Leukocytic cell sources of airway tissue kallikrein

Isabel T. Lauredo, Rosanna M. Forteza, Yelena Botvinnikova, and William M. Abraham

1Division of Pulmonary and Critical Care Medicine, University of Miami School of Medicine at Mount Sinai Medical Center, Miami Beach 33140; and 2Division of Pulmonary and Critical Care Medicine, University of Miami School of Medicine, Miami, Florida 33136

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Lauredo, Isabel T., Rosanna M. Forteza, Yelena Botvinnikova, and William M. Abraham. Leukocytic cell sources of airway tissue kallikrein. Am J Physiol Lung Cell Mol Physiol 286: L734–L740, 2004. First published December 5, 2003; 10.1152/ajplung.00129.2003.—Lung tissue kallikrein (TK) is a serine proteinase that putatively plays a role in the pathophysiology of asthma by generating kallidin and bradykinin, mediators that contribute to airway hyperresponsiveness. In previous studies we observed biphasic increases in TK activity in bronchoalveolar lavage fluid following airway allergen challenge in allergic sheep. Although glandular TK is likely a major source of the initial increase in TK, the sources of the late increases in TK that are associated with the development of airway hyperresponsiveness may be dependent on activated resident and recruited inflammatory cells including alveolar macrophages (AMs) and neutrophils (PMNs). These cells increase concomitantly with the late increases in TK activity. To test this hypothesis, we obtained AMs from bronchoalveolar lavage fluid and PMNs and monocytes (precursors of AMs) from sheep blood and determined whether these cells contained TK and whether these same cells could release TK upon activation. Using confocal microscopy, immunocytochemical techniques, and enzyme activity assays, we found that all three cell types contained and secreted TK. All three cell types demonstrated basal release of TK, which could be increased after stimulation with zymosan. In addition, PMNs also released TK in the presence of phorbol ester, suggesting multiple secretory pathways in these cells. Furthermore, we showed that human monocytes also contain and secrete TK. We conclude that in the airways, monocytes, PMNs, and AMs may contribute to increased TK activity. Knowing the sources of TK in the airways could be important in understanding the mechanisms of inflammation that contribute to the pathophysiology of asthma and may help in the development of new therapies to control the disease.

inflammation; serine proteases; asthma; airway hyperresponsiveness; neutrophils; macrophages

Tissue Kallikreins (TK) (EC 3.4.21.35) are a multiple family of serine proteinases that generate kallidin (lysyl-bradykinin) from low- and high-molecular-weight kininogen. In the lung, the generation of kallidin and its cleavage product bradykinin can contribute to the pathophysiology of asthma (5, 14, 33, 34, 38, 39, 51). Both peptides cause vasodilatation (47) and increases in vascular permeability (6) and bronchoconstriction (2, 42). Furthermore, studies in asthma patients and animal models of asthma indicate that elevated TK and kinin levels in bronchoalveolar lavage fluid (BALF) may mediate airway hyperresponsiveness (AHR), a hallmark of the disease (13, 18, 40, 42). TK has also been shown to be an important activator of type IV collagenase (92-kDa gelatinase) (16), which is involved in the degradation of extracellular matrix components and, therefore, could be important in airway remodeling seen in chronic asthma (7). These links between elevated TK and asthma symptoms suggest that increases in TK levels and/or activity may be critical factors in disease pathogenesis. Therefore, understanding the sources of TK and its regulation in the airways could be important therapeutically.

Airway challenge with antigen is associated with both an acute (<30 min) increase and a late (6–24 h) increase in BALF TK activity (3, 5, 14, 18, 40–42). The source(s) of the immediate increase in TK activity is most likely from submucosal gland cell secretion triggered by secretagogue mediators such as chymase and/or elastase (3, 5, 13, 14, 21, 41, 46) and from previously secreted TK that is bound to hyaluronan on the apical surface of the ciliated epithelium. This bound TK is inactive, but in the presence of oxygen radicals that are generated in the airways following allergen challenge (30), the TK-hyaluronan bond can be broken, thereby activating the TK (22, 23). Although glandular and epithelial-bound TK may contribute to the immediate increase in airway TK following allergen challenge, these may not be the primary sources for the more prolonged increases in TK/kinin levels seen 6–24 h after antigen challenge that are linked to AHR.

Our previous studies indicate that recruited inflammatory cells may be one important source for this late, more sustained increase in airway TK activity. Increases in the numbers of polymorphonuclear leukocytes (PMNs) and alveolar macrophages (AMs) in BALF coincide with the late increases in BALF TK activity, and inhibition of this cellular inflammatory response with adhesion molecule inhibitors prevented the increase in TK activity and the associated AHR (1, 3). Collectively, these studies suggest that PMNs and AMs could be potential sources of the enzyme.

There are data to support the hypothesis that both PMNs and macrophages can generate kinins. PMNs have been studied extensively and have been shown to generate kinins (28). Subsequent findings confirmed the presence of TK mRNA and protein expression in these cells (10, 19, 20), and more recent work indicates that PMN-associated TK can be released at sites of inflammation. Williams and coworkers (51) found that PMNs obtained from the synovial fluid of patients with rheumatoid arthritis had reduced TK activity compared with circulating PMNs from the same patients, which suggests that the PMNs released TK at some point in transit from the vasculature to the inflammatory locus. Such a mechanism would be consistent with PMNs contributing to the late antigen-induced increases in BALF TK activity.

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Macrophages may also be a source of TK (15, 44). Macrophages obtained from rat intestine granulomas contain and secrete TK (44), therefore it is possible that AMs also contain and secrete TK. This fact cannot be assumed, however, because tissue-resident macrophages adapt to their local environment to perform specific functions (29). TK mRNA was found to be present in promyelocytic cultures of an HL-60 cell line induced to differentiate toward monocytes (MONOs) (37), indicating that MONOs could also contain and secrete TK. Because these cells eventually migrate to the lung and become AMs, it is possible that both cell types could contribute to lung TK activity.

In this study, then, we formally test the hypotheses that AMs, MONOs, and PMNs contain and secrete TK. To do this, we used specific enzyme activity assays and immunocytochemistry to verify the presence of TK in both MONOs and AMs, and we used confocal microscopy to assess the localization of TK in PMNs by double labeling TK and elastase (which is present in the azurophilic granule of PMNs). In addition, we determined the functional aspects of TK release in these cells after stimulation with phorbol ester (PMA) and opsonized zymosan (OZ), two secretagogues that affect the secretory mechanisms of these cells differently (4, 11, 26, 32, 45, 48, 50, 52). Our findings show that AMs and, for the first time, MONOs (obtained from both sheep and human peripheral blood) contain and release TK. We also corroborate studies demonstrating that PMNs secrete TK and described the secretory pathways involved in TK release by these cells. Colocalization studies also revealed that, unlike elastase, TK is not stored in azurophilic granules.

MATERIALS AND METHODS

In this study, the PMNs and MONOs from blood and the AMs from BALF were obtained from sheep. This study was approved by the Mount Sinai Medical Center Animal Research Committee, which is responsible for ensuring the humane care and use of experimental animals. Peripheral blood from human volunteers was also obtained for the isolation of MONOs. This procedure was determined to be exempt from informed consent by the Mount Sinai Medical Center Institutional Review Board.

All chemicals and reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated. For stimulation studies, we prepared OZ by boiling the zymosan for 15 min in 0.85% saline solution and then washing it twice by centrifugation. We achieved opsonization by incubating the zymosan with 5 mg/ml of sheep serum in a rotator shaker for 1 h at 37°C. After incubation, the solution was centrifuged, and the pellet was washed twice and then resuspended in phosphate-buffered saline (PBS) without Ca2+ and Mg2+ at a concentration of 100 mg/ml. Aliquots were frozen at −70°C until used.

Isolation of MONOs from peripheral blood. Monocytes were isolated from anticoagulated blood (0.5 ml of 4.3% EDTA/ml for sheep blood and 10 units of heparin/ml for human blood) by density gradient centrifugation according to the method of Boyum (9). Briefly, blood was diluted 1:1 with PBS without Ca2+ and Mg2+. Histopaque-1077 was layered at the bottom of the tube, and then the preparation was centrifuged at 450 g for 30 min at room temperature. After centrifugation, a distinct layer at the plasma-histopaque interface was formed, which contained the mononuclear cells. To maximize the collection of MONOs, we collected the entire overlying layer and interface. The collective fraction was centrifuged at 300 g, and the pellet was washed with PBS twice at room temperature. This method has recently been substantiated to increase the yield of MONOs to >60% (36). Cell viability, assessed by trypan blue exclusion, was between 90 and 95%.

Cell differentials with Wright-Giemsa stain showed that for the various experiments between 50 and 70% of the cells in the interface were MONOs, with the remainder being lymphocytes. Because lymphocytes are not phagocytic cells and because neither mature lymphocytes (37) nor lymphocytic cell lineages have been shown to contain or express TK (37), further attempts to separate MONOs and lymphocytes were not pursued to avoid cell loss. For each experiment, we resuspended the mononuclear cell preparation (MONOs plus lymphocytes) at a concentration of 3–5 × 106 cells/ml in RPMI 1640 and then adjusted it to a concentration of 106 MONOs/ml by correcting for the cell differential in each experiment. TK values were reported as ng/106 MONOs.

Isolation of PMNs from peripheral blood. PMNs were obtained by the density gradient centrifugation preparation described above. Because of their density, PMNs are found in the bottom layer along with the erythrocytes after centrifugation. PMNs were separated from the red blood cells as previously described (12). Cell viability, assessed by trypan blue exclusion, was between 90 and 99%, and cell differentials were 95–99% PMNs, with eosinophils being the other cell present. PMNs were resuspended at 1 × 107/ml in RPMI 1640, and TK values were reported as ng/106 PMNs.

Isolation of AMs from BALF. Conscious sheep were restrained in a modified shopping cart, and BAL was performed as previously described (12). Briefly, the distal tip of a specially designed 80-gauge fiber optic bronchoscope was wedged into a subsegmental bronchus of one lung. BAL was done with 90 ml of PBS without Ca2+ and Mg2+ in three different airways. The BALF was filtered through one single layer of gauze and then centrifuged at room temperature for 15 min at 300 g to collect the cell pellet. The pellets from the three airways were combined, washed twice with PBS, and resuspended in Hanks’ balanced salt solution without Ca2+ and Mg2+ (HBSS). The washed cells were resuspended in 2 ml of HBSS with 100 units of penicillin and 0.1 mg/ml of streptomycin. The cell suspension was then carefully layered on top of two layers of Percoll (densities of 1.04 and 1.06 g/ml, respectively) in sterile, 15-ml polypropylene tubes and was centrifuged at 500 g for 30 min at room temperature. The cells were collected at the interface of 1.06 g/ml, resuspended in PBS, and washed twice by centrifugation at 300 g at room temperature. Cell counts, viability test by 0.5% trypan blue solution, and a cytoospin for cell differentiation were performed. Cell viability for these preparations was >90%, and cell differentiation showed purity between 80 and 90%, with lymphocytes being the main contaminating cell type.

**PMN stimulation.** To assess TK release from PMNs, we incubated 107 cells/ml at 38°C in a rotator water bath in the presence of 10 ng/ml of PMA or 1 mg/ml of OZ. The doses selected for these experiments were those that gave the maximum response in dose-response curves performed for each agent (data not shown). PMNs incubated with medium alone served as controls. In all experiments, samples were placed on ice for 5 min after incubation to stop the reaction and then centrifuged at 250 g for 10 min at 4°C. Supernatants were separated into aliquots, and in those where neutrophil elastase (NE) was measured (see below), aliquots were adjusted to a final concentration of 0.5 M NaCl. Samples were frozen at −70°C until assayed (see below).

**Stimulation of AMs and MONOs.** One milliliter of 106 AMs/ml or 106 MONOs/ml in RPMI 1640 was incubated in a rotator water bath at 38°C (sheep) and 37°C (human) for 1 h with OZ (1 mg/ml), PMA (10 ng/ml), or medium alone (control). The supernatants were collected, centrifuged at 250 g for 10 min at 4°C, and then frozen at −70°C until assayed.

**Enzyme activity assays.** Enzyme activities were measured using chromogenic substrates DL Val-Leu-Arg pNA for TK as described (21, 25) and N-methoxy-succinyl-Ala-Ala-Pro-Val pNA for NE as previously described (24, 35). Values were obtained from a standard curve done in parallel with the samples and reported as ng/106 cells.

When the release of TK and NE was compared, values were reported in mU/106 PMNs with 1 mU being equal to 1 mmol of substrate degraded per min at 22°C.
RESULTS

Stimulated sheep AMs, MONOs, and PMNs release TK. Under control conditions, all sheep cells released TK. AMs showed the largest basal release per cell, followed by PMNs and MONOs. Stimulation of all three cell types with OZ resulted in significant increases in TK activity. As illustrated in Fig. 1, TK activity increased in OZ-stimulated PMNs from a control value of 2.5 ± 0.3 ng/10^6 cells (n = 6) to 46 ± 7.7 ng/10^6 cells (n = 4, P < 0.01). MONOs stimulated with OZ increased TK activity from 1.7 ± 0.4 ng/10^6 cells to 18.4 ± 8.2 ng/10^6 cells (n = 4, P < 0.03), and TK activity in OZ-stimulated AMs rose from a control value of 110 ± 19 ng/10^6 cells to 1,558 ± 340 ng/10^6 cells (n = 4, P < 0.025). In contrast to OZ, PMA only significantly stimulated TK release in PMNs: control 2.5 ± 0.3 ng/10^6 cells (n = 6) to 5.1 ± 0.8 (ng/10^6 cells n = 5) after PMA stimulation. The release of granule constituents from PMNs is known to occur sequentially, with the contents of the specific granules being released before the contents of the azurophilic granules, which contain primarily NE (52). Because OZ causes exocytosis of both granule types, following the time course of TK and NE release from OZ-stimulated PMNs should give an indication as to the potential location of TK in these cells (8). Figure 2 shows that in supernatants from OZ-stimulated PMNs, TK activity reaches a maximum after 15 min, whereas NE activity is low at this time. NE activity then continues to increase during the 60-min observation period. These data, in conjunction with the fact that PMA only causes the release of specific granule (53) contents, suggest that TK is not likely contained in azurophilic granules.

Confocal microscopy demonstrates the presence of TK in sheep PMNs. Visualization of TK (red fluorescence) and NE (green fluorescence) supports the functional release studies,
which indicate that NE and TK are stored in different granule populations (Fig. 3A). There is clear definition in the intracellular granules between red and green fluorescence with little colocalization. Control slides where the primary antibody was omitted show no fluorescence (Fig. 3B).

**Sheep AMs and peripheral blood MONOs contain TK.** Immunoperoxidase staining of AMs and MONOs indicate that both cell types contain TK. TK is present in AMs (Fig. 4B) as evidenced by positive labeling (brown). There is no staining in the control cells, where the primary antibody was omitted (Fig. 4A). Peripheral blood MONOs (Fig. 4D) show staining characteristics similar to the AMs. Likewise, controls where the primary antibody were omitted show no labeling (Fig. 4C), confirming the specificity of TK staining. These data support the enzyme assays and functional release studies, demonstrating that both AMs and MONOs release TK.

**Human peripheral blood MONOs contain and secrete TK.** In two separate experiments from two different volunteers, human MONOs obtained from peripheral blood were found to secrete TK under basal and stimulated conditions. Basal TK release was 7.6 ± 3.5 ng/10⁶ MONOs and increased to 15.2 ± 5.4 ng/10⁶ MONOs after stimulation with OZ. As was observed with sheep MONOs, PMA failed to stimulate TK release. That the cells that contain and secrete TK are MONOs is supported by the positive immunostaining seen in Fig. 5B. The identity of these cells was confirmed by the presence of CD14 by immunocytochemistry (Fig. 5B), a marker for MONOs (27, 43). Similar to the sheep studies, control cells (where nonimmune rabbit serum was used instead of the first antibody) were negative (Fig. 5A).

**DISCUSSION**

The results of this study show that TK is present in and can be released from sheep PMNs, MONOs, and AMs. In addition we provide novel evidence that human MONOs contain and secrete TK. All three cell types show basal release of TK, and this release is increased following stimulation with OZ. In addition, PMNs stimulated with PMA also show increased TK release. Because these cell types are increased in the lung in various inflammatory conditions such as asthma, our findings support the hypothesis that these cells may act as a source of TK in the lungs and, in doing so, may contribute to the functional consequences (e.g., AHR) associated with increased TK activity as shown by us previously (3, 21, 41, 42).

We used two different agonists, i.e., OZ and PMA, to better understand the cellular pathways involved in stimulated TK release from these three cell types (4, 11, 26, 31, 32, 45, 48–50, 52). We found that OZ, a particulate stimulus of phagocytosis in granulocytes, stimulates the release of TK in all cells studied, whereas PMA, a protein kinase C stimulator, only stimulates release from PMNs. As discussed below, the difference in release patterns between OZ- and PMA-stimulated PMNs, AMs, and MONOs is important for identifying the possible cellular location of TK in these three cell types.

Human PMNs have four distinct granules: azurophilic, specific, tertiary, and secretory vesicles, whereas the ruminant PMNs used in this study contain only three. Two of the ruminant granules resemble the azurophilic and specific granules of human PMNs, whereas the third granule, termed a “large granule,” is unique to ruminants (4, 11, 26). Exocytosis of the distinct granule populations may occur independently (48), since discharge of the different granule types may be dependent on the stimulus and/or surfaces the PMNs contact during migration. Unlike azurophilic granules, which are hardly mobilized, specific and tertiary granules are readily exocytosed on cell activation (52). This may explain why the specific and tertiary granules contain many of the components involved in neutrophil adhesion and extravasations including adhesion molecules, extracellular matrix proteases, and enzymes implicated in the generation of soluble mediators of inflammation (17). On the basis of the differential secretory response of PMNs to OZ and PMA (both time course and stimulus specificity) and the confocal microscopy data, our findings indicate that TK and NE are contained in different
PMN granules. Previous studies have shown that PMA induces the exocytosis of specific and large granules but not azurophilic granules (4, 11, 26, 52), whereas OZ causes the release of all granule types, but with specific granules being released before the azurophilic granules (8). Our functional data showing the selective release of TK in PMA-stimulated PMNs and TK release preceding NE release in OZ-stimulated PMNs suggest that TK is likely localized in the specific and/or large granules and not azurophilic granules. These functional results are consistent with the confocal microscopy images, which show clear separation of TK- and NE-containing organelles.

We also demonstrate that AMs contain and release TK. Unlike PMNs, however, only OZ stimulated TK release in these cells. Because PMA is a poor stimulator of macrophage lysosome secretion (50), our findings would suggest that TK is contained in AM lysosomes. Our demonstration of TK in AM is consistent with findings of Stadnicki and coworkers (44), who reported TK activity in macrophages present in submucosal intestinal granulomas. These investigators also provided evidence that these macrophages secrete TK and that this TK contributes to the intestinal inflammatory response in a rat model of experimental enterocolitis. The results of our study
confirm and extend these observations to include not only AMs, but also peripheral blood MONOs, the cellular precursors of AMs. Our data clearly show that peripheral blood MONOs from both sheep and humans contain and secrete TK. Our findings that TK is present in and can be secreted by MONOs (both sheep and human) as well as AMs, in addition to those of Stadnicki et al. (44), differ from those of Figueroa et al. (19), who report that TK was present only in peripheral blood PMNs and not other blood leukocytes. Differences in the immunocytochemical methods used by these investigators may account for this discordance. Our immunocytochemical and functional data, in addition to the fact that TK mRNA is present in promyelocytes, argue strongly that MONOs do contain and secrete TK. This observation has important implications for inflammatory responses in the lung, where AMs increase in inflammatory conditions, e.g., resulting from allergen exposure. Thus the inflammatory-induced migration of peripheral blood MONOs to the lung provides another source of TK.

Although we did not specifically examine the relationship between increased TK activity and AHR in this study, we have extensively described this association in earlier studies and, in fact, these previous findings were the impetus for us to search for additional sources of the enzyme (1, 3). The results of the present study indicate that cells that migrate to the airways following an inflammatory insult, such as allergen challenge, have the potential to perpetuate the inflammatory state and its pathophysiologic consequences, e.g., AHR, by providing a continued localized source of TK. The importance of this cycle is further highlighted by our previous studies showing that interrupting this cascade with inhibitors of cell migration, e.g., selectin inhibitors, blocks the leukocyte recruitment to the lung, the increase in TK activity, and the AHR (1).

In conclusion, we showed that PMNs, AMs, and MONOs contain and secrete TK. Therefore, these cells, which are increased in inflamed lungs, may be likely sources contributing to the sustained increases in lung TK activity observed in airway disease.

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REFERENCES


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