主 論 文

Inter-alpha inhibitor proteins maintain neutrophils in a resting state by regulating shape and reducing ROS production

(インターアルファインヒビタータンパクは好中球形態を制御し、活性酸素分子種産生を抑制することによって好中球の静穏状態を維持する)

Introduction

Sepsis remains a critical problem with significant morbidity and mortality even in the modern era of critical care management. Experimental and clinical data have demonstrated that increased activity of neutrophil-derived serine proteases play a prominent role in sepsis-related tissue damage. Administration of protease inhibitors has been proposed as a therapeutic strategy to restore the balance between proteases and protease inhibitors in sepsis. Inter-alpha inhibitor protein (IAIP) is a serine protease inhibitors found in relatively high concentrations in human plasma (300-600 μg/mL). It is composed of heavy and light chain polypeptide subunits and the light chain, also called bikunin. The purpose of the present study was to examine the effects of IAIP and bikunin on the function of neutrophils, specifically focusing on their morphology, capillary passage, adhesion on endothelial cells, and ROS production.

Materials and methods

Isolation of neutrophils

Human neutrophils were isolated from peripheral blood obtained from healthy volunteers under a protocol approved by the institutional review board and in accordance with The Declaration of Helsinki. Human polymorphonuclear neutrophils (PMNs) were isolated by density gradient centrifugation over polymorphprep (Axis-Shield, Oslo, Norway).

Neutrophil shape assay

Purified human neutrophils ($5x10^4$ cells/well) pre-stained with Hoechst 33342 (nuclei) and calcein-AM (cytosol) were aliquoted in a volume of 100 μL to a 96-well microtiter plate. The incubation started with HSA (1μM), BSA (1 μM), different concentrations of IAIP or bikunin (25, 50, 100, 200, and 400 μg/mL) for 1 h at 37°C. The cell shape and cell size were analyzed by using an In Cell Analyzer 2000 (GE Healthcare/Life Sciences, Tokyo, Japan). The form factor (maximum diameter/ minimum diameter) and the cell size (area) were determined in each group.

Actin distribution and cell surface structure in neutrophils

The neutrophils were stained for actin after fixation with 4% paraformaldehyde (PFA) and treatment with 0.1% Triton X-100 by staining with phalloidin–Alexa 568 (F-actin), DNaseI–Alexa 488 (G-actin), and DAPI (nuclei). The samples were observed using aconfocal microscope (LSM 780; Carl Zeiss, Oberkochen, Germany). The samples were fixed in 4% paraformaldehyde, then post-fixed using 1% osmium tetroxide for 1 h at 4 °C for scanning electron microscopy.

Binding of IAIP on neutrophil surface

The neutrophils were incubated for 30 minutes at 37 °C with 100 or 200 μg/mL of fluorescein-labeled IAIP in the absence or presence of 400 μg/mL of unlabeled IAIP. After washing and fixed with 0.5 % PFA and applied for fluorescence-activated cell sorter (FACS) analysis.

Micro channel array flow analyzer (MC-FAN)

The isolated human neutrophils were treated with HBSS, IAIP (100 and 200 μg/mL), bikunin (100 μg/mL and 200 μg/mL) or HSA (1 μM) were forced to flow through artificial silicon microchannels. The time required for the passage of a 100 μL of the suspension of neutrophils through the MC-FAN was quantified.

Neutrophil adhesion assay

Neutrophil adhesion was evaluated by counting the residual cell numbers by In Cell Analyzer 2000 (GE Healthcare/Life Sciences, Tokyo, Japan) before and after the wells were gently washed twice with phosphate buffered solution (PBS) to remove any non-adherent cells from EA. hy926 monolayer of vascular endothelial cells.

Determination of ROS production and $p47^{phox}$ immunoblotting

Neutrophil extracellular ROS production was determined by the measurement of isoluminol chemiluminescence intensity using Flexstation 3 (Molecular Devices, Sunnyvale, CA). For detection of p47 phosphorylation in Ser 328 in neutrophils, 50 μg of protein was analyzed with SDS-PAGE and immunoblotted with specific antibodies.

Results

The morphological changes induced by IAIPs as spherical shape changes in concentration- dependent manner which was evaluated by the average cell areas and form factor, smaller in size and loss of irregularity of the shape. Scanning electron microscopic observation confirmed the loss of surface microvilli structures on neutrophils in HRG- and IAIP-treated groups in a concentration-dependent manner. The neutrophils treated with buffer alone, HSA or BSA showed the polymorphic shapes with many microvilli or folds on their surface.

F-actin was dominant in HRG treated and cytosolic G-actin was dominant in IAIP treated neutrophils in cytochemical staining. The spherical shape-inducing effects of IAIP were inhibited by the addition of mouse monoclonal Ab against IAIP, but not by control IgG. These results confirmed the specificity of the IAIP effects on neutrophil morphology. Using fluorescein-labeled IAIPs, we directly detected the binding of IAIPs on neutrophils that was

antagonized by the presence of unlabeled IAIPs, indicating the specificity of the binding. However, we observed that concentrations of bikunin similar to those of IAIPs did not affect the morphology of the neutrophils. There were trapping of irregular shaped neutrophils observed before or on the microcapillary slits of MC-FAN in HBSS, HSA and bikunin groups, but not in the IAIP treated groups. IAIP induced rounding of the neutrophils were also associated with reductions in the adhesion of neutrophils to the plastic well as well as the monolayer of vascular endothelial cells.

The extracellular ROS production was inhibited in a concentration-dependent manner by IAIPs when determined at 15 min after the start of incubation. A significant effect was even detected at 1.5 μg/mL. We performed Western immunoblot analysis of whole-cell lysates from neutrophils and the results showed that serine 328 phosphorylation occurred during the incubation with HBSS or HSA and that IAIPs clearly inhibited $p47^{phox}$ phosphorylation on Ser328 in neutrophils.

IAIPs (100 μg/mL) inhibited the expression of CD 162 on neutrophils both in the presence and absence of three agonists (C5a, IL-8 and fMLP). Treatment of neutrophils with IAIPs (100 μg/mL) inhibited LPS (10 ng/mL)- or calcium inophore (A 23187) (1 μM and 5 μM)-induced NET formation compared with HBSS- and HSA- treated groups. IAIPs (100 μg/mL) significantly inhibited the staining of DNA by Sytox suggesting the loss of integrity of plasma membrane and prevention of spontaneous death of isolated neutrophils.

Discussion

PMNs are the primary line of defense against invading microorganisms and are recruited as the first cells to inflammatory sites. The rheological properties of neutrophils appear to be defective during sepsis and septic shock. IAIP concentrations were decreased in patients with sepsis and the reduced levels predicted mortality rates. In the present study, we

demonstrated that exposure to IAIP induced changes in neutrophils such that the shape was more spherical, the size was smaller and there was a loss of microvilli on the surface of the neutrophils. Moreover, these changes were accentuated by increasing concentrations of IAIPs. The round shape with smooth surface in the presence of IAIPs facilitated the prompt passage of neutrophils through the microcapillary system and prevented neutrophil entrapment in the in vitro capillary system. The effects of IAIPs on the morphological features of the neutrophils and resultant rapid passage through the in vitro capillary system were saturable at 100 μg/mL in the present study. The normal concentration of IAIP in human plasma is 300-600 μg/mL. Therefore, the salutary effects of IAIP could be considerably reduced when endogenous IAIP levels decrease below 100 μg/mL during significant episodes of infection.

IAIP is composed of heavy and light chains and the free light chain is also termed bikunin. The results of our study demonstrated that a wide range concentrations of bikunin did not affect neutrophil morphology or function compared with those induced by similar concentrations of the complex molecules of IAIPs. Therefore, we postulate that heavy chains of IAIPs or heavy chains in combination with the light chain could be responsible for inducing the morphological changes, facilitating the passage through the microcapillary system and suppressing the tenacity of the attachment of neutrophils to the vascular endothelial cells. The exact mechanism by which IAIPs affect neutrophils remains to be determined. However, the beneficial effects of IAIPs on neutrophils could improve systemic and microcirculatory blood flow and, thereby, prevent potential immune-thrombosis during sepsis and septic shock like conditions.

IAIP inhibited extracellular ROS production in neutrophils was dependent upon the concentration of IAIPs and even very low levels of IAIPs were capable of inhibiting the release of ROS. Furthermore, IAIPs inhibited the $p47^{phox}$ phosphorylation on Ser328 among the different sites of phosphorylation on $p47^{phox}$. Consequently, these results suggest that IAIPs

suppressed ROS production under resting conditions in intact neutrophils and $p47^{phox}$ phosphorylation on a specific serine site.

In our current study, coincubation of neutrophils with a confluent inactivated vascular endothelial monolayer showed that IAIPs suppressed the adhesion of neutrophils on the vascular endothelial cells. One possible explanation could be that the spherical shape of neutrophils probably minimized the surface contact area and physical contact between the neutrophils and endothelial cells of the microvasculature. The ability of IAIPs to suppress ROS production could also be beneficial to the protection from endothelial injury. Consequently, the ability of IAIP to inhibit neutrophil adhesion to endothelial cells could represent an important component in the prevention of endothelial injury.

CD 162 (PSGL-1) is the major counterreceptor of P-selectin for neutrophil initial attachment and rolling on activated vascular endothelium. The inhibitory regulation of CD 162 expression by IAIPs demonstrated in the present study suggests that the suppressive effects of IAIP on the interaction between neutrophils and other effector cells attenuates the inflammatory response. NETosis is 1 form of cell death pathway of neutrophils and IAIPs inhibited the NETosis is consistent with the concentration-dependent inhibitory effects of IAIPs on ROS production. Taken together, IAIPs may inhibit immunothrombosis during polymicrobial infections. The inhibitory effects of IAIPs on spontaneous neutrophil death suggests that IAIPs might be an important plasma factor in the regulation of neutrophil survival.

Conclusion

IAIPs, but not bikunin, strongly regulates the human neutrophil shape, facilitating easy and rapid passage through capillaries and inhibiting the adhesion to the vascular endothelial cells. IAIPs also renders neutrophils quiescent by suppressing the release of ROS. These effects observed *in vitro* could represent a component of the beneficial effects of systemically administered IAIPs in vivo in animal models of sepsis.