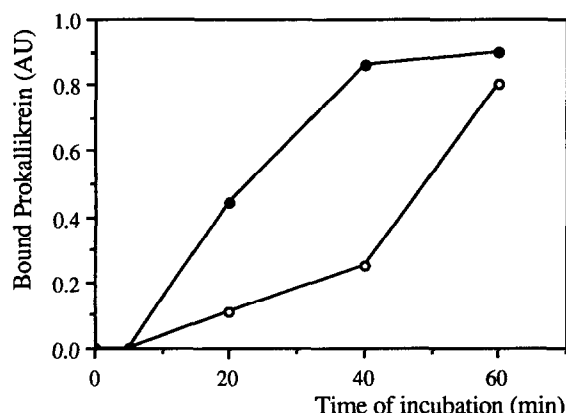
Fig. 2. Time course of binding of human recombinant prokallikrein to neutrophils. Isolated human neutrophils (3×10^7/ml) were incubated in the presence of 12 nM (o) or 24 nM (●) salivary-gland prokallikrein, respectively and total binding was determined as described in the text.

from human urine which accounts for between 40% and 60% of the total kallikrein [13].

To show that the binding observed was specific for neutrophils, and was not due to some common property of cell membranes, binding of the available kallikrein species to washed lymphocytes were studied (Fig. 1). Equal numbers of lymphocytes and neutrophils were used. Although the volume of neutrophil cells is approximately twice as big than the volume of unstimulated lymphocytes, the differences in the surface areas of both cell types are insignificant. In respect of this assumption, nine times as much recombinant proenzyme bound in the neutrophil samples as in the lymphocyte samples, while binding of recombinant kallikrein was undetectable in any case. Thus, the binding of prokallikrein appears to be specific for neutrophils. The values for binding of urinary kallikrein to lymphocytes are attributed to the above-mentioned prokallikrein portion of the enzyme preparation. The kallikrein–neutrophil interaction is restricted to the association of the proenzyme but it remains to be elucidated whether the short activation peptide of seven amino acids or an epitope exposed by the three-dimensional structure of the zymogen is responsible for cell binding.

To draw preliminary conclusions about the binding type, the association kinetics of prokallikrein to human neutrophils was investigated. Binding assays were performed with prokallikrein concentrations of 12 and 24 nM, respectively, and sampled at various times. Fig. 2 shows that the binding of prokallikrein to neutrophils was time-dependent and apparent equilibrium for saturable binding was not reached before one hour of incubation in the case of 24 nM prokallikrein. Lower prokallikrein concentrations prolonged the time course of association. The observed slow-binding kinetic is con-

trary to the association time course of the kallikrein substrate HMWK to neutrophils reaching the plateau of binding after 15–20 min [6]. Together, these data suggest that neutrophil binding of tissue prokallikrein does not occur through a preshaped receptor molecule but requires the complex formation with an acquired cofactor. This proposed binding mechanism is shown in the case of plasma prokallikrein where HMWK acts as the zymogen carrier on the circulating human neutrophil [8].

In summary, our results demonstrate for the first time that tissue prokallikrein specifically binds to intact human neutrophils and structural features of the zymogen are required for the interaction with unoccupied sites on the neutrophil surface. The presented data preclude a direct binding of mature kallikrein to neutrophils modifying the previously published hypothesis [9] of kallikrein endocytosis by neutrophils with regard to an internalization step of prokallikrein. The observed preference of the kallikrein precursor is a strong indication that indispensable proenzyme conversion to activated kallikrein plays an important role in the regulation of kallikrein bioactivity.

References

[1] MacDonald, R.J., Margolius, H.S. and Erdös, E.G. (1988) Biochem. J. 253, 313–321.

[2] Angermann, A., Rahn, H.-P., Hektor, T., Fertig, G. and Kemme, M. (1992) Eur. J. Biochem. 206, 225–233.

[3] Takada, Y., Skidgel, R.A. and Erdös, E.G. (1985) Biochem. J. 232, 851–858.

[4] Bhoola, K.D., Figueroa, K.D. and Worthy, K. (1992) Pharmacol. Rev. 44, 1–80.

[5] Figueroa, C.D., MacIver, A.G. and Bhoola, K.D. (1989) Br. J. Haematol. 72, 321–328.

[6] Gustafson, E.J., Schmaier, A.H., Wachtfogel, Y.T., Kaufman, N., Kucich, U. and Colman, R.W. (1989) J. Clin. Invest. 84, 28–35.

[7] Figueroa, C.D., Henderson, L.M., Kaufmann, J., De La Cadena, R.A., Colman, R.W., Müller-Esterl, W. and Bhoola, K.D. (1992) Blood 79, 754–759.

[8] Henderson, L.M., Figueroa, C.D., Müller-Esterl, W., Stain, A. and Bhoola, K.D. (1992) Agents Actions 38, 590–594.

[9] Figueroa, C.D. and Bhoola, K.D. (1989) in: The Kallikrein-Kinin System in Health and Disease (Fritz, H., Schmidt, I. and Dietze, G. eds.) pp. 311–320, Limbach-Verlag, Braunschweig.

[10] Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T. and Sakakibara, S. (1977) J. Biochem. 82, 1495–1498.

[11] Greengard, J.S. and Griffin, J.H. (1984) Biochemistry 23, 6863–6869.

[12] Fiedler, F. (1979) in: Handbook of experimental pharmacology (Erdös, E.G. ed.) vol. 25, pp. 103–161, Springer, Berlin.

[13] Spragg, J. (1988) Methods Enzymol. 163, 96–102.