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ЭКСПЕРИМЕНТАЛЬНАЯ ПЕРЕОРИЕНТАЦИЯ *IN VITRO* ФЕНОТИПА 4 СУБПОПУЛЯЦИЙ НЕЙТРОФИЛЬНЫХ ГРАНУЛОЦИТОВ ИЗ ПРОВОСПАЛИТЕЛЬНОГО К ПРОТИВОСПАЛИТЕЛЬНОМУ У ДЕТЕЙ С ХИРУРГИЧЕСКОЙ ГНОЙНОЙ ИНФЕКЦИЕЙ МЯГКИХ ТКАНЕЙ

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Резюме. Лечение детей раннего возраста с атипичными или рецидивирующими гнойными инфекциями мягких тканей (ГЗМТ), которые не демонстрируют хорошего ответа на хирургическое лечение и антибактериальные препараты, является наиболее сложным. ГЗМТ возникают на фоне нарушений функционирования иммунной системы и в первую очередь системы нейтрофильных гранулоцитов (НГ). Векторный эффект иммунотропной терапии на конкретную субпопуляцию NG может позволить корректировать дисфункции NG без ущерба для защиты хозяина, включая стратегии усиления, подавления или восстановления их функций. Цель исследования: оценить в системе *in vitro* модулирующие эффекты влияния аргинил-альфа-аспартил-лизил-валил-тирозил-аргинин (ГП) на трансформированный фенотип 4 субпопуляций НГ, а также на функциональную активность НГ детей с гнойно-воспалительными заболеваниями мягких тканей.

Исследованы образцы периферической крови (ПК) детей раннего возраста 2-4 лет: 17 детей с нетипично протекающими острыми ГЗМТ и 10 условно здоровых детей. На I этапе проведена сравнительная оценка содержания и фенотипа 4 субпопуляций НГ $CD16^+CD62L^+CD63^-$, $CD16^+CD62L^+CD63^+$, $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$, фагоцитарной и микробицидной функции НГ. На II этапе в системе *in vitro* определены эффекты ГП на НГ детей с ГЗМТ по изучаемым параметрам. Методом проточной цитометрии (FC500, Beckman Coulter, (США), конъюгаты МкАТ Beckman Coulter International S.A. (Франция)), определялось относительное количество НГ исследуемых субпопуляций и плотность экспрессии рецепторов (MFI). Для оценки фагоцитарной функции НГ использован микробиологический метод с оценкой завершенности фагоцитоза со S. aureus (штамм 209). Активность NADPH-оксидазы НГ исследовали в НСТ спонтанном тесте (НСТсп.) и в нагрузочном в системе *in vitro* (НСТст.).

Сравнительное изучение образцов ПК условно здоровых детей и детей с ГЗМТ позволило выявить различные варианты трансформации фенотипа изучаемых субпопуляциях НГ, сопряженных с дефектами их функциональной активности. В системе *in vitro* были продемонстрированы эффекты ГП,

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проявляющиеся снижением количества $CD16^+CD62L^+CD63^+$НГ и повышением $CD16^+CD62L^+CD63^-$НГ, модуляцией негативно измененного фенотипа субпопуляций $CD64^-CD32^+CD16^+CD11b^+$НГ и $CD64^+CD32^+CD16^+CD11b^+$НГ, направленные на восстановление фагоцитарной функции и поддержания напряженности NADPH-оксидаз.

В результате исследования установлено иммуномодулирующее действие ГП, которое проявляется в переориентации НГ с провоспалительного фенотипа на противовоспалительный, что может быть использовано в будущем при создании персонализированной таргетной иммунотерапии. Направлена на коррекцию неполноценного функционирования НГ у детей раннего возраста, страдающих ГЗМТ.

Ключевые слова: нейтрофильные гранулоциты, субпопуляции, эксперимент in vitro, гексапептид аргинил-альфа-аспартил-лизил-валил-тирозил-аргинин, гнойные заболевания мягких тканей, дети

IN VITRO EXPERIMENTAL REWIRING OF 4 NEUTROPHILIC GRANULOCYTE SUBSETS FROM THE PRO-INFLAMMATORY TO THE ANTI-INFLAMMATORY PHENOTYPE IN CHILDREN WITH SURGICAL PURULENT INFECTION OF SOFT TISSUE

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Abstract. Treatment of young children with atypical or recurrent purulent soft tissue infections (PSTD) that do not respond well to surgery and antibiotics is most challenging. PSTD occurs against the background of impaired functioning of the immune system and, first of all, the system of neutrophilic granulocytes (NG). The vector effect of immunotropic therapy on a specific NG subsets may allow the correction of NG dysfunctions without compromising host protection, including strategies to enhance, inhibit or restore their functions. The aim of study: to evaluate *in vitro* the modulating effects of arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine (HP) on the transformed phenotype of 4 NG subsets, as well as on the functional activity of NG in children with purulent-inflammatory soft tissue diseases.

We studied samples of peripheral blood (PB) from young children 2-4 years old: 17 children with atypical acute PSTD and 10 apparently healthy children. At stage I, a comparative assessment of the content and phenotype of 4 NG subsets $CD16^+CD62L^+CD63^-$, $CD16^+CD62L^+CD63^+$, $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$, phagocytic and microbicidal functions of NG was carried out. At stage II, the *in vitro* system determined the effects of HP on NG in children with PSTD according to the studied parameters. By the method of flow cytometry (FC500 "Beckman Coulter" (USA), conjugates of MkAT "Beckman Coulter International S.A." (France)), the relative number of NGs of the studied subsets and the density of receptor expression (MFI) were determined. To assess the phagocytic function of NG a microbiological method was used to assess the completeness of phagocytosis with *S. aureus* (strain 209). The activity of NG NADPH oxidase was investigated in the NBT-spontaneous test (NBTsp.) and in the *in vitro* NBT-induced test (NBTind.). A comparative study of PB samples from conventionally healthy children and children with PSTD made it possible to identify various variants of transformation of the phenotype of the studied NG subsets, associated with defects in their functional activity. In the *in vitro* system the effects of HP were demonstrated, manifested by a decrease in the amount of $CD16^+CD62L^+CD63^+$NG and an increase in $CD16^+CD62L^+CD63^-$NG, modulation of the negatively altered phenotype of subsets $CD64^-CD32^+CD16^+CD11b^+$NG and $CD64^+CD32^+CD16^+CD11b^+$NG, aimed at restoring phagocytic function and maintaining the tension of NADPH oxidases.

As a result of the study it was found the immunomodulatory effects of HP, which is manifested in the reorientation of NG from the pro-inflammatory phenotype to the anti-inflammatory one, which can be used in the future when creating personalized targeted immunotherapy aimed at correcting defective functioning NG in early children, suffering from PSTD.

Keywords: neutrophilic granulocytes, subset, experiment in vitro, hexapeptide arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine, purulent soft tissue diseases, children

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Introduction

Infections of the skin and soft tissues in children are common in the pediatric practice and often require surgical intervention. Localized purulent bacterial soft tissue lesions are treated with surgical aids (incision and drainage) and treatment with systemic antibiotics [3, 8].

The most difficult is the treatment of young children with atypical or recurrent purulent soft tissue infections that demonstrating no good response to surgical treatment and antibacterial drugs. It is shown that such purulent infections, as a rule, occur in parallel with impaired immune system functioning [10].

Neutrophil granulocytes (NGs) play an important role in the direct combating against purulent bacterial infection and at the same time coordinate and regulate the general, immune and inflammatory responses. It is established that NG exert various specialized functions on destruction of bacterial agents, one of which is not only phagocytic activity, but also the extrusion of their genomic DNA in the form of extracellular neutrophil traps (NET) [5].

Numerous studies indicate a significant phenotypic and functional NG heterogeneity [2, 4, 7, 9] as well as existance of diverse subsets. NGs are incredibly plastic cells able to reshape their phenotype under microenvironmental influence and change their properties. It accounts for NG potential undergo to transformational change. At the same time, it is shown that altered normal functioning of neutrophil granulocytes, both quantitatively and qualitatively, exerts negative effect on the course of purulent bacterial infection, and contributes to occurrence of atypical disease course. The vector effect of immunotropic therapy on a specific NG subset may allow to correct NG dysfunctions without compromising host protection, including strategies to enhance, inhibit, or restore their functions [6, 7].

NG is a unique and attractive target for therapeutic intervention. Correction of NG dysfunctions in atypically emerging purulent inflammatory diseases via modulating NG subset phenotype is one of the main urgent tasks in immunology [6, 12].

In this regard, it is of interest to study *in vitro* possibilities for remodeling negatively transformed phenotype of $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$, $CD16^+CD62L^+CD63^-$, $CD16^+CD62L^+CD63^+$NG subsets in children with purulent surgical infection of deep skin and soft tissues. It should be emphasized that these NG subsets are functionally important because the surface membrane molecules that shape their phenotypes – CD64, CD32, and CD16 are receptors for the three Fcγ fragments of IgG (FcγRs), respectively – FcγRI, FcγRII, and FcγRIII, whereas the CD11b molecule is a receptor for the C3b complement component. CD64 (FcγRI) is able to bind all IgG subclasses practically not being expressed on non-activated peripheral blood (PB) NG in healthy subjects. In acute bacterial infection, CD64-expressing NG emerge in the PB. CD64 can activate the ROS formation to enhance phagocytosis and trigger NG ADCC via an oxygen-mediated mechanism. The CD32 receptor (FcγRIIA) in response to NG contact with pathogens enhances the processes of endocytosis, increases the ROS production, NG secretory activity, activates their cytotoxic mechanisms and immunomodulatory functions. The CD16 receptor (FcγRIIIB), binding to IgG, as well as to FcγRIIA with FcγRIIIB, enables cytotoxicity, including ADCC, enhances phagocytic function, degranulation, and oxidative burst [1, 7, 12]. High expression of CD16 molecules on the NG membrane is associated with their increased functional activity, while low expression suggest about NG immaturity or "reverse differentiation". When the CD16 receptor interacts with CD11b/CD18 molecules, FcγRII-mediated internalization is enhanced. The CD11b receptor is the signaling partner for all Fcγ receptors, and its blockade leads to compromised activation of Fcγ receptors and impaired NG phagocytic function. CD11b is a marker of NG activation: with an adequate response to various microbial antigens, its expression level elevates. CD11b regulates chemotaxis, migration, adhesion, phagocytosis, respiratory burst, and NG degranulation [11, 12].

CD62L molecules are expressed on the membrane surface of circulating NG. The CD62L – L-selectin receptor [LAM-1], is a cellular adhesion molecule and is involved in the NG "rolling " of NG on the surface of the endothelium during inflammatory process. When the expression level of CD62L receptors is reduced, NG undergo apoptosis. The CD63 receptor, a marker of activated NG, is a glycoprotein-3 (LAMP-3) being found in the granules of non-activated NG, and is expressed on the surface of active NG. An increased level of CD63 expression is associated with the processes of NG phagocytosis and degranulation [1].

Of particular interest is the *in vitro* study of the immunomodulatory effects of the hexapeptide (HP) – arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine on functionally significant NG subsets. HP is an immunotropic substance bearing an active center of the thymic hormone thymopoietin and exerts immunoregulatory and detoxifying properties, inactivates free radical and peroxide compounds, restoring the balance of redox reactions in infectious and inflammatory diseases.

The aim of study: to evaluate *in vitro* modulatory effects of arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine (HP) on the transformed phenotype of 4 NG subsets, as well as on NG functional activity in children with purulent-inflammatory soft tissue diseases (PSTD).

Materials and methods

The single-center, prospective, non-randomized study was conducted. Peripheral blood (PB) samples of young children aged 2-4 years were examined.

The study group 1 consisted of 17 young children aged 2-4 years (9 boys and 8 girls) with atypical acute purulent-inflammatory soft tissue diseases (abscesses, phlegmons)-PSTD and comparison group contained 10 apparently healthy young children aged 2-4 years (5 girls and 5 boys). At the first stage, a comparative assessment of the number and phenotype of 4 functionally relevant NG subsets $CD16^+CD62L^+CD63^-$, $CD16^+CD62L^+CD63^+$, $CD64^-CD16^+CD32^+CD11b^+$, $CD64^+CD16^+CD32^+CD11b^+$ was carried out in the samples of the both groups: the study group 1 and comparison group. NG phagocytic and microbicidal functions were tested. At the second stage, the HP effects influence on NG subsets and functions were studied *in vitro* in children with PSTD. PB samples were incubated for 60 min at T 37 °C with HP (at a concentration of 10^{-6} g/l). After that, 4 functionally relevant subsets, phagocytic and microbicidal functions were also tested. The flow cytometry was used (FC500 "Beckman Coulter" (USA) for immunophenotyping of NG subsets with conjugated McAT "Beckman Coulter International S.A." (France)), percentage of NG subsets and the density of receptor expression identifying the various phenotypes via mean fluorescence intensity index (MFI) were determined. To assess the NG phagocytic function, PB samples were incubated with *S. aureus* (strain 209), for 120 min, at T-37. The level of active-phagocytic NG (%PHAN); phagocytic number (PN), phagocytic index (PHI) were tested. The percentage of digestion (%PD), the index of digestion (ID) were determined to assess the killing activity. The NG NADPH-oxidase activity was studied in the NBT-spontaneous test (NBTsp.) and NBT-induced test (NBTind.), by assessing opportunity to exert cytotoxic and cytolytic potential. The average cytochemical index and percentage of formazan-positive cells were determined (MCIsp, %FPCsp and MCIind., %FPCind), whereas the coefficient of mobilization (CM) – %FPCind/%FPKsp was calculated.

Statistical data processing was carried out by using computer-based software Microsoft Excel 2016 and StatPlus 2010. The results were presented as a median (upper and lower quartile) – Me ($Q_{0.25}$-$Q_{0.75}$) The nonparametric Mann–Whitney U test was used to assess the significance level for differences. The significance level was set at $p < 0.05$.

We are grateful to NPP Bionox, Ltd for providing for our study pharmacological substance Hexapeptide – arginyl-alpha-aspartyl-lysyl-valyl-tyrosyl-arginine.

Results and discussion

A comparative study of PB samples from children with PSTD revealed lowered number of NG subsets $CD64^-CD16^+CD32^+CD11b^+$ comprising 86.5 (80.5-96.1) % *vs* 98.0 (96.9-98.7) % found in the comparison group ($p < 0.05$). A change in the NG phenotype for this subset was also found: a 1.6-fold decrease in CD16 expression density to 81.5 (64.9-97.3), increased CD11b MFI ($p < 0.05$), but no changes in CD32 expression level compared to those in the group of apparently healthy children ($p > 0.05$) (Table 1).

At the same time, a significantly higher number of NG subsets $CD64^+CD16^+CD32^+CD11b^+$NG was found – 14.5 (5.6-15.7) % *vs* 0.3 (0.2-0.4)% in the comparison group($p<0.05$). The $CD64^+CD16^+CD32^+CD11b^+$NG subset expressed similar level of CD16, CD32, CD11b receptor expression density (MFI index) as the subset, $CD64^-CD16^+CD32^+CD11b^+$NG in PSTD ($p_1 > 0.05$, $p_2 > 0.05$, $p_3 > 0.05$) (Table 1), but the expression level for examined receptors differed from those observed in the group of healthy children. Thus, a 2.4-fold decrease in MFI CD64 down to 3.4 (2.2-4.7) *vs* 7.9 (5.3-12.2) in the comparison group, a 7.6-fold increase in MFI CD16 to 71.2 (60.2-98.2) *vs* 9.4 (5.1-19.6) in the comparison group, and a 1.6-fold increase in MFI CD11b to 24.6 (17.7-37.1) *vs* 15.4 (4.9-15.9) in the comparison group ($p_1 < 0.05$, $p_2 < 0.05$, $p_3 < 0.05$) were found, whereas the CD32 level did not differ from its density in the comparison group ($p > 0.05$) (Table 1).

A significant 8-fold increase in the number of activated NG subset $CD16^+CD62L^+CD63^+$NG – 64.0 (11.3-74.1) % was observed in children with PSTD compared to t healthy children – 7.6 (0.3-16.5) %, due to a decrease in $CD16^+CD62L^+CD63^-$ NG to 28.8 (6.6-32.8) %, which in apparently healthy children was 89.5 (81.5-96.8) % ($p < 0.05$). At the same time, there was a decrease in the MFI of CD16 ($p < 0.05$), and the level of expressed CD62L and CD63 surface receptors determined in both subsets did not change nor significantly differed from those in the comparison group (Table 1). The revealed transitions in phenotype and percentage of the studied functionally relevant NG subsets in children with PSTD are associated with the revealed defects in NG functional activity (Table 1).

The NG phagocytic function in children with PSTD was studied. The data obtained demonstrated a quantitative shortage of actively phagocytic NG (%PHAN 49.0 (39.3-53.0) *vs* 55.2 (54.0-57.0) in the comparison group, $p < 0.05$). At the same time, parameters of NG absorption capacity were increased: PHN – 5.2 (3.9-5.6) *vs* 4.1 (3.7-5.7) ($p > 0.05$) and PHI-2.7 (1.8-3.6) *vs* 2,5 (1.8-3.2) in the comparison group ($p > 0.05$). The decrease in degradation processes was detected: %D-53,1 (42.5-57.7) *vs* 61.6 (57.9-62.9) %, ID -1,3 (0,7-1,3) *vs* 1.6 (1.4-1.9) in the comparison group ($p_1 < 0.05$, $p_2 < 0.05$). At the same time, group with PSTD showed significantly increased spontaneous and induced activity of the NADPH-oxidases ($p < 0.05$) without preserving protective microbicidal potential, as evidenced by low KM ($p < 0.05$).

The effect of HP on the NG subset phenotype from children with PSTD was revealed in 1.5-fold decrease in CD16 MFI ($p < 0.05$) and 1.6-fold decrease in CD11b MFI in the $CD64^+CD16^+CD32^+CD11b^+$NG subset ($p < 0.05$), that prominently tended to dec-

TABLE 1. EFFECT OF HEXAPEPTIDE ON THE PHENOTYPE OF FUNCTIONALLY SIGNIFICANT SUBSETS OF NEUTROPHIL GRANULOCYTES IN CHILDREN WITH PURULENT-INFLAMMATORY SOFT TISSUE DISEASES, Me ($Q_{0.25}$-$Q_{0.75}$)

%CD64$^-$CD16$^+$CD32$^+$CD11b$^+$		MFI CD64	MFI CD16	MFI CD32	MFI CD11b
Healthy children	98.0 (96.9-98.7)		129.5 (115.8-131.8)	5.7 (4.8-6.1)	16.4 (9.7-17.7)
PSTD	86.5 (80.5-96.4)		81.5* (64.9-97.3)	5.3 (4.0-5.9)	19.4* (18.9-28.9)
PSTD + HP	94.7 (83.8-97.0)		73.2* (57.5-79.1)	5.4 (4.2-7.2)	10.6 (7.8-14.5)
%CD64$^+$CD16$^+$CD32$^+$CD11b		MFI CD64	MFI CD16	MFI CD32	MFI CD11b
Healthy children	0.3 (0.2-0.4)	7.9 (5.3-12.2)	9.4 (5.1-9.6)	5.5 (5.2-6.3)	15.4 (4.9-15.9)
PSTD	14.5* (5.6-15.7)	3.4* (2.19-4.72)	71.2* (60.2-98.2)	6.1 (5.02-7.56)	24.6* (17.7-37.1)
PSTD + HP	6.1* (5.5-12.7)	4.04 (2.6-7.2)	48.1* (35.1-75.3)	6.93 (6.1-9.2)	15.1 (11.9-17.5)

%CD16$^+$CD62L$^+$CD63$^-$		MFI CD16	MFI CD62	MFI CD63
Healthy children	89.5 (81.48-96.8)	128.9 (115.8-130.1)	7.08 (5.4-11.3)	
PSTD	28.8* (6.6-32.8)	83.5* (65.0-91.3)	5.7 (4.9-8.7)	
PSTD + HP	35.4 (27.0-73.5)	78.4* (67.5-80.1)	5.0 (4.6-5.6)	
%CD16$^+$CD62L$^+$CD63$^+$		MFI CD16	MFI CD62	MFI CD63
Healthy children	7.6 (0.3-16.5)	128.9 (115.8-130.1)	4.6 (3.5-8.9)	2.2 (1.5-3.2)
PSTD	64.0* (11.3-74.1)	83.5* (65.0-91.3)	5.6 (4.4-6.8)	2.2 (1.8-2.6)
PSTD + HP	52.0* (24.5-75.3)	78.4* (67.5-80.1)	4.7 (4.2-5.2)	2.0 (1.6-2.8)

Note. *, significant differences from the indicators of conditionally healthy children of the comparison group $p < 0.05$.

rease in MFI CD16 and MFI CD11b in the CD64$^-$CD16$^+$CD32$^+$CD11b$^+$NG subset paralleled with unchanged CD32 membrane receptor expression in both subsets (Table 1.).

HP affected CD16$^+$CD62L$^+$CD63$^+$NG and CD16$^+$CD62L$^+$CD63$^-$NG subsets demonstrated in children suffering from PSTD revealed by redistribution of both subsets: decreased percentage (%) of CD16$^+$CD62L$^+$CD63$^+$NG and increased proportion (%) of CD16$^+$CD62L$^+$CD63$^-$NG along with unchanged CD16, CD62L and CD63 expression density assessed by MFI index (Table 1.).

The study examining HP effect on NG phagocytic and microbicidal NADPH-dependent function in children with PSTD revealed the effects associated with the modulation of the receptor apparatus aimed at restoring the % of PHAN, the functions of degradation and maintaining the tension of NADPH oxidases necessary for mounting response to infectious process. The NG phagocytic activity tended to recover, but the indicators of all parameters remained below the magnitude in apparently healthy children: %PHAN ($p < 0.05$), PHN ($p < 0.05$), PHI ($p < 0.05$), ID ($p < 0.05$). At the same time, the reserve NADPH-oxidase activity increased by 2-fold in CM compared to the baseline level, which was higher than in comparison groups ($p < 0.05$).

Conclusion

During the experimental stage of the *in vitro* study, the different modulating HP effect on the number and the phenotype of 4 NG subsets and functions were shown, as well as an opportunity of reprogramming NG subset phenotype that was changed during PSTD.

Thus, the study resulted in describing the immunomodulatory HP effects manifested in NG rewiring from the pro-inflammatory to the anti-inflammatory phenotype, which can be used in the future while creating personalized target immunotherapy aimed at correcting defective NG functioning in early children, suffering from PSTD.

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