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Short Communication

The role of calpain enzyme on neutrophils chemotaxis ability

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ABSTRACT

Neutrophils are highly specialized cells that form the first line of defense against foreign substances. One of the major functions of neutrophils is its ability to undergo chemotaxis following the chemoattractant gradients towards the inflammation sites. The aim of this study is to determine the role of the enzyme calpain on neutrophils ability to perform chemotaxis. This experiment was performed using Dunn Chemotaxis Chamber and ibidi® Chemotaxis and Migration Tool software. The results showed that calpain enzyme inhibitor was able to reduce the distance of neutrophils migration towards the chemoattractant (*N*-formyl-methionyl-leucyl-phenylalanine), and thus disrupting the cells ability to undergo chemotaxis. The results indicated that calpain has a role in neutrophils chemotaxis ability. Henceforward, studying neutrophils taken from the blood of patients with inflammatory disorder could help to further understanding the application of calpain enzyme inhibitor on neutrophils cellular functions.

1. INTRODUCTION

Neutrophils are one of the major cell types involves in the series of defense mechanisms and act as the first line of defense against pathogens. During an inflammatory response, neutrophils moves out from the blood circulation and migrate towards the site of infection. Neutrophils move towards the inflammatory sites by responding to the chemoattractants released from cells at the site of infection or from foreign microorganism[1]. Neutrophils begin to stick to the endothelium of blood vessel and begin to squeeze through the wall of blood vessel to reach the damaged area. Adhesin molecules (selectins and integrins) were implicated in the emigration of neutrophils from bloodstream into the interstitial fluid[2].

Calpain enzyme naturally exists in neutrophils and involves in shape changes that helps neutrophils to spread out in order to squeeze between the endothelium cells toward infection sites[3]. Calpain family consists of several calcium ions dependent cysteine proteases. The calpain enzyme is a proteolytic enzyme which is regulated by the concentration of calcium ions involves in remodeling the protein structures needed for cell movement[4]. Calpain enzyme inhibitor acts on the adhesin molecules and prevents calpain-2 from functioning even in the present of calcium. Consequently, this will disable neutrophils to change its normal spherical shape form in order to squeeze through the endothelium cells[5].

Excessive activation of neutrophils by immune complexes in the autoimmune disease is one of the mechanisms that cause damaged to the joints such as rheumatoid arthritis[6]. Patients suffering from autoimmune diseases fail to display immune system tolerance and as a result attack the person's own tissues. This study demonstrates the effect of inserting calpain enzyme inhibitor to isolated neutrophils and how it could disrupts chemotaxis ability.

2. MATERIALS AND METHODS

2.1. Neutrophils isolation from whole blood

Neutrophils were isolated from heparinized blood of healthy adult volunteers. The procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee following the rules of the Declaration of Helsinki 1975. All volunteers gave their informed consent for inclusion before they participated in this study. About 20 ml of heparinized blood was mixed with 5 ml Dextran (6% Balanced Salt Solution (BSS)) and allowed to sediment for 30-45 min at room temperature in order to separate the red blood cells (RBC) and white blood cells (WBC). After sedimentation, the middle layer containing white blood cells and plasma were carefully removed and transferred into clean tubes. The tubes were then centrifuged with Ficoll-Paque at 2000 rpm for 30 sec in order to isolate neutrophils from the blood samples. Excess RBCs were removed through hypotonic lysis with distilled water (dH_2O) and the osmolarity of the cells were restored after 10 sec by diluting it with 25 ml BSS. The supernatant was then discarded and the pellets containing isolated neutrophils ($2x10^5$) were carefully transferred by re-suspending it in Hepes Buffered Krebs (HBK) solution for the experimental procedure.

The isolated neutrophils were divided into three groups namely; the positive control, the calpain inhibited, and the negative control group. The positive control group was introduced to chemoattractant only, whereas the calpain inhibited group was introduced to chemoattractant and incubated with calpain enzyme inhibitor. The negative control group was not introduced to any chemoattractant or inhibitor.

2.2. Neutrophils incubation with calpain enzyme inhibitor

Calpain enzyme inhibitor ALLN is a cell-permeable molecule. 10 µl of 1 mM calpain ALLN inhibitor is incubated with 1 ml of isolated neutrophils for 20-30 min at room temperature. The incubation process allows the lipid soluble calpain enzyme inhibitor to pass through the lipid bilayer of neutrophils and inhibit the calpain enzyme[7]. The cells were kept on ice until further use.

2.3. Cell-tracking using Dunn chemotaxis chamber and analysis

Dunn Chemotaxis Chamber was set-up by filling both of the annular wells (inner well and outer well) with control medium and chemoattractant (1 mM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)). In this set-up only

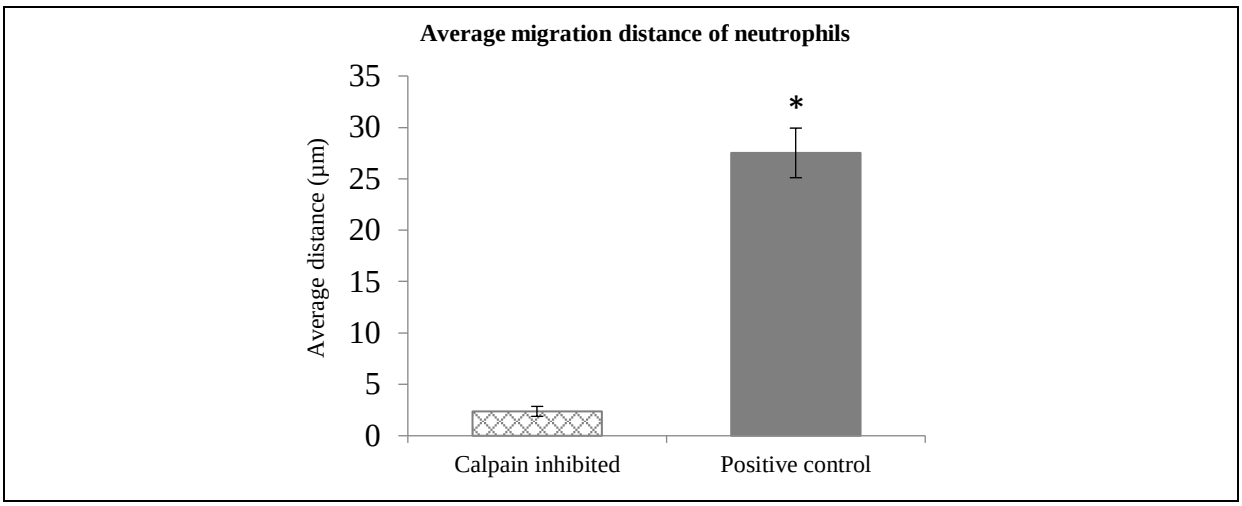

Figure 1. Average distance of isolated neutrophils migration (µm) from three different groups. The data is expressed as mean±S.E.M. (*, $p<0.05$).

neutrophils would be able to migrate towards the chemotactic gradient created by fMLP. Isolated neutrophils were seeded on a clean coverslip and the cells were left to attach on the surface[8]. The seeded coverslip was inverted onto the chamber in a counterbalance position. To create chemotactic gradient, the medium was drained from the outer well using a syringe with fine-bore needle and replaced with medium containing the chemoattractant. The chamber was placed on a temperature-controlled microscope stage for time-lapse recording of the cells migration towards the fMLP chemotactic gradient. The blind inner well of this chemotaxis chamber help to ensure the stability of the gradient. Chemotaxis was evaluated using ImageJ software and ibidi® Chemotaxis and Migration Tool software for plotting and analyzing the tracked cells.

3. RESULTS

Isolated neutrophils from the positive control group (stimulated by 1 mM fMLP) showed the highest average distance (27.52 µm ± 0.49) during migration as compared to the calpain inhibited neutrophils (2.37 µm ± 2.42) (Figure 1). No cells migration was recorded with the negative control group (no fMLP stimulation).

By comparing the migration plot, the positive control group demonstrated a more diverse distribution than other groups. Various directed movements of neutrophils from the center of axis indicated that the cells in positive control group have migrated towards fMLP (Figure 2a). The cross at the center of the plot represents the Center of Mass (COM) which illustrates the movement of cells from their starting point towards fMLP based on the recorded time.

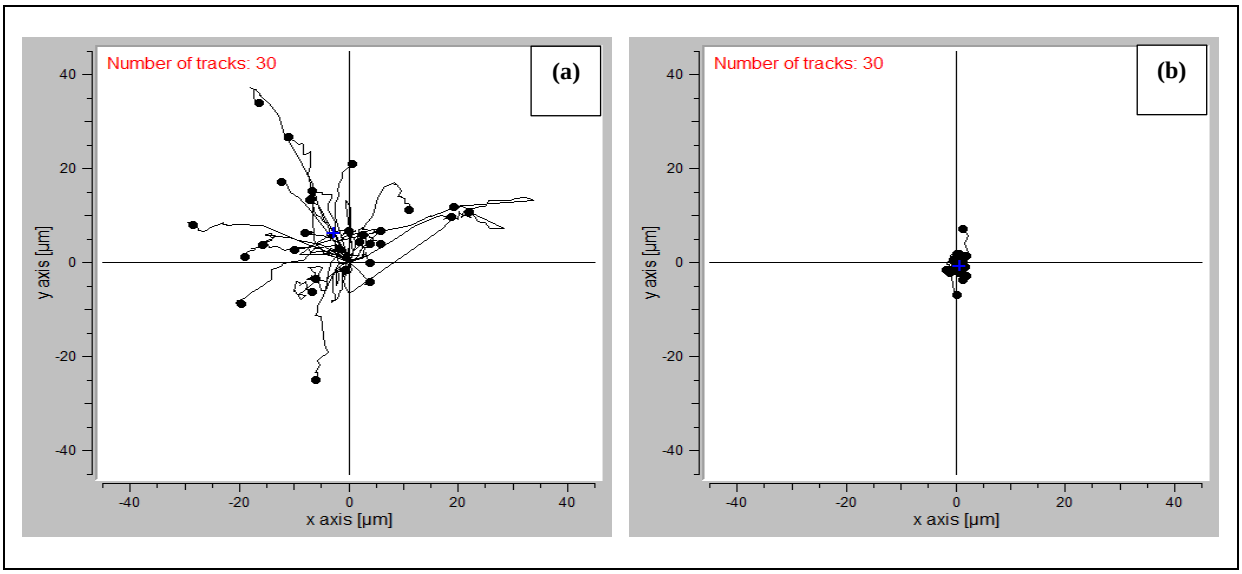

Figure 2. Migration plot representing 30 neutrophils of (a) positive control group and (b) calpain inhibited group using ibidi® chemotaxis and migration tool software.

A total of 30 individual neutrophils in the calpain inhibited group were observed and 17 neutrophils showed directed movement from the COM (Figure 2b), however, it appears that the cells movement towards fMLP was restricted or disrupted. The average accumulated distance for the positive control group is 27.52 µm with the velocity of 0.026 µm/sec (Table 1). The most active cell was able to travel towards fMLP with a distance of 59.46 µm whereas the shortest distance recorded was 6.80 µm. The average accumulated distance for the calpain (Table 1) inhibited group recorded a lower number. The farthest migrating calpain inhibited cell moved towards fMLP with a distance of 10.26 µm and averaging at 2.37 µm with a velocity of 0.003 µm/sec (Table 2).

Table 1. The measured distance and velocity of the positive control group.

	Minimum	Maximum	Average
Distance (µm)	6.80	59.46	27.52
Velocity (µm/sec)	0.007	0.058	0.026

Table 2. The measured distance and velocity of the calpain inhibited group.

	Minimum	Maximum	Average
Distance (µm)	0	10.26	2.37
Velocity (µm/sec)	0	0.010	0.003

The three colored lines represent neutrophils movement from its starting position towards the chemoattractant which is located on the right side of the image (Figure 3). The cells tracking showed longer lines traces for positive control group (Figure 3a) which suggests farther distance travelled. However, the migration ability of calpain inhibited group (Figure 3b) appeared to be limited and presented a shorter line traces.

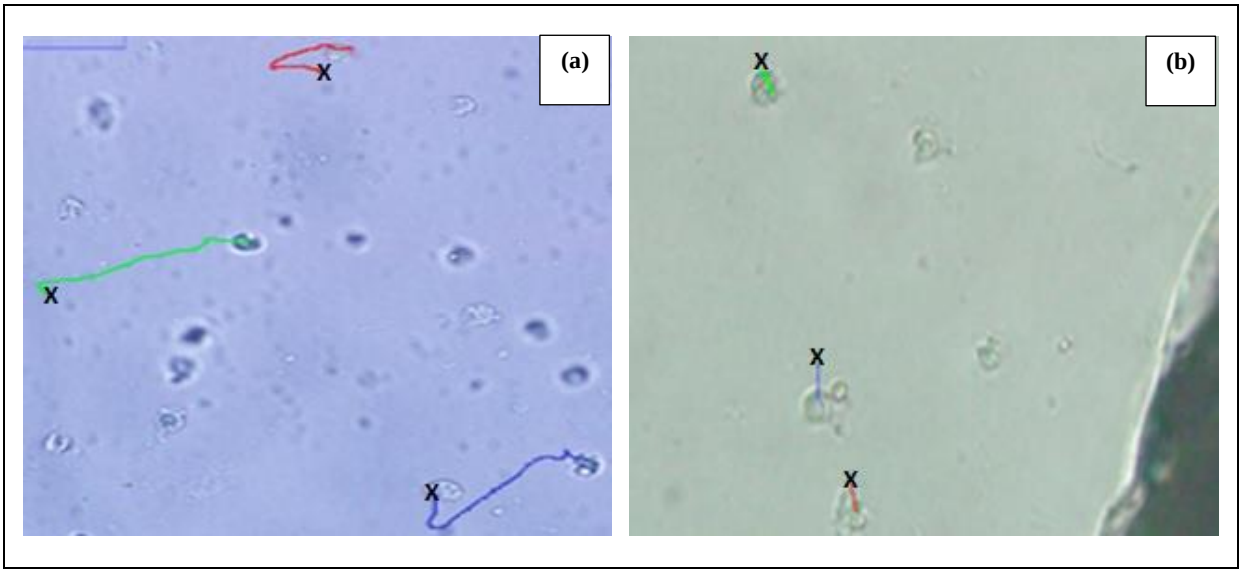

Figure 3. Neutrophils migration tracked using ImageJ software for (a) positive control group and (b) calpain inhibited group. "X" marks the starting points of neutrophils migration.

4. DISCUSSION

In normal physiological condition, neutrophils are triggered by chemoattractant released by inflammatory reaction and leave the blood vessel towards the inflammation site. The chemotatic stimuli include interleukin-8 and kinin which are the specialized products of damages tissues[9]. This phenomenon is called chemotaxis. In this study, fMLP was used as the chemoattractant whereas calpain enzyme inhibitor was applied in order to inhibit or reduce neutrophils chemotaxis ability. The calpain inhibited neutrophils showed a lower average accumulated distance as compared to normal neutrophils (positive control group), thus indicating disruption on their directed migration ability. The negative control group on the other hand showed no cells movement altogether. By inhibiting calpain activities, neutrophil chemotaxis and direction of these cells towards the chemoattractant (fMLP) were reduced and at the same time promotes random migration of neutrophils that leads to decreasing directional migration[10].

Based on the migration plot, the positive control group showed a more diverse migration than the other groups. This distribution of cells tracks indicates that the cells were able to move further away from its starting points towards the chemoattractant, each with different cellular behaviors and movements. Calpain naturally exists in neutrophils and acts as the regulator for actin cytoskeleton which allows neutrophils shape changes and cellular migration[12]. Calpain enzyme may have a role in neutrophils chemotaxis ability based on the decreased distance recorded by the calpain inhibited group. After the introduction of fMLP, only some of the calpain inhibited neutrophils were able to travel towards the chemoattractant. This appeared to have contradictory effects for the positive control group as the normal cells still have their sense of direction towards fMLP intact. There were also cells that demonstrated chemokinesis or backward-

forward movement. It is believed that this phenomenon showed cells with different behaviors and movements. The inhibition of calpain-2 enzyme in neutrophils leads to membrane protrusion and rapid chemokinesis13. Passive mechanical properties of neutrophils behavior that plays an important role in both the microcirculation and the immune system have shown that every cell even in the same groups has their own abilities and behavior towards certain processes14. The inhibition of calpain-activation prevents neutrophils to flatten normally15. In 'resting' state, neutrophils are in spherical form and the cells will spread out of its normal spherical shape before undergoing chemotaxis.

Chemotaxis involves two elements which are cells migration and gradient sensing. Cell migration is essentially a mechanical process that steadies the actin polymerization at the leading edge of the cell and provides the force required to push the cell forwards towards the chemotactic stimuli[16]. Polarization causes neutrophil shape changes (also termed elongation), creating a leading edge or lamellipodium and a distinct rear or also known as trailing end or uropod. The lamellipodium is characterized by the constant generation of a meshwork of F-actin, which pushes the cell forward[17]. The uropod represents the sites where old sites of neutrophil attachment to the substrate are being dissolved and allows net translocation of the cell by actomyosin contractility. Both of them able to activate neutrophils polarized morphology and is similar to the pseudopod extrusion towards the chemoattractant area.

This study indicated that the inhibition of calpain enzyme has reduced neutrophil's directional senses towards chemoattractant. This cellular behavior may have a consequential effect on the changes in calcium activities for adhesion and elongation of neutrophils. In normal physiological condition, calcium was released from their storage in the cell when triggered by the stimulation of chemoattractant. Therefore, inhibition of neutrophils chemotaxis ability could have distorted the calcium activities needed for cell migration towards the chemotactic stimuli.

5. CONCLUSIONS

Chemotaxis is one of the key mechanisms in inflammatory reaction. In order to reduce inflammation, it is important to understand how to control these spontaneous inflammatory reactions by knowing the elements or substances involved. In conclusion, inhibiting calpain enzyme has the possibility to potentially reduce neutrophils migration towards the inflammatory sites. It would be definitely fascinating to study the effects of calpain inhibitor on neutrophils isolated from patients with inflammatory disorder such as rheumatoid arthritis. This could perhaps give a deeper understanding about the role of calpain-1 and -2 enzymes on neutrophils chemotaxis ability.

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Conflict of interest

The authors declare that there are no conflicts of interests.

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Ethical approval

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REFERENCES

1. Franco SJ. Regulating cell migration: calpains make the cut. Journal of Cell Science. 2005;118(17):3829-38.
2. Gambardella L, Vermeren S. Molecular players in neutrophil chemotaxis-focus on PI3K and small GTPases. Journal of Leukocyte Biology. 2013;94(4):603-12.
3. Dewitt S, Francis RJ, Hallett MB. Ca2 and calpain control membrane expansion during the rapid cell spreading of neutrophils. Journal of Cell Science. 2013;126(20):4627-35.
4. Chodniewicz D, Alteraifi AM, Zhelev DV. Experimental Evidence for the Limiting Role of Enzymatic Reactions in Chemoattractant-induced Pseudopod Extension in Human Neutrophils. Journal of Biological Chemistry. 2004;279(23):24460-6.
5. Hanna RA, Campbell RL, Davies PL. Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. Nature. 2008;456(7220):409-12.
6. Dewitt S, Hallett M. Leukocyte membrane "expansion": a central mechanism for leukocyte extravasation. Journal of Leukocyte Biology. 2007;81(5):1160-4.
7. Katsube M, Kato T, Kitagawa M, Noma H, Fujita H, Kitagawa S. Calpain-mediated regulation of the distinct signaling pathways and cell migration in human neutrophils. Journal of Leukocyte Biology. 2008;84(1):255-63.
8. Lokuta MA, Nuzzi PA, Huttenlocher A. Calpain regulates neutrophil chemotaxis. Proceedings of the National Academy of Sciences. 2003;100(7):4006-11.

9. Chou JS, Impens F, Gevaert K, Davies PL. m-Calpain activation in vitro does not require autolysis or subunit dissociation. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics. 2011;1814(7):864-72.
10. Nuzzi PA, Senetar MA, Huttenlocher A. Asymmetric Localization of Calpain 2 during Neutrophil Chemotaxis. Molecular Biology of the Cell. 2007; 18(3):795-805.
11. Ishak R, Hallett MB. Defective rapid cell shape and transendothelial migration by calpain-1 null neutrophils. Biochemical and Biophysical Research Communications. 2018;506(4):1065-70.
12. Ortega-Gómez A, Perretti M, Soehnlein O. Resolution of inflammation: an integrated view. EMBO Molecular Medicine. 2013;5(5):661-74.
13. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: Is immunity the second function of chromatin? The Journal of Cell Biology. 2012;198(5):773-83.
14. Cicchetti G, Allen PG, Glogauer M. Chemotactic Signaling Pathways in Neutrophils: from Receptor to Actin Assembly. Critical Reviews in Oral Biology & Medicine. 2002;13(3):220-8.
15. Tarmizi NN, Dawam NL, Jais MR, Ismail EN, Ishak R. Role of Janus Kinase 1 (JAK1) on neutrophils spreading and directed migration ability. International Journal of Research in Pharmaceutical Sciences. 2018;9(4):1154-61.
16. Campbell JS, Hallett MB. Active calpain in phagocytically competent human neutrophils: Electroinjection of fluorogenic calpain substrate. Biochemical and Biophysical Research Communications. 2015;457(3):341-6.
17. Ishak R. Calpain-1: Investigating its role in murine neutrophils (Doctoral dissertation, Cardiff University) 2012.