The binding of tissue prokallikrein to isolated human neutrophils

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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 propeptide occurred by limited proteolysis of the proenzyme, 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 localization 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 occurred 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...
by centrifugation at 400 × g for 30 min at 20°C. The lymphocyte-
containing plasma of the upper band and the neutrophil-enriched frac-
tion 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 RPM11640 medium supplemented with 20% fetal calf serum,
resuspended in the same medium and used within 6 h after preparation.

This procedure yielded approximately 6 × 10⁶ 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
eclusion 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 × 10⁷
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 superna-
tant 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) accord-
ing to [10] by means of a Perkin Elmer LS 50 spectrofluorometer
(λₑₓ = 380 nm; λₑₘₘ = 460 nm). The amidolytic kallikrein activity was
measured directly or after trypsination 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 prokal-
lkrein expressed in recombinant baculovirus-infected in-
sect 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 intro-
duces an enzymatic assay based upon the selective tissue
kallikrein substrate H-Pro-Phe-Arg-AMC [10] to moni-
tor the amidolytic activity of unbound kallikrein using
sensitive fluorescence measurements. To assess misrepre-
sentations of fluorescence intensity which might occur by
the kallikrein content of freshly isolated cells, washed
neutrophils alone at a concentration of 3 × 10⁷ cells/ml
were incubated under assay conditions, and the ami-
dolytic kallikrein activity in the supernatant was meas-
ured 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 back-
ground from kallikrein activity in cell binding experi-
ments. Cell viability was not affected by the assay condi-
tions as can be proved, thus excluding the release of
cell 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-kal-
lkrein, 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 prokal-
lkrein increased, the level of binding increased until sat-
uration was observed at ~25 nM prokallikrein with
approximately 5 pmol prokallikrein bound per 10⁶ neu-
rophils, which corresponds to about 3 × 10⁹ molecules
of prokallikrein bound per cell. In contrast to prokal-
lkrein binding, no association of recombinant mature
cell 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 isoalted

![Graph](image-url)  # Fig. 1. Concentration-dependent binding of different human tissue kallikrein species to human white blood cells. Neutrophils (A) and lymphocytes (C) at a concentration of 3 × 10⁷ 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.
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 as 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 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 contrary 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.

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