Mutations in \textit{MEFV} cause alterations in neutrophil F-actin and phagocytosis dynamics

T. K. Davtyan, S. A. Avetisyan, G. S. Hakobyan

«Armenicum» Research Centre, CJSC Armenicum
37, Nalbandyan Str., Yerevan, Republic of Armenia, 0001

1Yerevan State Medical University named after Mkhitar Heratsi, Ministry of Education and Science of Republic of Armenia
2, Koryun Str., Yerevan, Republic of Armenia, 0025
tigdav@excite.com

\textbf{Aim.} To examine neutrophil F-actin, phagocytosis and macropinocytosis dynamics in patients with Familial Mediterranean Fever (FMF), in an effort to understand the mechanisms that regulate switch of neutrophil activation program.

\textbf{Methods.} Whole blood neutrophils obtained from 37 attack-free FMF patients and 20 normal donors (ND) were activated with N-formyl-Met-Leu-Phe (fMLP), phorbolmyristate acetate (PMA) or lipopolysaccharide (LPS) and cellular F-actin content, phagocytosis and macropinocytosis determined by flow cytometry. F-actin oscillation amplitude and period were calculated from the curves generated by mathematical simulation giving the assumption that in neutrophil F-actin oscillates about a fixed point in a harmonic motion.

\textbf{Results.} Unstimulated neutrophil F-actin content was markedly increased in FMF patients. fMLP- but not PMA- or LPS-stimulated and Col-pretreated neutrophils were characterized by different pattern of F-actin dynamics and delayed time period of F-actin oscillation during FMF. Neutrophils from FMF patients failed to induce chemoattractant receptor desensitization during repeated action of fMLP, while in ND it occurred with significant reduction of F-actin oscillation amplitude and period. In FMF patients we observed significant enhancement of phagocytosis but not macropinocytosis amplitude and frequency.

\textbf{Conclusions.} Impaired neutrophil F-actin, phagocytosis and macropinocytosis oscillations amplitude and frequency that tightly regulate switch of neutrophil activation program during its encounter with increasing concentration of chemoattractants may be a potential pathogenic mechanism causing aberrant resolution of inflammation during FMF.

Keywords: familial Mediterranean fever, neutrophil, F-actin, phagocytosis, macropinocytosis, N-formyl-Met-Leu-Phe, phorbolmyristate acetate.

\textbf{Introduction.} The discovery of mutations in the \textit{MEFV} (for MEditerranean FeVer) locus has changed considerably the understanding of the most common auto-inflammatory disease Familial Mediterranean Fever (FMF). This gene encodes a protein called pyrin, expressed primarily on the innate immune system cells, including neutrophils, and cytokine-activated monocytes [1]. While it is known that pyrin interacts with five cellular proteins: PSTPIP1 [2], 14-3-3 [3], Caspase-1 [4], ASC [5], and Siva [6], the exact function of pyrin in cell and the mechanism underlying the pathological effect of pyrin mutations are yet to be revealed. In transfected cells pyrin was observed to co-localize with actin in lamellar structures and in supranuclear ruffles, but not with stress fibers [7].

Since, actin plays a central role in biological motility as an essential constituent of cytoskeleton and a partner of intracellular signaling pathways associated with chemoattractant-receptor activation, here we investigated neutrophil F-actin, phagocytosis and macropinocytosis dynamics during neutrophil chemoattractant-dependent activation in FMF patients carrying homozygous or compound heterozygous mutations in the \textit{MEFV} locus.

\textbf{Materials and methods.} Peripheral blood samples were obtained from 37 attack-free FMF patients, diag-
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nosed according to the Tel-Hasomer criteria [8] and 20 sex- and age-matched normal donors (ND). MEFV mutations in exon 10 were identified in all patients (18 patients were homozygous for the M694V mutation, 19 remaining patients were compound heterozygous for the M694V and one of the V726A, M680l, E148Q, R761H and F479L mutations). The quantitative flow cytometry determination of intracellular F-actin content was performed [9]. Whole blood samples were incubated with 20 ng/ml phorbol-12-myristate-13-acetate (PMA) or 10–7 M N-formyl-Met-Leu-Phe (fMLP) or 1 µg/ml lipopolysaccharide (LPS) from Escherichia coli 026:B6 or 0.1–10 µg/ml colchicine (Col) for 1–10 min. Phagocytosis assay was performed using latex beads and dextran-FITC. Oscillation time-response curves were generated from calculated mean values ± SD data for F-actin content using the equation (1):

\[ Y = B + A \cdot \sin(F \cdot t + P), \]  

where \( Y \) represents a relative F-actin content, expressed as a mean channel number – MCN; \( B \) is a base line; \( A \) is an amplitude; \( F \) is a frequency and \( P \) is a phase shift of oscillation and \( t \) is time in min. \( A \) and \( F \) values were calculated using Graph Pad Prism v4.01 software and periods of oscillations (\( T \)) were calculated by equation (2) giving assumption that F-actin oscillates about a fixed point in a harmonic motion [10]:

\[ T = \frac{2\pi}{F}. \]

**Results and discussion.** Unstimulated neutrophils F-actin content in FMF patients was significantly higher (\( P_w < 0.01 \)) than in ND (Fig. 1, A). Pretreatment of whole blood with 10 µg/ml Col for 2 h increased neutrophil F-actin content in both FMF (\( P_w < 0.007 \)) and ND (\( P_t < 0.0001 \)). However, Col-pretreated neutrophils F-actin content in FMF patients was found to be significantly lower (\( P_w < 0.02 \)) than in ND (Fig. 1, A). The existence of several shifted maximums in F-actin dynamics of ND and FMF patient’s activated neutrophils led us to test if this is due to different pattern of actin cytoskeleton polymerization and depolymerization regular cycles. fMLP-induced neutrophil F-actin oscillation showed differences in baseline, but not in amplitude and frequency of F-actin oscillations (Fig. 1, B, C). The period of fMLP-induced neutrophil F-actin oscillation, for FMF patients was 4.08 ± 0.46 min and
4.09 ± 0.18 min for ND. As we expected, the period of F-actin oscillation in Col-pretreated neutrophil, activated by fMLP increased to 1.31 min in FMF patients (5.39 ± 0.13) and to 1.1 min in ND (5.19 ± 0.21). Thus, fMLP-stimulated and Col-pretreated neutrophils were characterized by different pattern of F-actin dynamics and delayed shift of maximums during FMF.

We found that fMLP-, LPS- or PMA-stimulated neutrophil F-actin dynamics in FMF patients is characterized by oscillations with different amplitude and periods, whereas in ND’s activated neutrophil F-actin dynamics has an undulating shape in the presence of fMLP only. Next, we analyzed how delayed F-actin oscillation period in FMF could contribute to altered F-actin dynamics during repeated fMLP action. We incubated ND’s and FMF patient’s whole blood neutrophils with fMLP for 10 min, then added equal amount of fMLP for 1–10 min and assayed the cellular F-actin content. We observed that repeated action of fMLP in ND (Fig. 2, A) induced significant reduction in both overall F-actin content and cellular F-actin oscillation amplitude (1751 ± 166 in single dose fMLP treated cells versus 725 ± 120 MCN in double fMLP treated cells, P = 0.03). In contrast to ND, FMF patients’ neutrophils failed to induce reduced F-actin dynamics during repeated action of fMLP (Fig. 2, B). Indeed, neither F-actin oscillation amplitude (1298 ± 104 in single dose fMLP treated cells versus 1442 ± 93 MCN in double fMLP treated cells) nor oscillation period (4.64 ± 0.09 min versus 4.66 ± 0.09 min, respectively) were changed in FMF patient’s neutrophils in the presence of single or double doses of fMLP.

The period of unstimulated neutrophil phagocytosis oscillation for FMF patients was 3.65 ± 0.03 min and 6.06 ± 0.12 min for ND (P = 0.02) with enhanced amplitude (274.9 ± 37 MCN versus 68.7 ± 19 MCN in ND, P = 0.04) and frequency of phagocytic uptake of latex beads. However, fMLP-stimulated neutrophil phagocytosis dynamics revealed oscillation behavior in FMF patients with two-fold increase in the period of latex beads phagocytic uptake (6.03 ± 0.05 min) and linear time-dependence in ND’s. Unstimulated neutrophil macropinocytosis dynamics revealed oscillation behavior in ND’s with the period of FITC-dextran uptake 7.21 ± 0.16 min, whereas the linear time-dependence was observed for FMF patients. The period of fMLP-stimulated neutrophil macropinocytosis oscillation for FMF patients was found to be 4.78 ± 0.03 min and 6.4 ± 0.01 min for ND (P = 0.04). Thus, the period of fMLP-stimulated neutrophil macropinocytosis oscillation in FMF patients was found to be much closer to the period of fMLP-stimulated F-actin oscillation (4.73 ± 0.13 min for F-actin and 4.78 ± 0.03 min for FITC-dextran uptake) compared with two-fold increase in the period of FITC-dextran uptake in ND (3.58 ±
± 0.17 min for F-actin and 6.49 ± 0.01 min for FITC-dextran uptake).

There are a number of unexpected findings described in this study. The first unexpected finding is the increasing of overall F-actin content in unstimulated neutrophils in FMF patients. Second, we found that repeated action of fMLP in ND induced significant reduction of both overall F-actin content and cellular F-actin oscillation amplitude, which paralleled with double increasing of neutrophil F-actin oscillation period.

The resolution of inflammation can now be regarded as an integral component of the neutrophil activation negative regulation programs [11]. The best known example is the phenomenon, known as neutrophil chemotactic receptors desensitization. When neutrophils encounter increasing concentration of chemotacticant, they gradually become nonresponsive to further stimulation by the same agent. In contrast to ND, FMF patients’ neutrophils failed to induce reduced F-actin dynamics during repeated action of fMLP, suggesting that neutrophils from FMF patients fail to induce chemotactic receptor desensitization. In conclusion we suggested that mutant MEFV-encoded pyrin may contribute to the decreasing of plasticity of cellular cytoskeleton and neutrophils failed to induce reduced F-actin or phagocytosis dynamics during repeated action of different chemotacticant or particles.

Т. К. Давтян, С. А. Аветисян, Г. С. Акопян
Мутации в гене MEFV приводят к нарушению содержания F-актин нейтрофилов и динамики фагоцитоза

Резюме
Цель. Изучение динамики внутриклеточного содержания F-актина, фагоцитоза и макропиноцизаза нейтрофилов у больных семейной средиземноморской лихорадкой (ССЛ) для выявления молекулярно-клеточных механизмов периодичной активации.
Методы. Нейтрофилы цельной крови 37 больных ССЛ вне приступа и 20 нормальных доноров (НД) активировали хемотрактантным пептидом N-формил-Мет-Лей-Фен (fMLP), L-формил-Мет-Лей-Фен (fMLP-), фибриногеназацетатом (PMA) или липополисахаридом (LPS). Содержание клеточного F-актина, фагоцитоз и макропиноцизаза оценивали методом проточной цитофлуориметрии. Амплитуду и период осцилляций F-актина вычисляли из кривых, полученных методом математической обработки данных, с допущением, что F-актин осциллирует гармоническими колебаниями вокруг фиксированной точки. Результаты. Содержание F-актина в не стимулированных нейтрофилах оказалось достоверно выше у больных ССЛ fMLP-, но не PMA- или LPS-стимулированые, и предварительно обработанные колхицином нейтрофили характеризовались разными динамическими изменениями в содержания F-актина с увеличенным периодом его осцилляции при ССЛ. При повторном воздействии fMLP нейтрофилы больных ССЛ не индуцировали десенсибилизацию хемотрактантных рецепторов, однако у НД десенсибилизация развивалась со значительным уменьшением амплитуды и периода осцилляции клеточного F-актина. У больных ССЛ выявлено существенное увеличение амплитуды и периода осцилляции фагоцитарной активности, но не макропиноцизаза нейтрофилов. Выводы. Нарушение динамических отношений фагоцитоза, макропиноцизаза и хемотрактации, регулирующих программы активации и десенсибилизации нейтрофилов в условиях непрерывного воздействия хемотрактантных, может быть потенциальным патогенетическим механизмом аберрантной резолюции воспаления при ССЛ.

Ключевые слова: семейная средиземноморская лихорадка, нейтрофил, F-актин, фагоцитоз, макропиноцизаза, N-формил-Мет-Лей-Фен, фибриногеназацетат.

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