Diabetes mellitus. 2014;(3):77–82

Функциональное состояние нейтрофильных гранулоцитов и маркеры апоптоза при сахарном диабете 1 типа у детей

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В последние годы доказано, что полиморфноядерные лейкоциты играют важную роль в развитии сахарного диабета (СД). Дисфункция нейтрофилов способствует повреждению ткани поджелудочной железы, а также повышенной восприимчивости к инфекции при СД 1 типа (СД1).

Цель. *Изучение функциональной активности нейтрофильных гранулоцитов (НГ) при СД1 у детей.*

Материалы и методы. *Обследовано 25 детей в возрасте от 7 до 15 лет. Для оценки программируемой клеточной гибели выявляли количество НГ, экспрессирующих маркеры апоптоза (CD95, CD95L, Bcl2). Функциональную активность НГ определяли по показателям фагоцитоза, уровню миелопероксидазы, лизосомальных катионных белков, активных радикалов кислорода.*

Результаты. *Установлено снижение бактерицидной активности НГ с дефицитом поглощения, секреции активных радикалов кислорода, функционального резерва. Показано увеличение готовности к апоптозу, что сопровождалось повышением экспрессии CD95, снижением – Bcl2. Выявлено увеличение цитотоксического потенциала нейтрофилов в виде повышения уровня миелопероксидазы и лизосомальных катионных белков.*

Заключение. *Увеличение апоптотического потенциала НГ на фоне функционально-метаболических изменений может являться отражением их активного вовлечения в иммунопатогенез заболевания.*

Ключевые слова: *сахарный диабет; нейтрофилы; фагоцитоз; миелопероксидаза; лизосомальные катионные белки; оксидазная активность; апоптоз*

Neutrophil granulocyte functional status and expression of apoptosis markers in children with type 1 diabetes

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In recent years, polymorphonuclear leukocytes have been proven to play an important role in the development of diabetes mellitus (DM). Neutrophil dysfunction contributes to pancreatic tissue damage as well as an increased susceptibility to infection in type 1 DM (T1DM) patients.

Objective. *The objective of this study was to investigate the functional activity of neutrophil granulocytes (NGs) in children with T1DM.*

Materials and methods. *This study involved 25 children aged 7–15 years. To evaluate programmed cell death, the number of NGs expressing apoptosis markers (CD95, CD95L and BCL2) was determined. The functional activity of NGs was determined in terms of phagocytosis and the levels of myeloperoxidase, lysosomal cationic proteins and active oxygen radicals.*

Results. *A reduction in the bactericidal activity of NGs with deficiencies in phagocytosis, secretion of active oxygen radicals and functional reserve was found. An increase in the apoptotic potential of NGs was demonstrated, which was accompanied by an increase in CD95 expression and a decrease in BCL2 expression. An increase in the cytotoxic potential of neutrophils in the form of enhanced levels of myeloperoxidase and lysosomal cationic proteins was revealed.*

Conclusions. *Therefore, an increase in the apoptotic potential of NGs associated with functional and metabolic changes may reflect the active involvement of NGs in the immunopathogenesis of T1DM.*

Keywords: *diabetes mellitus; neutrophils; phagocytosis; myeloperoxidase; lysosomal cationic proteins; oxidase activity; apoptosis*

DOI: 10.14341/DM2014377-82

In recent years, neutrophil granulocytes (NGs) have been found to play an important role in the development of diabetes mellitus (DM) [1, 2, 3]. The involvement of neutrophils in the destruction of pancreatic β-cells and the pathogenesis of microvascular damages has been demonstrated [4]. A decrease in the number of peripheral polymorphonuclear leukocytes (PMNLs), preceding disease onset and accompanying the development of type 1 DM (T1DM), has been demonstrated, which may be related to PMNL accumulation in the exocrine pancreas [3].

It has been found that in the early stages of the development of insulitis, directed migration of leukocytes to the pancreas occurs, and these cells are capable of local secretion of proinflammatory cytokines as well as cytotoxic enzymes and active oxygen radicals that trigger apoptosis and necrosis of β-cells [5]. Hyperglycaemia is considered to be one of the factors that increases the destructive potential of NGs [6]. The role of PMNLs in the development of diabetic angiopathy and retinopathy has been demonstrated [7, 8]. Data on the involvement of neutrophils in the pathogenesis of diabetic nephropathy have been obtained. In patients with diabetes, tubular and mesenchymal renal cells were found to produce a large amount of chemokines, potentiating the influx of leukocytes, their adhesion and subsequent synthesis of proinflammatory cytokines; this led to glomerular sclerosis and fibrosis of the interstitium [9].

In T1DM patients, studies have detected defective chemotaxis, low bactericidal activity of neutrophils, abnormal production of superoxide and leukotrienes, secretion of lysosomal enzymes and also changes in the basal level of intracellular calcium [10, 11]. Very little is known regarding apoptosis of PMNLs in T1DM. Early studies revealed an increase in the neutrophil apoptosis rate [12] and were focused on the inability to delay apoptosis of these cells in T1DM patients on stimulation with a lipopolysaccharide [12, 13]. Hyperglycaemia was suggested to increase apoptosis of NGs [1]. Later, a reduction in apoptosis of PMNLs was demonstrated in clinical and experimental studies. This reduction likely promotes the accumulation of neutrophils in inflammatory exudates, leading to tissue damage as well as predisposing T1DM patients to prolonged staphylococcal infections [1, 2].

NGs are the main cells involved in innate immunity, and a change in their structural or metabolic status is the basis of low resistance to infection observed in DM patients and thus their susceptibility to infectious diseases [2, 3, 14]. A frequent cause for seeking medical care among DM patients is an infection of the ear, nose and throat and respiratory tract, genito-urinary and gastrointestinal tracts or skin and soft tissues [14].

Despite the obvious significance of the problem, the role of PMNLs in the development of T1DM in children has not been established definitively. Moreover, the involvement of NGs in the genesis and development of the disease is still debated. The available literature demonstrates impairment of neutrophil cell death in terms of both activation and inhibition. Further investigation of apoptosis markers expressed by peripheral blood neutrophils and their functional status is of great clinical importance, because their impairment promotes pancreatic tissue damage as well as an increased susceptibility to infections in T1DM patients.

Aim

The objective of this study is to investigate the functional activity of and apoptosis markers expressed by NGs in children with T1DM.

Material and methods

Twenty-five children with T1DM between the ages of 7–15 years were examined (Table 1). Patients were divided into groups depending on disease duration. Group I included 12 children with a disease duration of less than 3 years. The mean age of patients in Group I was 9.7 ± 0.42 years, and the mean disease duration was 1.7 ± 0.49 years. Group II included 13 children with a DM duration of more than 3 years. The mean age of children in Group II was 13.3 ± 0.99 years, and the mean disease duration was 7.1 ± 0.88 years. The glycated haemoglobin level reached 11.3 ± 0.90% in Group I and 10.0 ± 0.71% in Group II. The control group included 15 healthy children between the ages of 7 and 15 years. All patients or their parents provided written informed consent for participation in the study.

To assess programmed cell death, the numbers of NGs expressing apoptosis markers were determined. Neutrophils were isolated via a Ficoll–Paque and Ficoll–Urografin (GE Healthcare, Sweden) double density gradient. The cell suspension was washed three times with RPMI-1640 medium (Vector-Best, Russia). In neutrophil cultures, the number of cells expressing CD95, CD95L and BCL2 receptors was evaluated by flow cytometry using monoclonal antibodies (Invitrogen, USA).

The phagocytic index was determined by the ability of NGs to absorb melamine–formaldehyde latex particles. The level of the oxygen-dependent bactericidal activity of NGs was evaluated using the nitroblue tetrazolium test (NBT) according to the number of formazan-containing cells (formazan-positive cells). The level of lysosomal cationic protein (LCP) was determined according to the Shubich method, and the myeloperoxidase (MP) level was determined by the Graham–Knoll method. The mean cytochemical coefficient was calculated according to the method of Astaldi.

The assessment of comorbid infectious and inflammatory diseases was performed on the basis of clinical examination at a specialised department and an analysis of medical history records from a children's polyclinic, taking into account the results of dispensary observation by the pediatrician, otolaryngologist and dermatologist.

The Primer of Biostat 4.0 and Attestat 10.5.1 software packages were used for the statistical analysis of data. To assess intergroup differences, a repeated measures analysis of variance with calculation of the Newman–Keuls and Dunn criteria was used. An analysis of the qualitative characteris-

Table 1

Characteristics of clinical groups

Parameter	Group I (n = 12)	Group II (n = 13)
Mean age, years	9.7 ± 0.42	13.3 ± 0.99
Mean disease duration, years	1.7 ± 0.49*	7.1 ± 0.88
HbA_{1c}, %	11.3 ± 0.90	10.0 ± 0.71

HbA_{1c} – haemoglobin A1c; * p < 0.05 – significantly different compared with Group II (Newman–Keuls test, Dunn test)

Table 2

Parameters of functional activity of neutrophil granulocytes in children with type I diabetes mellitus

Parameter	Group I (n = 12)	Group II (n = 13)	Control group (n = 15)
Phagocytosis, %	82.6 ± 2.33	72.9 ± 2.35* **	81.0 ± 1.65
CP, a.u.	1.8 ± 0.04*	1.7 ± 0.05*	1.4 ± 0.03
MP, a.u.	2.5 ± 0.03*	2.7 ± 0.07* **	2.1 ± 0.02
NBT, %	3.0 ± 0.41*	3.6 ± 0.58*	7.7 ± 0.7
SI NBT, a.u.	2.5 ± 0.37	1.7 ± 0.36* **	2.6 ± 0.05

CP – cationic proteins, MP – myeloperoxidase, NBT – formazan-positive cells, SI NBT – stimulation index in the NBT test; *p < 0.05 – significantly different compared with the control group, **p < 0.05 – significantly different compared with Group I (Newman–Keuls test, Dunn test).

Table 3

Expression neutrophil granulocyte apoptosis markers in children with type I diabetes mellitus

Apoptosis marker	Group I (n = 12)	Group II (n = 13)	Control group (n = 15)
CD95, %	77.6 (71.15–83.99)*	87.93 (84.24–91.63)* **	58.43(54.95–1.90)
CD95L, %	9.5 (8.14–10.92)*	12.1 (10.22–13.96)* **	7.3 (6.46–8.09)
BCL2, %	3.99 (2.9–5.08)	2.78 (2.36–3.19)*	5.38 (4.21–6.55)

CD95 – the apoptosis-mediating receptor, CD95L – a ligand of the CD95 receptor, BCL2 – the receptor of apoptosis resistance; *p < 0.05 – significantly different compared with the control group; **p < 0.05 – significantly different compared with Group I (Newman–Keuls test, Dunn test).

tics was performed using the χ^2 criterion. Quantitative values with a normal distribution are presented as mean ± the standard error of the mean (X ± sx); parameters characterised by an abnormal distribution are presented as the median and the interquartile (25th and 75th percentiles) range [Me (Q1–Q)]. The differences were considered significant at $p < 0.05$.

Results

The study of the functional activity of PMNL revealed a reduction in phagocytosis in children with a disease duration of more than 3 years (72.9 ± 2.35%, $p < 0.05$). In patients with a T1DM duration of less than 3 years, phagocytosis parameters did not differ from those in the control group (Table 2).

According to the spontaneous NBT test, NG oxidase activity parameters were significantly decreased, and the functional reserve level (SI NBT) was reduced in children with a T1DM disease duration of more than 3 years compared with that in the control group.

A comparison of NG functional activity parameters revealed lower phagocytosis ($p < 0.05$) and lower SI NBT test ($p < 0.05$) parameters (Table 2) in children with a T1DM duration of more than 3 years compared with those in children with a disease duration of less than 3 years.

Patients in both groups were found to show increases in cytological and enzyme chemical parameters, such as the levels of MP and LCPs, compared with the control group, with MP levels being higher in children with a disease duration of more than 3 years.

An analysis of apoptosis markers revealed an increase in the expression of pro-apoptotic CD95 markers and a decrease in the expression of anti-apoptotic BCL2 markers in children with T1DM (Table 3). The maximum expression of CD95 was detected in children with a disease duration of more than 3 years.

An increase in the percentage of PMNLs expressing CD95L on their surface was found, and the highest expression was detected in children with a disease duration of more than 3 years.

The development of comorbid infectious and inflammatory diseases was observed in 72% of children enrolled in the study, and this percentage was higher than the percentage of the control group with respect to similar characteristics (20%; $p < 0.05$)' or 'and this percentage was higher than the frequency of similar characteristics in the control group (20%; $p < 0.05$).

Frequent respiratory infections were detected in children in both Groups I and II (Table 4). Chronic tonsillitis was diagnosed in 33.4% of children in Group I and in 30.8% of children in Group II. Chronic maxillary sinusitis was observed only in children in Group II (23.1%). Urinary tract infections were confirmed in a few cases. Cystitis was detected in 8.3% of children in Group I and in 7.7% of children in Group II. Chronic pyelonephritis was diagnosed only in children in Group II (15.4%). Skin diseases occurred in 16.7% of children in Group I and in 41.7% of children in Group II. The pathologic structure included streptodermas in 16.7% of children in Group I and in 15.4% of children in Group II, recurrent herpes infections in 23.1% of children in Group II and allergic dermatitis in 8.3% of children in Group I and 23.1% of children in Group II. In single cases, children in Group I (8.3%) were diagnosed with candidiasis of the oral mucosa. In children with a T1DM duration of more than 3 years, repeated episodes of stomatitis (23.1%) were observed.

A comparison of the frequency of infectious and inflammatory diseases depending on disease duration revealed higher frequencies in children with a T1DM duration of more than 3 years. However, significant intergroup differ-

Table 4

Comorbid diseases in children with type I diabetes mellitus depending on disease duration

Type of infection	Pathology	(%, percentage of children)		
		Group I (n = 12)	Group II (n = 13)	Control group (n = 15)
URT infections	Frequent acute respiratory viral infections	58.3 (7)	53.8 (7)	20.0 (3)
	Recurrent bronchitis	16.7 (2)	23.1 (3)	-
ENT infections	Chronic tonsillitis	33.4 (4)	30.8 (4)	13.3 (2)
	Chronic maxillary sinusitis	-	23.1 (3)	-
Diseases of the skin and subcutaneous tissue	Allergic dermatitis	8.3 (1)	23.1 (3)	-
	Pyodermia	16.7 (2)	15.4 (2)	-
	Mucocutaneous herpes	-	23.1 (3)	6.7 (1)
	Chronic pyelonephritis	-	15.4 (2)	-
	Cystitis	8.3 (1)	7.7 (1)	-
Urinary tract infections	Intestinal giardiasis	8.3 (1)	-	-
	Functional dyspepsia	16.7 (2)	15.4 (2)	6.7 (1)
	Mucosal candidiasis	16.7 (2)	15.4 (2)	-
	Stomatitis	-	23.1 (3)	-

URT – upper respiratory tract, ENT – ear, nose and throat

ences were not obtained, which was probably due to small sample sizes.

Discussion

The structural and metabolic status of PMNLs is known to be inextricably related to their physiological functions and to represent the sum of their activation, adhesion, chemotaxis, antigen uptake and their killing and breakdown. We found a reduction in the number of phagocytic cells in children with a T1DM duration of more than 3 years, which is consistent with the results of most studies on phagocytic activity in T1DM [2, 10, 11].

Hyperglycaemia and hyperketonemia are known to be able to contribute to the impairment of the phagocytic and chemotactic function of PMNLs in DM, causing neutrophils to become spherical and to largely lose their ability to form pseudopodia. These changes result in reduced adhesion and migration of NGs and hindered phagocytosis and capping [4].

The present study demonstrated a reduction in the parameters of the spontaneous and stimulated NBT test in children with T1DM that may be associated with the impaired degranulation process, which is a coagulation of specific and azurophilic granules of PMNLs with their phagosomes. Degranulation is known to be an energy-consuming process. Under experimental conditions, a significant reduction in the level of glycogen, inhibition of its synthesis and a decrease in the activity of key enzymes of the anaerobic glucose oxidation and pentose cycle were found in PMNLs in DM, leading to a decrease in the intracellular level of ATP [15].

A comparison of NG functional activity parameters in the group of children with a T1DM duration of more than 3 years revealed lower phagocytosis and SI NBT test parameters compared with those in children with a disease duration of less than 3 years, which is probably related to disease duration and functional depletion of NGs [16].

Patients in both groups were found to exhibit an increase in cytological and enzyme chemical parameters, such as the levels of MP and LCPs, compared with the control group, with MP levels being higher in children with a disease duration of more than 3 years. Our findings likely indicate an increase in the bioaggressive potential of peripheral blood neutrophils in children with T1DM due to the increased production of hypochlorous acid and cationic proteins. The most cytotoxic products of MP are known to be hypochlorous acid and hypochlorite anions, which are the major damaging agents in biological systems [17].

We found that the progression of DM in children is accompanied by an increase in the percentage of neutrophils expressing apoptosis markers (CD95) and a decrease in the proportion of cells expressing anti-apoptotic proteins on their surface. The most pronounced activation of apoptosis was observed in the group of children with a disease duration of more than 3 years. According to the literature, the intensity of neutrophil apoptosis in DM may be low [18, 19] or high [12, 13, 20]. PMNLs of DM patients did not demonstrate a reduction in apoptosis induced by bacteria and a lipopolysaccharide [13]. It is believed that impairment of glucose and glutamine disposal may be a factor predisposing PMNLs to apoptosis [2]. However, there are also data in the literature demonstrating a decrease in the rate of NG apoptosis in DM that likely initiates chronic inflammation processes, causing damage to the pancreatic tissue [18, 19].

The present study revealed an increase in the percentage of cells expressing CD95L on their surface, and this increase may contribute to the enhanced programmed cell death among pancreatic islet β-cells infiltrated with leukocytes. The highest expression levels were observed in children with a disease duration of more than 3 years. Therefore, an increase in the NG apoptotic potential associated with functional and metabolic changes is a reflection of the active involvement of NGs in the immunopathogenesis of T1DM. Overexpression of certain markers of the biocide capacity of PMNLs in the form of an increase in the levels of MP and LCPs was found

to be accompanied by an increase in their apoptosis that may be associated with a change in the glutamine metabolism in NGs on the development and progression of DM.

At the same time, a reduction in the bactericidal activity of neutrophils with deficiencies in phagocytosis, secretion of active oxygen radicals and functional reserve was found. The degree of functional insufficiency depended on disease duration, and the greatest increases were observed in children with a T1DM duration of more than 3 years.

PMNLs are known to represent a link between innate and adaptive immunity and to play a leading role in antibacterial protection. Impairment of their functional competence in children with T1DM is one of the factors predisposing these patients to the development of infectious diseases. T1DM patients in the present study had an increased susceptibility to infectious diseases, which is consistent with the findings of previous studies [2, 3, 14].

Conclusions

1. NGs in children with T1DM are characterised by a high readiness to apoptosis and low bactericidal activity with deficiencies in phagocytosis, production of active oxygen radicals and functional reserve.
2. An increase in CD95L expression by NGs in TIDM patients may contribute to an increase in programmed cell death among pancreatic islet β-cells infiltrated by immunocompetent cells.
3. Signs of the antigenic stimulation of PMNLs and an increase in their cytotoxic potential in T1DM are identified in the form of activation of their metabolic activity with an increase in the levels of MP and LCPs.

Information on funding and conflicts of interest

This work was performed under the Federal Target Scientific Program. The state registration number is 01200954364.

This study was approved by the Local Ethics Committee of the Stavropol State Medical University and the Ministry of Health of the Russian Federation. Protocol №16 from May 14, 2012

The authors declare no apparent or potential conflicts of interest related to this research and publication of this article.

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