Экспериментальная и профилактическая медицина

UDK 577.122.352+616.379-008.64

Changes in the Activity of Adenosine Deaminase Isoforms in the Blood Plasma in Young Patients with Type 1 Diabetes

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Key words: adenosine deaminase 1, adenosine deaminase 2, blood plasma, glycated hemoglobin, type 1 diabetes mellitus

Introduction

Type 1 diabetes mellitus (T1DM), one of the most common and serious chronic diseases in children and adolescents, is increasing worldwide [9, 13].

An increasing amount of evidence highlights the critical role for the adenosine system in regulation of glucose homeostasis, the pathophysiology of diabetes mellitus, including both types T1DM and T2DM, as well as the associated complications [34].

Adenosine level undergoes regulation by rapid phosphorylation to AMP by adenosine kinase and deamination to inosine by adenosine deaminase [2,12].

Adenosine deaminase (ADA, EC 3.5.4.4) is a key enzyme of the purine salvage pathway catalyzing deamination of (deoxy)adenosine to (deoxy)inosine. Playing an important role in regulation of adenosine concentration, ADA participates in the development of immune system [21]. Its crucial role in regulation of cellular immunity was shown by the severely decreased T and B cell numbers in patients affected by genetic deficiency of ADA [8]. The enzyme is required in the differentiation and proliferation of lymphocytes and monocyte/macrophage system. In mammalian ADA presented by two genetically and catalytically different isoformes – ADA1 and ADA2. The gene of the more widely studied ADA1, characterized with Km = 0.02 mM, is located on human chromosome 20q.11.33. It exists in two molecular isoforms: a monopeptide of molecular mass of 35-40 kDa and its 280-300 kDa complex with ADA-binding

protein identical to the cluster of differentiation 26 (CD26). The latter is known as a multifunctional enzyme dipeptidyl peptidase IV (DPPIV). The ADA2 gene, previously named cat eye syndrome chromosome region 1 (CECR1), coding the poor studied isoenzyme ADA2 (molecular mass 110 kDa, characterized with Km = 2 mM), is located on chromosome 22q11.1. Ubiquitous ADA isoenzymes are considered as suitable markers of cell-mediate immunity and have been used for monitoring severe diseases associated with immune system disorders [6,10,29].

A number of reports are devoted to the studies of ADA in T2DM. An increase in the ADA activity level of, which is mainly due to ADA2 isoform, was observed in the blood plasma of diabetic patients compared to healthy controls. In diabetes ADA activity correlated with the level of the glycated hemoglobin (HbA1c) and other clinical parameters. It has been reported that in the majority of patients the elevated ADA2 but not ADA1 activity decreased following blood glucose control [23]. Total ADA activity correlates with HbA1c, and ADA2 activity in patients with high HbA1c was found to be much higher than that in patients with low HbA1c [25].

Despite of efforts made, there was slow progression in the T1DM research over the years, and the pathogenesis of T1DM remains largely unknown. The role of adenosine in regulating of T cell functions at onset of T1DM is not completely understood. Moreover, the data on ADA studies in children with T1DM are scarce and controversial.

In T1DM several genetic factors are associated with β-cell autoimmunity onset and clinical progression. A predisposition to T1DM in children was studied depending on the alleles of the ADA1 polymorphic gene, and variability for different alleles of ADA1 were shown [17]. Comparison of ADA2 activity in the peripheral blood serum in healthy children and at different pediatric inflammatory and immune-mediated diseases showed significant elevation in the level of ADA2 activity in children with systemic juvenile idiopathic arthritis, an autoimmune disease, complicated with macrophage activation syndrome. As ADA2 isoenzyme is secreted mainly by monocytes/macrophages, it was proposed as a marker of macrophage activation syndrome [27].

Our study showed differences in the activity of two ADA isoenzymes at T1DM. The normative range of ADA2 activity in peripheral blood of healthy pediatric population was established. We demonstrated specific sex- and pubertal changes in the activity of the ADA1 isoform in the plasma of control healthy subjects and patients with T1DM, regardless of their glycemic status, the changes in which are mainly associated with the ADA2 isoform. Our findings demonstrated the importance of alterations in the ADA activity in T1DM. Therefore, testing of the ADA activity may be useful in assessing the severity of the disease and the effectiveness of the therapy, helping in developing new approaches to the prevention and treatment of autoimmune diabetes.

Material and Methods

Chemicals Adenosine and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other reagents were of high purity degree.

Patients The young subjects with T1DM and healthy subjects (case/control study) were patients at the Center «Muratsan» of the Yerevan State Medical University (YSMU) after Mkhitar Heratsi, Department of Endocrinology. All subjects or their legal representatives gave their informed consent to participate in the study in accordance with Good Clinical Practice (GCP) standards and the WMA Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects [35]. Patients with T1DM were diagnosed according to guidelines of American Diabetes Association (ADA) [7]. The study was approved by the Ethical Committee of the YSMU.

The study involved 59 patients (27 girls and 32 boys), in the age from 2 to 22 years with T1DM of different duration. Fifty four (54) sex and age matched healthy volunteers (25 girls and 29 boys) were enrolled as controls. Individuals with any kind of infection or inflammatory diseases, which lead to increase in ADA, were excluded. The exclusion criteria were applied in the macrovascular and microvascular (retinopathy, nephropathy) diseases, acute or chronic liver, kidney or cardiac diseases, malignancy, arterial hypertension, and pregnancy. The absence of macrovascular disease was controlled by the absence of a cardiovascular event or procedure, angina, ischemic ECG abnormalities. At the time of T1DM diagnosis at least two types of antibodies, associated with the development of T1DM, including the islet cell autoantibodies to insulin (ICA) and the glutamic acid decarboxylase (GADA), were positive.

Procedures were performed at the Department of Endocrinology of «Muratsan» university Hospital (Yerevan, Armenia). All patients received daily insulin injections. The absence of microalbuminuria was controlled by measurement of urinary albumin/creatinine ratio. All the participants were examined early in the morning, fasted, having avoided caffeinated beverages, cigarettes and strenuous exercise since the previous evening.

Isolation of peripheral blood plasma Freshly obtained venous blood was drawn into 3.8% sodium citrate anticoagulant, dissolved in phosphate buffered saline (1.15 mM NaCl, buffered with 0.02 M phosphate buffer, pH 7.4). After centrifugation at 6000 rpm for 20 min at 4°C in the supernatant, plasma, free of platelets, was obtained and immediately used in the assay.

Plasma HbA1c was measured at «Muratsan» Hospital clinical laboratory by a latex turbidimetric assay and estimated as a percentage (%) [11].

ADA assay The total ADA activity in plasma was assessed by measuring of ammonia, produced in the catalyzed reaction of adenosine deamination at incubation at 37^{o}C for 40 min of assay mixture, containing in 0.5 ml: 0.04 M K-phosphate buffer, pH 7.0, 6 mM adenosine and an aliquot of a sample. The

enzymatic reaction was stopped by addition per 1 ml of phenol-nitroprusside and hypochlorite reagents [3]. The intensity of the developed color was measured at 630 nm against blank with all the reagents, excluding adenosine. The ammonia content was evaluated using ammonium sulfate as a standard. The ADA activity was expressed as μmole of produced ammonia per 1 L of plasma for 1 minute (U/L). ADA2 activity was obtained in identical experiment conducted in the presence of 0.04 mM EHNA, a selective inhibitor of ADA1. ADA1 activity was calculated by subtracting ADA2 activity from the total ADA activity. All samples were duplicated.

Statistical analysis The data were analyzed using GraphPad Prism 3 (GraphPad Prism Software Inc. San Diego, CA, USA) [26]. The results were expressed as the mean (M) ± standard error of the mean (SEM). The non-parametric Mann–Whitney test was used for statistical analysis of variables. Spearman's correlation coefficient (r) assessed. The differences among groups considered as statistically significant when the two-tailed P values were < 0.05.

Results and Discussion

The following groups of the patients with T1DM included in this study: 1) new-onset (NO), who was diagnosed with the first manifestation of diabetes and the patients, who received insulin up to one year, an average of 0.5-0.6 year; 2) long term (LT), the patients with long-term T1DM, who received insu-

Table 1

Clinical data for children/preadolescents (subgroup I) with new-onset (NO) and long-term (LT) type 1 diabetes mellitus and of healthy volunteers

Variables	NO	LT	Healthy
Number of subjects	18	7	22
Male/female	8/10	3/4	9/13
The first manifestation/up to a year/Male//female	10/8//7/11	-	-
Age (years), median (range)	7.4 [6.0-11.0]	8.4 [6.0-11.0]	8.5 [5.0-11.0]
Age of debut (years), median (range)	7 .9 [6.0-11.0]	5.9 [5.9-9.4]	-
Diabetes duration (years), median (range)	0.6 [0.01-1.0]	2.3 [1.2-5.0]	-
HbA1C (%), median (range)	9.0 [5.9-12.8]	7.3 [5.0-9.4]	4.7 [4.0-5.3]
Cholesterol (mg/dL), median (range)	169 [141-192]	175 [145-197]	155 [137-175]
Triglycerides (mg/dL), median (range)	89 [71-105]	97 [72- 117]	79 [67-99]
Creatinine clearance (mL/min), median (range)	115 [95-128]	113 [89-133]	117 [99-133]

lin more than one year, an average of 5.3-5.9 years. Patients distributed into two groups by sex: girls (G) and boys (B); and subgroups by age: (I) children and preadolescents under 11 years old; (II) adolescents and young adults over 11 years old. The information regarding the sex, age and treatment of T1DM for the patients and healthy volunteers of subgroup I and II presented in Tables 1 and 2, respectively. The data for sex- and age-matched healthy subgroups compared.

Table 2

Clinical data of adolescents/young adults (subgroup II) with new-onset (NO) and long-term (LT) type 1 diabetes mellitus and healthy volunteers

Variables	NO	LT	Healthy
Number of children	13	21	32
Male/female	8/5	14/7	19/13
The first manifestation/up to a year/Male//female	14.3/13.9//8/5	-	-
Age (years), median (range)	14.1 [12.0-20.0]	14.5 [12.2-21]	15.9 [12.0-23.0]
Age of debut (years), median (range)	13.9 [10.2-21.2]	9.3 [3.0-13.0]	-
Diabetes duration (years), median (range)	0.31 [0.1-1.0]	5.4[1.5-12.0]	-
HbA1C (%), median (range)	9.4 [6.7-11.8]	9.1 [5.7-13.7]	4.8 [4.1-5.4]
Cholesterol (mg/dL), median (range)	168 [141-193]	177 [143-199]	159 [133-179]
Triglycerides (mg/dL), median (range)	85 [65-107]	92 [69-115]	81 [63-107]
Creatinine clearance (mL/min), median (range)	113 [94-127]	111 [91-132]	116 [98-129]

The total activity of ADA and the activities of its isoenzymes ADA1 and ADA2 in the blood plasma of T1DM patients and healthy controls are presented in Table 3 for the G, B and combined groups, separately for age groups I and II.

The data in Table 3 show, that both the tADA and ADA2 activities in T1DM patients (9.69±0.81 and 6.08±0.41U/L, respectively) were two times higher than in healthy controls (4.98±0.4 and 3.0±0.29 U/L, respectively), $p < 0.0001$. The estimated increase of ADA1 activity was about 1.5 times at values of 2.78±0.38 in patients with T1DM vs 1.71±0.35 in the healthy controls. The difference is statistically significant ($p < 0.0232$), but less pronounced.

The comparison of ADA activity in the sex groups (B and G) and age subgroups (I and II) showed the difference in ADA1 activity between B and G in II subgroup for healthy control subjects. In the B group ADA1 activity was higher (2.74±0.55), than in the G group (1.32±0.36, p value is 0.0278). This observation might be attributed to the differences of puberty of boys and girls in this age subgroup.

Table 3

Serum adenosine deaminase activity in patients and healthy volunteers

Subjects	Num-ber	Adenosine deaminase activity (*U/L*)		
Healthy volunteers		tADA	ADA1	ADA2
Total (boys and girls)	54	4.98 ± 0.4	1.71 ± 0.35	3.0 ± 0.29
Boys	29	5.00 ± 0.44	2.44 ± 0.43	2.75 ± 0.35
Children / preadolescents (I)	10	4.53 ± 0.81	1.64 ± 0.38	3.02 ± 0.69
Adolescents / young adults (II)	19	5.24 ± 0.77	2.84 ± 0.61	2.40 ± 0.42
Girls	25	4.71 ± 0.54	1.53 ± 0.33	3.36 ± 0.44
Children / preadolescents	12	4.8 ± 0.79	1.53 ± 0.54	3.41 ± 0.63
Adolescents / young adults	13	4.62 ± 0.77	1.32 ± 0.36	3.36 ± 0.62
T1DM patients				
Total (boys and girls)	59	9.69 ± 0.81***	2.78 ± 0.38*	6.08 ± 0.41***
Boys	32	10.0 ± 1.04***	3.60 ± 0.59$^{\#}$	5.85 ± 0.81***
Children / preadolescents	10	10.0 ± 1.5***	3.82 ± 0.74*	6.13 ± 1.19***
Adolescents / young adults	22	9.00 ± 1.54***	3.43 ± 0.90$^{\#}$	6.1 ± 0.56***
Girls	27	9.47 ± 0.66***	2.36 ± 0.25*	6.85 ± 0.72***
Children / preadolescents	15	9.47 ± 0.94***	2.08 ± 0.31$^{\#}$	7.18 ± 0.80***
Adolescents / young adults	12	8.77 ± 1.03***	2.75 ± 0.4*	5.89 ± 0.81***

Data are presented as *mean* ± *SEM* and ***p < 0.0001, *p< 0.05, $^{\#}$p > 0.05 - between control and patients with T1DM groups.

We did not observe regular differences of the tADA and ADA2 activity levels between sex and age groups as well as between groups of T1DM patients divided by the duration of the disease and treatment with insulin; as well as between T1DM patients with and without ketoacidosis (data not shown).

Lee and colleagues [27] and Gao and colleagues [19] reported strong correlation between tADA and ADA2 activities in the serum of healthy individuals and patients with immune-mediated diseases (including rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, myasthenia gravis and autoimmune liver diseases). We also presented correlations between tADA and ADA2 activities in the plasma of T1DM patients and healthy controls. Total ADA highly correlated with ADA2 in healthy individuals (Fig. 1A; r = 0.7388, p <0.0001). Correlation coefficient between tADA and ADA2 in patients with T1DM (Fig. 1B; r = 0.8776, p <0.0001) was higher than in healthy children, which coincides with data of cited authors.

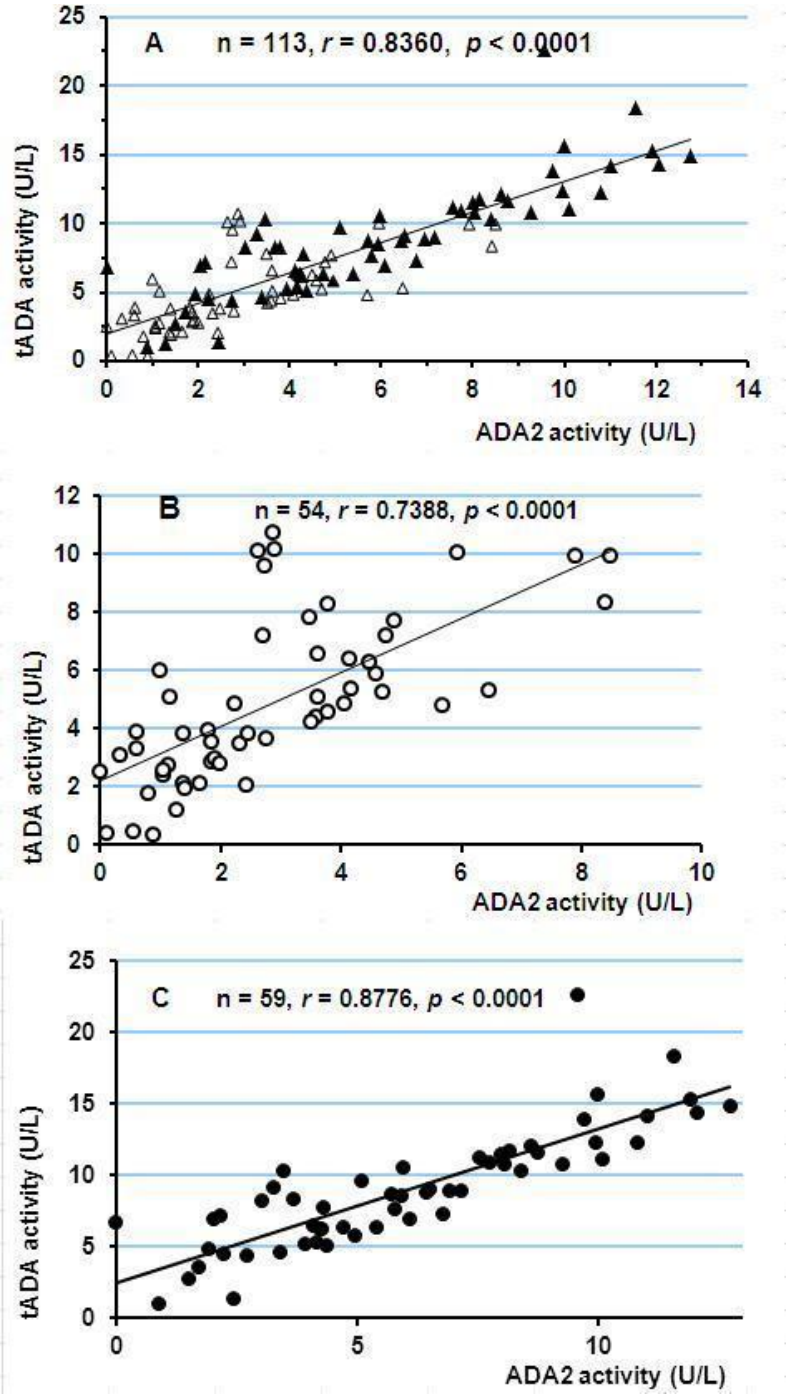

Fig. 1. Correlation between plasma tADA and ADA2 activity.
A – in all 113 individuals (Δ – healthy controls, ▲– T1DM patients); B – in 54 healthy controls, C – in 59 T1DM patients. r – Spearman's correlation coefficient.

In our studies, we did not find elevation of the ADA activity in blood plasma of children compared to adults; the data presented in our previous publications [4, 5]. Nevertheless, the other group of authors reported much higher level of ADA isoforms in children in comparison with this parameter in adults [27].

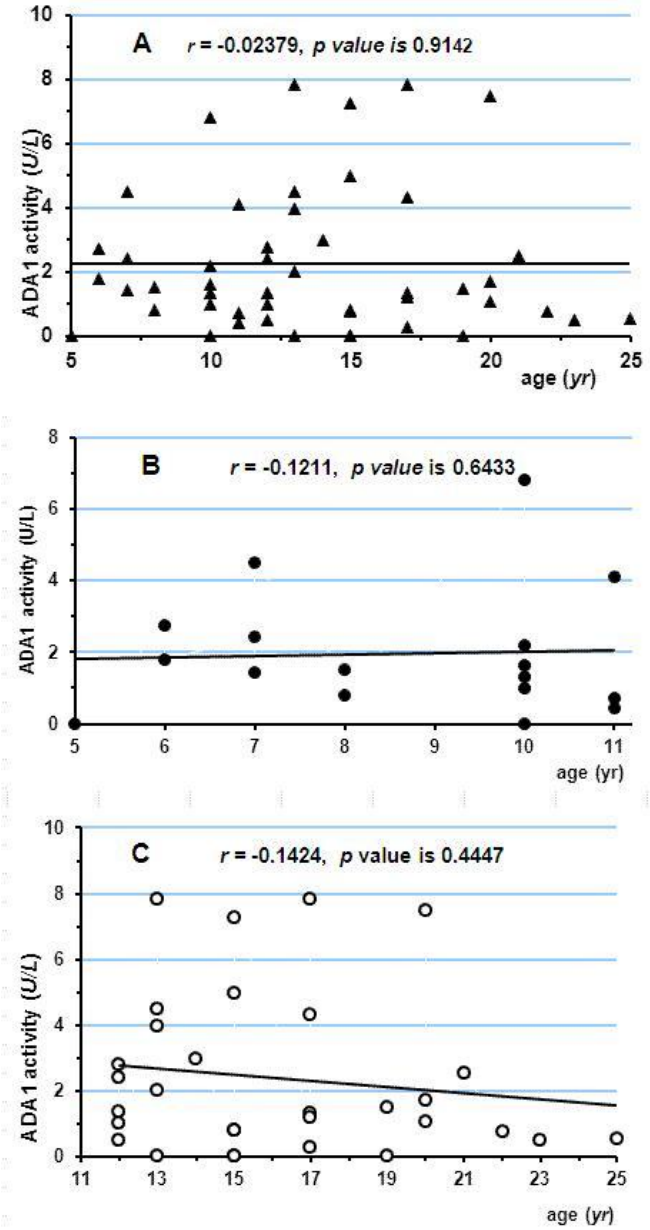

Fig. 2. The correlations of ADA1 activity and age of healthy controls: A – all participants, B – group I (<11years) and C – group II (>11 years).

Fig. 2 represents correlations between the ADA1 isoform activity and age of participants in the healthy control group.

Calculated data did not reveal any difference in ADA1 and ADA2 activities between age groups of children.

The Spearman correlation between the plasma ADA activity in T1DM patients and the level of glycated hemoglobin shown in Fig. 3.

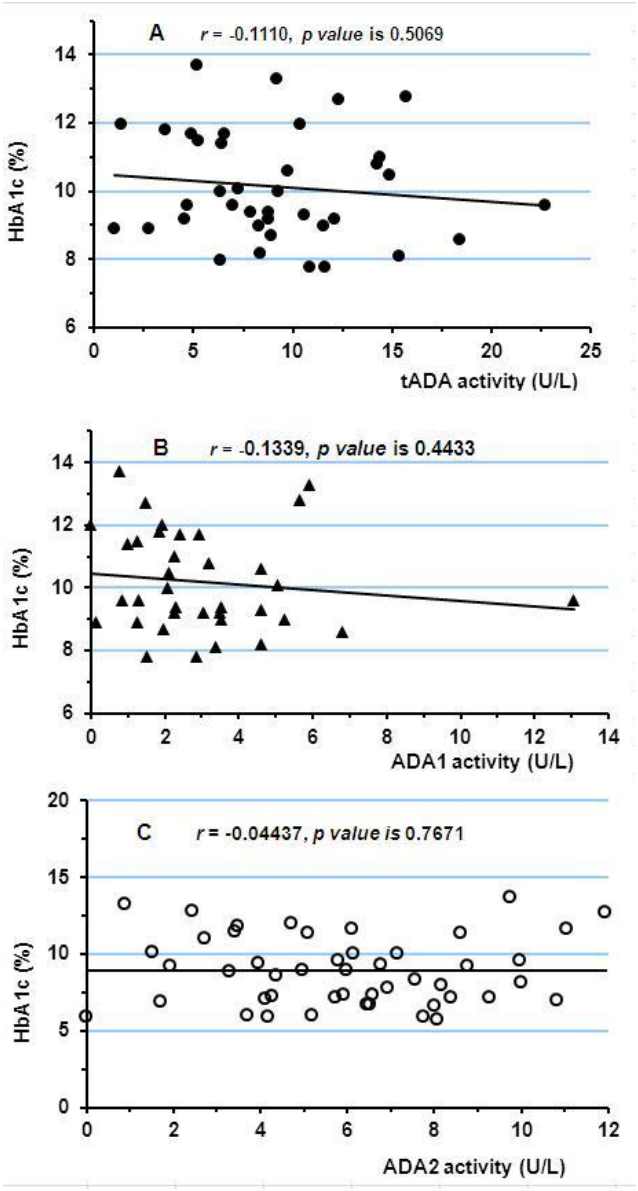

Fig. 3. The correlations of ADA activity (tADA, ADA1, ADA2) and HbA1c in blood plasma, of patients with T1DM

There was no correlation between the levels of ADA activities and of glycated hemoglobin in the blood plasma of patients depending of compensation, Fig. 3.

Fig. 4. represents the experimental points of tADA activity in plasma of T1DM patients and healthy controls.

Based on the data obtained, it can be assumed that the threshold of tADA activity in plasma for patients with T1DM is about 5 U/L.

Autoimmune diseases characterized by the abnormal immune response against self-tissue, which caused by the failure of immune homeostasis nature. The role of ADA1 in the cellular immunity was first identified in patients with severe combined immune- deficiency [22]. The pathogenesis of ADA2 deficiency includes lymphoproliferation, cytopenia, and variable degrees of immunodeficiency, and still is poorly understood [29]. The high activity of ADA was considered to be a reflection of immunological disturbance observed in such severe diseases, as tuberculosis [4-5,35], infectious mononucleosis and other diseases [18].

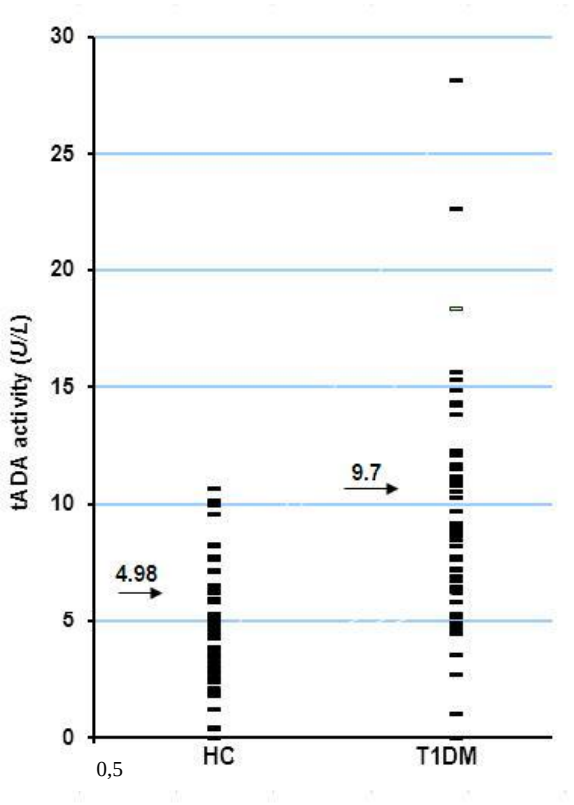

Fig. 4. Total ADA activity in blood plasma of patients with T1DM and healthy controls. Arrows show the mean values of tADA activity.

Elevated ADA activity has been reported in T2DM, and it correlated with glycemic control in T2DM patients. It was shown that Hb1Ac is increased in T2DM patients depending on the duration of the disease, and a positive correlation between the levels of HbA1c and ADA2 activity was observed [23, 25].

Our study aimed to evaluate the plasma level of ADA activity in the T1DM pathogenesis in young patients living in Armenia. Our results have shown that ADA activity increased in T1DM patients compared to healthy controls (Table 3). This increase occurred mainly due to elevation of ADA2 activity. The tADA and ADA2 activity increased significantly for almost two times in the T1DM cases ($p < 0.0001$). ADA1 was also elevated, nearly by 1.5 times ($p < 0.0232$), but less pronounced.

However, contrary to the reported in the literature data on T2DM, we did not observe a positive correlation between the increasing activity of ADA and the level of glycated hemoglobin in type 1 diabetic young patients (Fig 3).

Interestingly, in the nonobese T2DM patients, characterized by low inflammation, the serum ADA was higher, having strong positive correlation with fasting plasma glucose, but not with HbA1c levels [24]. On the contrary, it has been shown that in adolescent T1DM patients correct glycemic control improved with the duration of diabetes [14].

The increase of ADA activity observed was mainly due to elevation of the ADA2 isoenzyme activity. The elevated ADA2 but not ADA1 activity decreased following blood glucose control in the majority of poorly controlled T2DM patients [11].

Diabetes mellitus often accompanied with complications of vision, and it alters the expression of the activity of adenosine metabolizing enzymes in the retina. The increased expression of ADA2 but not of ADA1 isoenzyme, had been observed in the retina of diabetic patients [15]. In children and adolescents with T1DM, the development of diabetic retinopathy depends on compensation and occurs several times more often in children with poor compensation. At HbA1c >7.5% the incidence of diabetic retinopathy significantly increases. This indicator is critical for the development of diabetic retinopathy in children and adolescents with T1 diabetes [1].

It was shown that activated monocytes/macrophages and dendritic cells (DC) at sites of inflammation and in the microenvironment of tumor are thought to be the major source of ADA2 in plasma and pleural fluids [16]. Last years, evidence has accumulated indicating the active participation of DC in the pathology of autoimmune diseases, including diabetes [31, 30]. DCs are

specialized, potent antigen-presenting cells being key regulators of both innate and adaptive immune responses, effectors and tolerance. They have a key role in all stages of the autoimmune diabetes development: in the activation of T cells and maintaining the immune tolerance. It was shown on animal model that tolerogenic DCs are explored as promising players in the T1DM therapy. Up-regulation of ADA expression in DC obtained on mice model is accompanied by robust and spontaneous activation of DC, which suggests that ADA promotes DC activation by removing the suppressive effect of adenosine and thus contributing to the onset of autoimmune T cell dysregulation [20,36]. It was shown that monocytes, differentiating into DC and macrophages, secrete ADA2 into the culture medium, and the level of ADA2 secretion by monocytes differentiating into DC was higher than of monocytes differentiating into macrophages. It was suggested that only ADA2 isoenzyme of ADA is secreted by activated DC [33].

Interestingly, the very high doses of adenosine, achievable only in the presence of inhibitors of ADA, are toxic to murine chondrocytes and induce chondrocyte apoptosis [28]. The affinities of ADA1 and ADA2 isenzymes for substrate, adenosine, differed strongly (Km for ADA2 100 times higher than Km for ADA1), and ADA2 is adapted to maintain the high, but controlled levels of adenosine in pathologic conditions, in ADA1 activity absence. This suggests a regulatory role for ADA2 in inflammation and a potential of ADA2 as a diagnostic biomarker for inflammatory diseases. The significance and specific role of ADA2 isoenzyme needs to be explored in further studies. Although the mechanisms involved in this phenomenon are yet unclear, measurement of serum ADA activity is important for better understanding of the clinical aspects of DM. Thus, adenosine regulation may be a potential approach of intervention in T1DM.

Conclusions

The results demonstrated significant differences between ADA activity levels in the blood plasma of T1DM patients and healthy controls. This increase was mainly due to a twofold increase in the activity of the ADA2 isoenzyme.

We determined the normative range of peripheral blood plasma ADA2 activity in healthy young volunteers and proved the alteration in activity of ADA enzyme implication in T1DM pathogenesis. The measurement of plasma ADA activity is important for understanding the clinical aspects of T1DM and

may be useful in predicting the glycemic and immunological status of patients with T1DM.

Our study revealed also the sex- and puberty-associated differences in ADA1 activity in adolescents/young adults group of healthy individuals, which may be associated with changes during puberty in children.

Competing Interests

None declared.

Acknowledgments

The authors gratefully acknowledge the cooperation of all the people included in the study.

Accepted 05.02.21

Изменения в активности изоформ аденозиндезаминазы в плазме крови у молодых пациентов с сахарным диабетом 1 типа

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В данной работе мы исследовали общую активность аденозиндезаминазы (tADA) и ее изоформ (ADA1 и ADA2) в плазме крови молодых пациентов разного возраста с сахарным диабетом 1 типа, включая детей и подростков. Мы установили нормальный диапазон активности ADA в плазме периферической крови в контрольной группе и подчеркнули различия в активности изоформы ADA1 у подростков: в группе мальчиков активность ADA1 была выше, чем в группе девочек, вероятно, из-за различий в половом созревании у мальчиков и девочек одного возраста. В плазме периферической крови пациентов с сахарным диабетом 1 типа общая активность аденозиндезаминазы tADA (9,69 ± 0,81 U/L) и активность изоформы ADA2 (6,08 ± 0,41 U/L) были в два раза выше, чем у здоровых лиц контрольной группы (4,98±0,4 и 3,0±0,29 U/L, tADA и ADA2 соответственно; p <0,0001). Увеличение активности изоформы ADA1 у пациентов с сахарным диабетом 1 типа (2,8±0,3 U/L), по сравнению со здоровыми контрольными субъектами (2,09±0,27 U/L), также было статистически значимым, но менее выраженным (p<0,027). В заключение отметим, что изменения активности изоферментов ADA свидетельствуют об их значении в патогенезе сахарного диабета 1 типа и важной роли в формировании иммунологического статуса больных детей и подростков.

Ադենոզինդեամինազի իզոձևերի ակտիվության փոփոխությունները 1-ին տիպի շաքարային դիաբետով երիտասարդ պացիենտների արյան պլազմայում

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Այս աշխատանքում մենք ուսումնասիրել ենք ընդհանուր ադենոզինդեամինազի (tADA) և դրա իզոձևերի (ADA1 և ADA2) ակտիվությունը 1-ին տիպի շաքարային դիաբետով տարբեր տարիքի հայ երիտասարդ հիվանդների արյան պլազմայում, ներառյալ երեխաներն ու դեռահասները: Տվյալներից դուրս է բերվել ստուգիչ խմբի ծայրամասային արյան պլազմայում ADA-ի ակտիվության բնականոն տիրույթը: Ցույց են տրվել դեռահասների մոտ ADA1 իզոձևի ակտիվության տարբերությունները. տղաների խմբում ADA1-ի ակտիվությունն ավելի բարձր էր, քան աղջիկների խմբում, հավանաբար միևնույն տարիքի տղաների և աղջիկների սեռական հասունացման տարբերությունների պատճառով:

1-ին տիպի շաքարային դիաբետով հիվանդների ծայրամասային արյան պլազմայում ADA-ի ընդհանուր ակտիվությունը՝ tADA (9,69 ± 0,81 U/L) և ADA2 իզոձևի ակտիվությունը (6,08 ± 0,41 U/L) կրկնակի բարձր էին, քան առողջ ստուգիչ խմբում (tADA և ADA2, համապատասխանաբար՝ 4.98 ± 0.4 և 3.0 ± 0.29 U/L, p <0.0001): ADA1 իզոձևի ակտիվության աճը 1-ին տիպի շաքարային դիաբետով հիվանդների մոտ (2.8 ± 0.3 U/L), համեմատած առողջ ստուգիչ անհատների հետ (2.09 ± 0.27 U/L), նույնպես վիճակագրորեն նշանակալի էր, բայց քիչ արտահայտված (p < 0,027):

Այսպիսով, ADA ֆերմենտի իզոձևերի ակտիվության փոփոխությունները ցույց են տալիս դրանց կարևոր նշանակությունը 1-ին տիպի շաքարային դիաբետի պաթոգենեզում և դերը հիվանդ երեխաների ու դեռահասների իմունաբանական կարգավիճակի ձևավորման գործում:

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