Immunovisualization of High (HK) and Low (LK) Molecular Weight Kininogens on Isolated Human Neutrophils

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An immunocytochemical study was performed to examine the cellular localization and the subcellular distribution of kininogens in human blood cells. Kininogens were visualized using the immunogold-silver staining method and confocal scanning laser microscopy. We confirmed the existence of high molecular weight kininogen in human neutrophils and describe for the first time the presence of low molecular weight kininogen on these cells. Both high and low molecular weight kininogens were restricted to the neutrophils where they localized as clusters of immunogold particles on the cell membrane. No labeling was observed intracellularly in organelles such as mitochondria, endoplasmic reticulum, and membranes of platelets, cultured human endothelial cells, and neutrophils. Residues (556 to 595) on the light chain of HK are essential for the binding of plasma prekallikrein and both enzyme and substrate circulate as a complex. When HK is coupled to receptors on the neutrophil, then the conversion of prekallikrein to kallikrein could occur on the surface of the neutrophil cell membrane. Once activated, plasma kallikrein bound to HK might cause localized kinin release, stimulate elastase secretion, and induce neutrophil aggregation.

HUMAN KININOGENS are endogenous protein substrates for tissue and plasma kallikrein, which by proteolytic cleavage form vasoactive kinin peptides. In addition, kininogens contain domains that function as cysteine proteinase inhibitors and act as cofactors for contact activation of blood coagulation. These proteins are synthesized and secreted into the circulation by hepatocytes. For many years, the presence of both high (HK) and low (LK) molecular weight kininogens was thought to be restricted to extracellular fluids. However, more recent studies have shown that these molecules are present in several other cell types. Thus, kininogens have so far been identified in the collecting ducts of the human kidney, sweat glands, endothelial cells, and human neutrophils. Until the present study, the precise ultrastructural localization of the kininogens has remained elusive. Experiments with platelets indicate that when they are activated HK is translocated from the α-granules to the external surface of the cell membrane.

The binding of kininogens to cell membranes most probably involves specific acceptor proteins exposed on the outer face of cells. Specific, saturable, and reversible binding sites for HK have been identified on the cell membranes of platelets, cultured human endothelial cells, and neutrophils. Residues (556 to 595) on the light chain of HK are essential for the binding of plasma prekallikrein and both enzyme and substrate circulate as a complex. When HK is coupled to receptors on the neutrophil, then the conversion of prekallikrein to kallikrein could occur on the surface of the neutrophil cell membrane. Once activated, plasma kallikrein bound to HK might cause localized kinin release, stimulate elastase secretion, and induce neutrophil aggregation.

LK has not been demonstrated on neutrophils. Whereas the presence of HK in neutrophils has been reported, the source of HK remains unclear. Thus, in neutrophils HK could be synthesized in situ, captured by emiocytosis from plasma, and transported into the cell by pinocytotic vesicles or be simply bound to its putative receptor on the surface. As a first step to the precise localization of the two forms of kininogens on human blood cells, the present immunocytochemical study was initiated. We provide using light, confocal laser scanning, and electron microscopy, evidence for the co-existence of HK and LK on the surface of human neutrophils. The enzymic action of kallikrein on kinogen may result in the circumscribed release of kinins in close proximity to neutrophils marginalizing on endothelial cells.

MATERIALS AND METHODS

To achieve a precise subcellular localization of the kininogens, visualization was performed with conventional, confocal scanning laser and immunogold-electron microscopy. When conventional light microscopy was used, immunolocalization was achieved through the immunogold silver enhancing technique.

Neutrophil isolation. Human neutrophils were isolated from anticoagulated whole blood provided by volunteers at The Medical School, University of Bristol, England, UK, and at The Regional Hospital, Valdivia, Chile. Blood, 18 mL, collected in 2 mL of 105 mmol/L sodium citrate, was mixed with 20 mL of dextran (6% wt/vol, average molecular weight 266,000) and 60 mL of phosphate-buffered saline (PBS: 10 mmol/L sodium phosphate, 2.7 mmol/L potassium chloride, and 137 mmol/L sodium chloride, pH 7.4; containing 0.4% wt/vol tri-sodium citrate). The mixture was left to stand for 45 minutes at room temperature to allow red blood cells to sediment. When this was completed, the upper leukocyte-enriched plasma (8 mL) was gently layered over Ficoll-Hypaque (16 mL) (Pharmacia-LKB Biotechnology, Uppsala, Sweden) and
centrifuged at 400g for 20 minutes at 23°C. The cell pellet was resuspended in an erythrocyte lysis buffer (155 mmol/L NH₄Cl, 2.7 mmol/L KHCO₃, and 3.7 mmol/L EDTA, pH 7.4). The cells were centrifuged at 500g for 10 minutes and the pellet was washed three times in PBS-trisodium citrate buffer. This procedure yielded a cell preparation that contained about 95% granulocytes with a viability of about 98%.

**Antibodies.** Five different antibodies to kininogens were used: (A) rabbit antiserum (III-279) to the whole of the LK molecule (recognizes both LK and HK); (B) monoclonal antibody (MoAb) (2B5) directed to the common 64-Kd heavy chain of kininogens (recognizes both HK and LK); (C) MoAb (C11C1) directed to an epitope on the unique 56-Kd light chain of HK (recognizes only HK); (D) MoAb (LKL3) directed to an epitope at the unique carboxyterminal segment (amino acids 389 to 409) of LK (recognizes only LK); (E) polyclonal antibody (R7) directed to the same portion of LK as above (recognizes exclusively LK). Antibodies D and E were raised against a synthetic peptide (NH₂-Cys-Gly-Try-Lys-Gly-Arg-Pro-Pro-Lys-Ala-Gly-Ala-Gln-Pro-Ala-Ser-Glu-Arg-Glu-Val-Ser-COOH) corresponding to residues 389 to 409 of the LK light chain. The peptide was coupled to keyhole limpet hemocyanin by maleimidobenzoyl-N-hydroxysuccinimide ester. Both the monoclonal and polyclonal antibodies (D and E, respectively) were specific for LK and showed no significant crossover with HK either in enzyme-linked immunosorbent assay or immunoblots using purified LK and HK from human plasma.

**Immunogold silver staining method.** Immunostaining was performed on smears prepared using white blood cells after dextran sedimentation and neutrophils isolated by centrifugation on Ficoll-Hypaque gradients. Smears were air-dried and fixed for 30 seconds to 1 minute with 2% (wt/vol) paraformaldehyde in PBS (pH 7.4) or 4% (vol/vol) formaldehyde in acetone, buffered to pH 6.7 with phosphate buffer. The fixed smears were washed twice in PBS for 5 minutes and then immunostained in accordance with the following procedure: (1) 5% (vol/vol) nonimmune goat serum in PBS with the complement system destroyed by heating at 60°C for 30 minutes; (2) rabbit (1:500) or mouse (1:50 to 1:100) antikininogen antibody overnight at 20°C; (3) gold (10 or 30 nm)-labeled probe (goat antirabbit or goat antimouse IgG, diluted 1:100) (Janssen Biotech, Olen, Belgium) for 1 hour; (4) silver enhancer for 10 to 20 minutes at room temperature. The silver enhancer reagent (Janssen Biotech) was prepared by mixing equal amounts of enhancer and initiator solutions according to the manufacturer's instructions. Finally, the slides were washed thoroughly with distilled water, counterstained with hematoxylin and eosin, and mounted in DPX (BDH, Poole, UK) mounting medium.

**Confocal scanning laser microscopy.** Confocal microscopy has been developed recently as solution to the out of focus, blurred image witnessed in conventional light microscopy. This method has several additional advantages over other microscopic techniques, including noninvasive optical sectioning of intact, fixed, and non-fixed cells. The smears fixed as indicated previously were washed...
with PBS (pH 7.4) containing 1% (wt/vol) bovine serum albumin (BSA) to inhibit nonspecific binding and 1% (vol/vol) nonimmune goat serum to block Fc receptors on the neutrophil membrane. The next step involved incubation with: (1) antibody to HK or LK as above and (2) fluorescein-labeled F(ab), fragments (sheep anti-mouse IgG; Sigma Chemicals, St Louis, MO) diluted 1:32 for 30 minutes. F(ab), fragments were used to exclude the nonspecific binding of the label to Fc receptors. Both fixed and nonfixed immunostained cells were observed under a confocal fluorescence microscope (Bio-Rad, Hemel-Hempstead, UK) equipped with an excitatory laser light.

**Immunoelectron microscopy.** Some aliquots of isolated neutrophils (10⁶ cells/mL) were fixed for 15 minutes with 1% (wt/vol) paraformaldehyde buffered to pH 7.4 with 0.1 mol/L phosphate buffer. Before immunostaining, some of these fixed neutrophils were partially permeabilized by incubation with either 0.01% and 0.1% Triton X-100 (Sigma) for 10 minutes at room temperature. The permeable state was verified by visualizing immunoreactive human neutrophil elastase in azurophilic granules with a specific polyclonal antibody to the enzyme (obtained from Serotec, Oxford, UK) and protein A coupled to 10-nm gold particles.

The fixed, permeabilized, and nonpermeabilized neutrophils were washed three times for a duration of 5 minutes with PBS that contained 1% (wt/vol) BSA and 1% (vol/vol) nonimmune goat serum. Some aliquots of nonfixed, nonpermeabilized cells were incubated directly with antikininogen antibodies in the presence of 0.2% sodium azide to prevent internalization of the antibody by the cells. The fixed but as yet not immunostained cells were incubated with rabbit or murine antikininogen antibodies in the BSA-goat serum-PBS buffer for 4 hours at room temperature under gentle agitation. After washing with PBS, all of the cells, immunostained with the primary antibody, were incubated for 2 hours at room temperature with gold-labeled probes (goat antirabbit IgG labeled with 30 nm gold particles or goat antimouse IgG coupled to 10 nm gold particles) diluted 1:4 in the same PBS. On completion of this procedure, the cells were washed three times with PBS for 5 minutes and fixed with 5% (vol/vol) glutaraldehyde in PBS for 1 hour at room temperature. Finally, the cells were postfixed with 1% (vol/vol) osmium tetroxide and embedded in epon-araldite as previously described. Ultrathin sections were counterstained with lead citrate and examined under a Philips EM-300 electron microscope (Philips, Eindhoven, Holland).

**Immunocytochemical controls.** Controls included replacement of the kininogen antibody by nonimmune serum or IgG of the same species and also by omission of the first antibody. Additional controls were prepared by pre-absorption of the primary antibody with 50 μg/mL of the respective purified antigen.

**RESULTS**

This is the first study in which LK and HK have been immunolocalized using antibodies that specifically discriminate between the two molecules. Of the different blood cells, HK was visualized on neutrophils and platelets whereas LK was seen only on the neutrophils.

All the antibodies used in this study recognized immunoreactive kininogens on the isolated neutrophils. When the immunogold-silver staining method was performed on blood cell smears, prepared after dextran sedimentation, and incubated with a polyclonal antibody (III-279) that recognized both LK and HK by conventional microscopy, it was evident that kininogens were restricted to the neutrophils while other blood cells such as eosinophils, lymphocytes, and monocytes remained unstained. The immunoreactive kininogens appeared as black dots, and therefore the
KININOGENS ON NEUTROPHIL CELL MEMBRANE localization of LK (Fig 1) and HK (Fig 2) could only be distinguished by using MoAbs specific to each molecule. Of the two kininogens only the antibody to HK reacted with platelets (Fig 2b).

Conventional light microscopy is limited in its ability to demonstrate many of the details of cellular structure. Therefore, we used the new powerful imaging technique of confocal scanning laser microscopy in which fluorescein-labeled F(ab), fragments were used, with the color gradient from blue (nil) to red (maximal) providing an estimate of the amount of kininogen on the cell. The light microscopy localization of LK and HK was confirmed with the confocal imaging (Figs 3 and 4). Furthermore, this method permitted the optical scanning of fluorescence in nonfixed neutrophils that showed the label as a ring on the neutrophil cell membrane (Fig 4). Progressive optical sectioning of the cells confirmed this observation and failed to show a distribution of the fluorescent label in the center of the neutrophil.

The next question to answer was the precise ultrastructural site at which the kininogens were sequestered. At the electron microscopy level, immunoreactive kininogen appeared as clusters of gold particles restricted to the external surface of the neutrophil cell membrane; no gold labeling was observed intracellularly in organelles such as mitochondria, endoplasmic reticulum, and azurophilic or specific granules (Fig 5, a and b). This pattern of immunostaining remained unchanged after permeabilization of the neutrophils with 0.01% or 0.1% Triton X-100 (Fig 5, c and d), a procedure that permitted the visualization of elastase in the granules (Fig 5, e).

![Figure 5](image-url)

**Figure 5.** Immunogold electron micrographs: Immunoreactivity to LK and HK is restricted to the neutrophil membrane only (see arrows), observed using heavy-chain, rabbit polyclonal Ill-279 as the primary antibody and antirabbit IgG labeled with 30-nm gold particles as the second antibody. (a and b) Immunostaining performed on nonfixed, nonpermeabilized cells directly incubated with the antikininogen antibody. (a) Low power original magnification ×25,000. Enhancement, by counterstaining with lead citrate, of the small, dense cytoplasmic grains that correspond to glycogen β particles (gly). (b) High power original magnification ×90,000. No counterstaining with lead citrate. The granules (g) show no gold particles. (c and d) The neutrophils were permeabilized. The cells were treated with 0.1% Triton X-100 at 21°C. The immunoreactivity is still restricted to the cell membrane (arrows), with no evidence of any localization in the granules (g) or the cytosol. Original magnification × 85,000. (e) Visualization of neutrophil elastase (using protein A-10 nm gold particles bound to anti-elastase antibodies) in the granules of neutrophils permeabilized by the same procedure as in (c) and (d). Original magnification × 100,000.
relationship between each kininogen molecule and its receptor on the neutrophil membrane cannot be determined from our morphologic study, but should become evident from binding experiments. Both of the kininogens associated with the neutrophil membrane are likely to have originated from the circulating plasma pool, although the possibility that kininogens may be synthesized by the neutrophil and then translocated to the cell membrane cannot be totally discarded. However, were this the case immunogold particles should have been evident in the Golgi apparatus and in the granule populations, a phenomenon never observed in our localization studies. Definitive evidence to clarify whether neutrophils de novo express LK and HK must await in situ hybridization or polymerase chain reaction experiments with the appropriate cDNA probes to detect the specific mRNA for each of the kininogens.

In a recent study Gustafson et al. showed that human neutrophils contain HK and possess specific, reversible, and saturable binding sites for this molecule. Similar high-affinity binding sites have been reported for endothelial cells and platelets. In platelets, competition between HK and LK for the same binding sites may occur, suggesting that either the whole or part of their common segment encompassing the heavy chains, the kinin moiety, and a small proportion of the light chains was involved in the binding to the receptor. Kininogens anchored to specific cell receptors may serve two functions: (1) in the case of platelets, surface-bound kininogen together with prekallikrein and factor XI, which bind to the HK light chain, could locally trigger the endogenous coagulation cascade, and (2) in the case of neutrophils, proteolytic processing of surface-bound LK and HK by kininogenases (tissue and plasma kallikreins, respectively) could form kinins, which by opening junctions between endothelial cells may promote the local diapedesis of neutrophils and the extravasation of plasma constituents. Future studies should be aimed at the identification of the pathways by which cleavage of the surface-bound kininogens on the neutrophil membrane results in a circumscribed release of kinins.

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