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The effect of calpain inhibitor on neutrophils spreading ability

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ABSTRACT

Neutrophils spreading are a key event in the development and progression of inflammation, which is signaled by cytokines. In order to undergo inflammation process, neutrophils will migrate towards the site of infection by adhering along the endothelium and undergoing a major morphological transition from a spherical shape to a flattened shape, which is known as neutrophils spreading. Neutrophils transmigrate, whether by migration between the endothelial cells or through an endothelial cell. Calpain are involved in several key aspects of migration, including adhesion and spreading. Neutrophils were isolated from whole blood and observed under the microscope in order to study their spreading behavior. The results of neutrophils spreading were recorded and measured in terms of the cells surface area of spreading after stimulation with chemoattractant. This study showed the inhibition of calpain, which is triggered by Ca^{2+} influx has prevented neutrophils from changing its shapes and spread to its optimal. fMLP induced calpain inhibitor-treated neutrophils demonstrated smaller spreading areas ($116.09\mu m^2 \pm 2.58$) as compared to the positive control cells ($219.88\mu m^2 \pm 3.73$). Therefore, a calpain inhibitor may play an important part in preventing neutrophils from spreading, which could halt the progression of inflammatory disorders.

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INTRODUCTION

A neutrophil is a type of matured white blood cell that circulates the bloodstream. Neutrophils are essential in protection against disease and infections by removing and destroying some types of bacteria and foreign substances through a process called phagocytosis. Once engulfed, the pathogen is killed by the chemicals inside its granules. The function of phagocytic cells is identifying eliminating pathogens that might cause infection (Janeway, 2001). Neutrophils are identified by its three to five-round sections of the nucleus called lobes that are connected by thin threads known as chromatin. Neutrophils are made by the bone marrow and sometimes made outside of the marrow as well (Zakaria *et al.*, 2018). After neutrophils are formed, it is released into the circulating blood. Neutrophils are the first immune cells to arrive at a site of infection, through a process known as cell spreading as well as chemotaxis (Ishak and Hallett, 2018). Neutrophils are short-lived and endure immediate death after ingesting a pathogen. When not activated, the cells has a half-life of between four to ten hours. Neutrophils are abundant and responsible for the main part of an immune response. The cells circulates the bloodstream until signaled to a site of infection through chemical cues in the body. Neutrophils are activated and recruited from the

circulating blood due to the presence of chemical signals called chemoattractant. Chemoattractant is released from tissue injuries and bacterial infections at the sites of inflammation, and it produced a chemical gradient for neutrophils to migrate to the site of infection. Neutrophils are fast-acting and arrive at the site of infection within one hour (Tarmizi *et al.*, 2018). Neutrophil has the capability to change its morphology during adhesion and phagocytosis (Dewitt and Hallett, 2007). Neutrophil spreading is the key factor in determining the rate of neutrophil extravasation. After rolling along the endothelium, neutrophils flatten onto the endothelial surface at the sites of inflammation.

Over-activation of neutrophils can result in autoimmune conditions such as rheumatoid arthritis (Ishak, 2012). Calpains are Ca^{2+}-dependent cysteine proteases that function as regulatory enzymes and have been associated with several major cellular functions, including exocytosis, cell differentiation, cellular fusion, signal transduction, and apoptosis. The roles of calpain have been reported in studies involving cell motility, cell spreading, fibroblast cells, and myoblast fusions of skeletal muscles. To overcome this, the treatment of these cells with calpain inhibitors could reduce spreading. The influx of immune cells into inflammation sites could be decelerated or inhibited by targeting μ-calpain. Inhibition of calpain 2 has reduced the influx of inflammatory cells in the animal model of experimentally induced inflammation and extravasation (Dewitt and Hallett, 2002). Animal models of collagen-induced arthritis are potently inhibited by calpain inhibition (Yoshifuji *et al.*, 2005), and in an experimental model of rheumatoid arthritis induced by antitype collagen mAb, calpain inhibition using the calpain inhibitor, E64d, produce a dramatic reduction in joint inflammation (Chatterjee *et al.*, 2005). Therefore, autoimmune conditions could be controlled by studying the function and behaviors of neutrophils. Thus, this work describes the effects of calpain inhibitor on neutrophils spreading ability and its potential for anti-inflammatory therapy.

MATERIALS AND METHODS

Preparation of reagents and chemical solutions

Hepes Buffered Krebs (HBK) was prepared by adding NaCl (107g/L), $NaHCO_3$ (32.26g/L), KCl (5.96g/L), $MgCl_2$ (3.25g/L), $CaCl_2$ (5.17g/L), and 10mM HEPES (1.9g/L) in 650ml distilled water. 5L of Balance Salt Solution (BSS) was made by dissolving NaCI (40g), KCI (1g), Na_2HPO_4 (5.75g), and KH_2PO_4 (1g) in double-distilled water, and the pH was adjusted to 7.4 with NaOH. fMLP (formyl-Methionyl-Leucyl-Phenylalanine) was weighed (1mg) and dissolved in dry DMSO (2.3ml) to make a 1mM stock solution. Dextran Solution (6%) was prepared by adding 15g Dextran and 2.25g NaCI into 250ml distilled water.

Blood collection

Blood was obtained from healthy adult volunteers and collected with the informed consent of participants. About 10 ml of blood was withdrawn using a butterfly needle in order to ensure that neutrophils are less or not activated during the collection process. The blood collected was transferred into a universal container with 10μl of heparin to prevent coagulation.

Isolation of neutrophil from whole blood

Neutrophils were isolated from the heparinized blood of healthy volunteers according to the methodology described by (Hallett *et al.*, 1990). About 10ml of heparinised blood was mixed with 6% Dextran and allowed to sediment at 37ºC for 30 to 45 minutes. After sedimentation, the layer containing white cells and plasma were taken and centrifuged at 2000rpm for 60 seconds. Then, the erythrocytes residue was removed by hypotonic lysis. In this step, the cells will be resuspended in double-distilled water for 10 seconds. The osmolarity of neutrophils was restored by adding 25ml BSS (pH 7.4) and then centrifuged at 2000rpm for 60 seconds. After centrifugation, the supernatant was removed, and the pellet containing the neutrophils was resuspended in 1ml of HBK solution. The isolated neutrophils were kept on ice until further use.

Incubation of neutrophils with calpain inhibitor

The calpain inhibitor was purchased from Santa Cruz Biotechnology Inc. The ALLN calpain inhibitor used in this study is an inhibitor of calpain 1 and 2. It inhibits neutral cysteine proteases and proteasome. The cell-permeable calpain inhibitor was introduced to the isolated neutrophils and labelled as a treated group. 1μl of the calpain inhibitor (25mg/ml) was added to the isolated neutrophils and incubated at 37ºC for about 30 minutes. Isolated neutrophils for the positive and negative groups were also incubated at the same temperature and duration, respectively.

Neutrophils spreading and data collection

The spreading capability of neutrophils was performed in a six wells multi-dish (Thermo Fisher Scientific) with a glass bottom. Neutrophil spreading was determined by recording the changes in cells shapes and morphology under the microscope.

HoloMonitor^{TM} M3 microscope was used to record neutrophils spreading in real-time. 100μl isolated neutrophils (1x10^6) were loaded in the six wells multi-dish and allowed to adhere for about 3 minutes onto the glass bottom. Then, 10μl of 1mM fMLP (diluted in DMSO) was added to the cells. The surface area of neutrophils spreading was calculated and analyzed using ImageJ software based on the continuous still images of the cells. The surface area size was calculated according to the area of circle formula,

$$Neutrophils\ area\ of\ circular = \pi r^2$$

*Where π = 3.142, and 'r' is the radius of neutrophils

Statistical analysis

The data is expressed as mean $\pm$ S.E.M and analyzed using One-way ANOVA single factor and Student's t-test. P <0.05 is considered as significantly different.

RESULTS AND DISCUSSION

Neutrophils spreading area

Neutrophils spreading area was determined by measuring the surface area of the cells when it changes shape and becomes flat. The isolated neutrophils were divided into three different groups, which includes a calpain inhibitor-treated group, positive control, and negative control. Each group was represented by 100 randomly selected neutrophils and the results for the spreading area were expressed as mean$\pm$S.E.M. The area of cells spreading for the negative control group was measured after neutrophils have attached onto the glass bottom multi-dish, whereas the measurement for the positive control and calpain inhibitor-treated group were performed after the introduction of fMLP to the attached neutrophils.

The negative control group recorded the smallest spreading area of 73.34μm$^2\pm$0.62 as compared to the fMLP induced calpain inhibitor-treated group (116.09μm$^2\pm$2.58) and a positive control group (219.88μm$^2\pm$3.73) (Figure 1). Adding fMLP caused neutrophils to change its cellular morphology and undergo spreading with a visible multi-lobed nucleus. eutrophils spreading area was calculated by measuring the length of the X-axis and Y-axis (Figure 2 a-c). In general, most neutrophils in the negative control group showed similar morphology with diameter measurement in the range of 8μm to 9μm. The large surface area was visible for neutrophils in the positive control group after the introduction of fMLP. The lipid-soluble calpain inhibitor was incubated with neutrophils and allowed to permeate in order to determine its effect. fMLP was added after the neutrophils treated with calpain inhibitor has attached onto the glass bottom multi-dish. Evidently, the surface areas of calpain inhibitor-treated neutrophils spreading were smaller as compared to the positive control group with the longer time required to complete spreading.

The ability to spread on endothelial cell wall is one of neutrophils main physiological characteristics. The presence of chemoattractant during inflammation triggers neutrophils to undergo a major morphological transition from its spherical shape to flatten cells and eventually leaving the blood vessels towards the target site. fMLP induces degranulation and adhesion (Stevenson *et al.*, 2004), and is known to induce calcium flux and chemotaxis (Sogawa *et al.*, 2011). Neutrophils produce adhesive molecules that allow the cells to attach and spread on a glass surface (Bertram *et al.*, 2012). In this study, the modified glass-bottom multi-dish was used to substitute the role of the endothelial cell wall. The surface area of cells refers to the breadth of neutrophils morphological changes that spreads on the glass bottom multi-dish.

Neutrophils, in its original form, are circular-spherical cells, and if the cells are not stimulated, it appears to be in a spherical shape (Selz, 2011). Without the presence of chemoattractant, negative control neutrophils still retain its spherical shape and loosely attached onto the glass surface. Unstimulated circulating neutrophils are round cells of about 8μm to 9μm in diameter, whereas spreading neutrophils have bigger cells surface area as compared to cells that did not spread. The spreading ability of calpain inhibited neutrophils appeared to be disturbed but was not totally lost after the cells were exposed to fMLP. By contrast, the positive control neutrophils have generally lost their spherical form and complete the spreading process after the introduction of fMLP. The surface area of the spread neutrophils can be clearly measured along the X- and Y-axis. When neutrophils flatten out onto the endothelial cells, the morphological transformation of neutrophils was dramatic (Dewitt and Hallett, 2007). The surface area of the cells that increases during flattening onto the endothelium was more than 100% from its original surface area.

Doubling of surface area means that neutrophils have numerous surface wrinkles and folds, and the surface area of neutrophils could stretch greatly in excess from a sphere with the same diameter (Ishak, 2012). These could explain about how neutrophils are able to spread with its minimum volume. Literally, the membrane expansion of neutrophils occurred without the insertion of the new

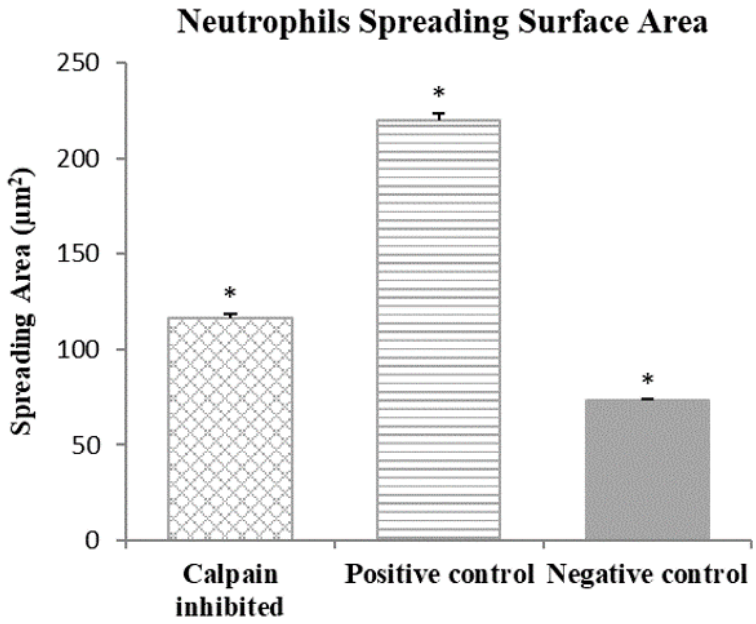

Figure 1: Average spreading areas of neutrophils after fMLP stimulation. The data is expressed as mean ± S.E.M. (*p<0.05).

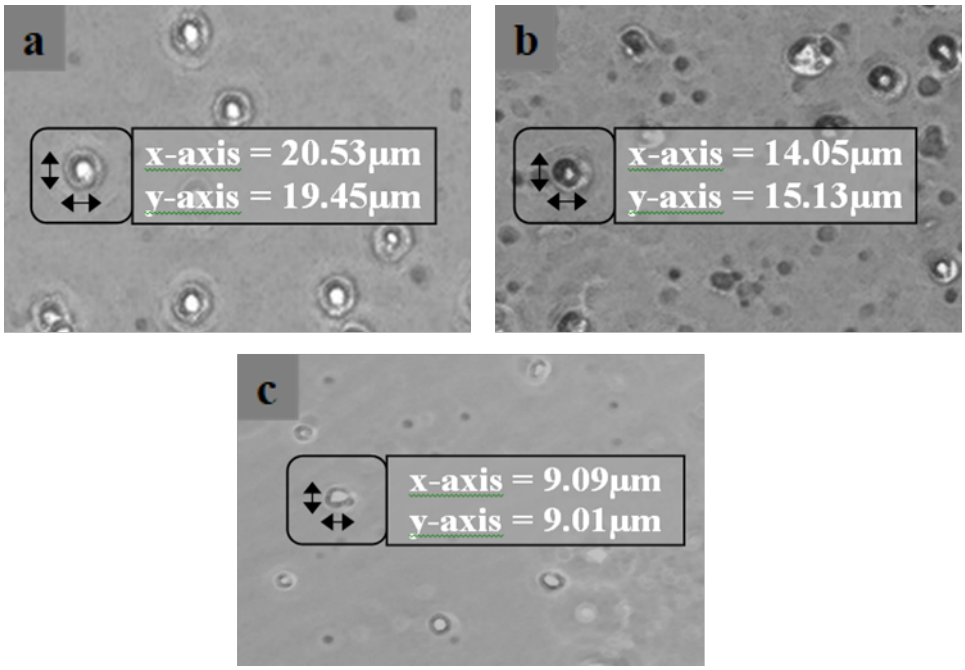

Figure 2: The size of neutrophils for (a) positive control group, and (b) calpain inhibitor-treated group after fMLP stimulation. (c) The negative control group without chemoattractant.

membrane but resulted from the wrinkled plasma membrane.

Houk *et al.* (2012) reported that the characteristic of the plasma membrane, the cell spreading, and membrane expansion depended on the activation of the Ca^{2+} activated protease, calpain, whereas the cytosolic IP_3 was an initiator of Ca^{2+}, which stimulates the neutrophils flattening process. IP_3 was generated by the activation of phospholipase C (PLC) and was also involved in neutrophils spreading as inhibition of PLC activity showed an inhibitory effect on the cells (Dewitt *et al.*, 2013). Ca^{2+} influx is generated physiologically for rapid neutrophil spreading (Franco *et al.*, 2004). In other cell types, there is growing evidence for a role of calpain-2 in focal adhesion complexes and slow motility. This indicated that activation of Ca^{2+} influx and calpain stimulates the morphological transitions during neutrophils spreading. Activation and regula-

tion of calpain have been identified for calcium and phospholipid binding, autolysis, phosphorylation, and inhibition by calpain inhibitor (Franco, 2005). In addition, calpain inhibition prevents full extension of pseudopodia during phagocytosis by neutrophils and spreading by lymphocytes (Hillson and Hallett, 2007). There has been significant evidence that calpain inhibitors are effective in preventing neutrophils extravasation in experimental inflammation in animal models (Tissier *et al.*, 2004).

CONCLUSIONS

In conclusion, the chances of halting inflammatory reaction could be attained by manipulating neutrophils behaviours. Calpain is one of the potential target enzymes involved in reorganizing the cytoskeleton system in neutrophils and other cells. The use of a calpain inhibitor has disturbed neutrophils ability to spread by reducing its cellular surface area, and this may consequently stop the cells from migrating to the inflammation site. However, limited capabilities of neutrophils to change its shape and spread suggested that the cells could have an alternative mechanism for the signaling pathway or the involvement of other enzymes to offset the effect. Over-activation of neutrophils often results in inflammatory disorders such as rheumatoid arthritis, and treatment with calpain inhibitor may prove to be beneficial to fight this condition. This finding offers a valuable understanding on calpain involvement in neutrophils behavior and has great potential to be used for anti-inflammatory therapy.

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