Transformation of conventional T cells into regulatory T cells in children with metabolic syndrome

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Abstract

Background: Much research has been done in the recent years to establish an association between obesity, metabolic syndrome and the immune system. Numerous data suggest that the decreased number and/or function of regulatory T cells (Treg cells) can lead to chronic minimal inflammation present in patients with obesity and trigger formation of atherosclerotic plaque.

Aim: To generate Treg cells from the peripheral blood in children meeting the diagnostic criteria of metabolic syndrome.

Methods: A total of 25 children with metabolic syndrome and 25 controls were enrolled in the study. Peripheral blood was collected, $CD4^+/CD25^-$ cells were separated and cultured for 4 weeks in the presence of a Treg expander (CD3/CD28) and interleukin-2. The expression of the transcription factor FoxP3 as a Treg marker was assessed before and after culture using reverse transcriptase polymerase chain reaction (RT-PCR) and flow cytometry.

Results: Before the culture we observed a slightly lower percentage of Treg cells in children with metabolic syndrome vs controls. After the culture we noted a significant increase in mRNA expression and in the percentage of FoxP3-positive cells. We observed no differences in the results between the children with metabolic syndrome and the controls.

Conclusions: Our study shows that it is possible to generate Treg cells from peripheral blood of children with metabolic syndrome. In future, these findings could be used to develop a model of immunotherapeutic intervention for patients at risk of cardiovascular disease.

Key words: metabolic syndrome, obesity, regulatory T cells, children

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INTRODUCTION

Metabolic syndrome (MS) is a constellation of certain cardiovascular (CV) risk factors, i.e. central obesity, dyslipidaemia, elevated blood pressure and hyperglycaemia. The incidence of MS in developed countries continues to be high and rising. This is also the case in children and adolescents [1]. Although this phenomenon is most pronounced in the United States, it has also reached the proportions of an epidemic in Europe, including Poland [2, 3]. Interventions affecting the environment, dietary habits and lifestyle, including physical activity, are needed to reduce CV risk [4]. Attempts are also being made to elucidate the pathogenesis of obesity and MS, so that effective treatments can be implemented. Much focus has been placed in the recent years on the immune basis of

atherosclerosis and obesity [5]. It has been postulated that the presence of chronic minimal inflammation may underlie insulin resistance induced by obesity. The formation of adipokines by the adipose tissue may play a significant role here [6]. Chronic inflammation accompanying obesity may also accelerate endothelial dysfunction and the development of atherosclerotic changes in blood vessel walls, leading in consequence to CV disease.

Following the reactivation (in the mid-1990s) of the theory of suppressor T cells, which are currently referred to as regulatory T cells (Treg cells), they are being extensively researched in terms of effects on immune system homoeostasis, including their role in the development of obesity and atherosclerosis [7, 8]. Very little data have been accumulated so far on this topic and the research has mainly been carried out on an animal model, namely the apolipoprotein E-deficient mice [9]. These findings offer an opportunity to implement immune intervention as a therapy to cause a suppression or regression of changes leading to myocardial infarction or stroke. The results of our experiments suggest the presence of abnormal number and/or function of Treg cells present in obese children with MS [10, 11]. Our study was an attempt to generate Treg cells from the peripheral blood of children meeting the diagnostic criteria of MS. These cells could be of use in the treatment of immune abnormalities associated with obesity.

METHODS

We prospectively enrolled 25 children between 12 and 18 years of age (median age: 16 years) with the diagnosis of MS established according to the IDF 2007 criteria (central obesity plus at least two of the following criteria: hypertension, hypertriglyceridaemia, low HDL-cholesterol and fasting hyperglycaemia/diabetes mellitus) [12]. The reference group (n = 25) consisted of patients hospitalised at the Department of Paediatrics, Endocrinology and Diabetes with a Cardiology Unit, Medical University in Bialystok, Poland, without obesity, without metabolic, endocrine or immune disorders, infections or chronic infections (with the most common diagnosis being a history of arrhythmia or haemodynamically insignificant congenital heart disease). In each of the subjects we determined height, body mass, body mass index (BMI), waist circumference and centile values of all these parameters. The following laboratory parameters were determined in both groups of patients: fasting plasma glucose, lipid parameters (total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol), uric acid, urea, creatinine, aminotransferases, TSH and 24-h cortisol profile. Additionally, patients in the study group underwent oral glucose tolerance test with measurement of glucose and insulin at 0 and 2 h and the calculation of the HOMA index as the product of the fasting plasma insulin level and the fasting plasma glucose level, divided by 22.5. Blood pressure was assessed by performing 3 measurements at rest. Patients in either group received no treatment. The experiment was carried out in accordance with the principles laid down in the Declaration of Helsinki and had been approved by the Bioethics Committee of the Medical University in Bialystok, Poland. An informed consent was obtained from the parents/caregivers of each child.

A 5-mL heparinised blood sample was collected from each patient, mononuclear cells were isolated (Histopaque, Sigma) and subsequently non-regulatory T cells (i.e. CD4$^+$/ /CD25$^-$ cells) were isolated (using the equipment and reagents manufactured by Miltenyi Biotec: CD4$^+$/CD25$^+$ Regulatory T Cell Isolation Kit). These isolated cells were cultured (37°C, 5% CO_2) for 4 weeks in the presence of a culture medium (RPMI + FBS), Treg expander (the so-called artificial CD3/CD28 dendritic cells manufactured by Dynal) and interleukin-2 (300 IU/mL on day one, followed by 100 IU/mL) (modified procedure according to Longhi et al. [13]). Control cultures with the same medium were also established. Before and after the culture, the cells were analysed for expression of the transcription factor FoxP3 at the level of mRNA (using real-time reverse transcriptase polymerase chain reaction [RT-PCR]) and at the level of the protein (using flow cytometry). The following subpopulations were assessed: CD4$^+$, CD4$^+$CD25$^-$ and CD4$^+$CD25^{high} CD127^{low}FoxP3$^+$. Detailed descriptions of flow cytometry and real-time RT-PCR have been reported previously [14, 15]. The assessment of the regulatory T cell subpopulation by flow cytometry is illustrated in Figure 1 and an example of a RT-PCR reaction for the transcription factor FoxP3 compared to the reference gene GAPDH is given in Figure 2.

Statistical analysis

The results of clinical examinations and flow cytometry did not show a normal distribution (the Kolmogorov-Smirnov and Shapiro-Wilk tests), therefore were analysed using non-parametric tests and are presented as median or percentage values. The data before and after the culture (dependent data) were compared using the Wilcoxon test, while the data obtained for the study group were compared with those obtained for the reference group using the U Mann-Whitney test (independent data). The analysis was conducted using Statistica 9.0. A p value < 0.05 was considered statistically significant. The changes in mRNA expression are presented as a relative change in mRNA content *vs* mRNA content before the culture while the quantities of mRNA in the control culture and the quantities of mRNA in the reference group are presented as values normalised to the reference gene GAPDH. Values between 0.75 and 1.25 reflect no change in mRNA expression, values below 0.75 reflect a lower or reduced expression, while values above 1.25 reflect a higher or increased expression of mRNA for a given gene.
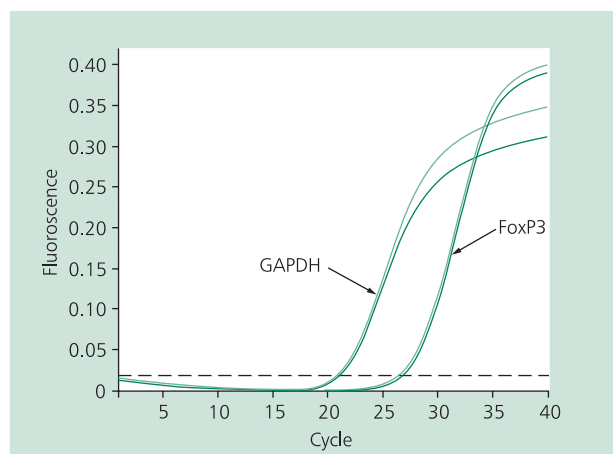
RESULTS
Clinical examination results

The study and the reference groups did not differ between each other in terms of age and gender, although there were
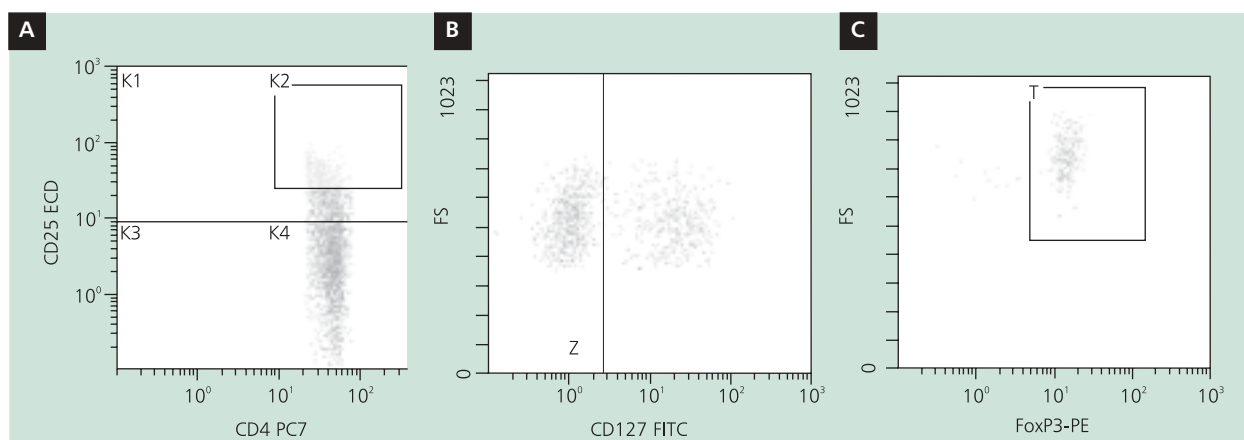
Figure 1. Example flow cytometry data showing the method of assessment of regulatory T cells among mononuclear cells; **A.** $CD4^+CD25^{high}$ cells; **B.** $CD127^{low/-}$ cells; **C.** $FoxP3^+$ cells; FS — forward scatter

Figure 2. An example of expression of messenger ribonucleic acid — mRNA for the transcription factor FoxP3 compared to the control gene GAPDH form the software of the equipment for real-time reverse transcriptase polymerase chain reaction

significantly higher body mass values (including centile values), BMI values, waist circumference values and blood pressure values in the study group (Table 1). In addition, children with the diagnosis of MS had higher glucose levels (both fasting levels and levels following glucose loading), higher total cholesterol levels and lower HDL-cholesterol levels than children in the reference group. There were no differences between the groups in height, levels of uric acid, urea, creatinine, aminotransferases, TSH or the 24-h cortisol profile.

Peripheral lymphocyte percentage counts in children with metabolic syndrome vs children in the reference group

There were no differences in percentage counts of lymphocytes, including the $CD4^+$ and the $CD4^+CD25^-$ subpopulations, between the group of children with MS and the reference group with median counts of 35.0% *vs* 36.5% for $CD4^+$ and 94.2% *vs* 92.9% for $CD4^+CD25^-$, respectively (p > 0.05), although there were slightly lower percentage counts of Treg ($CD4^+CD25^{high}CD127^{low}FoxP3^+$) cells in the peripheral blood of children with MS compared to the children in the reference group (median percentage counts of 2.5% *vs* 3.1% with borderline statistical significance; p = 0.05). The FoxP3 mRNA expression was lower in the mononuclear cells isolated from the peripheral blood of children with MS compared to the children in the reference group (relative expression: 0.5).

Generation of regulatory T cells after the culture

Among the separated $CD4^+CD25^-$ cells before the culture there was a low percentage of regulatory T cells ($FoxP3^+$ expression below 1%, with no difference between the study and the reference groups). Similarly, the presence of mRNA for the transcription factor FoxP3 was observed in small amounts: relative (GAPDH-normalised) expression of FoxP3 was 0.01 in the study group and 0.03 in the reference group.

Following the 4-week culture of $CD4^+CD25^-$ cells with Treg expander and interleukin-2 a significant increase in FoxP3 expression was noted both at the mRNA and the protein levels. The percentage count of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ regulatory T cells after the culture ranged from 65% to 90% of the cells (median values in the study and the reference groups: 76.2% *vs* 82.9%; p > 0.05) compared to the range of 1–3% in control cultures (the medium alone without stimulation). Similarly, an increase in FoxP3 mRNA quantity was observed: a relative increase in expression in the study group compared to before-culture values (4.8 *vs* 5.1 in the reference group).

DISCUSSION

Treatment with regulatory T cells offers hope for redressing the immune balance in disorders caused by immune system

Table 1. The clinical characteristics of the children in the reference group and the children with the diagnosis of metabolic syndrome

	Reference group	Metabolic syndrome	P
Body mass [kg]	69.5	83.1	< 0.0001
Body mass index [kg/m²]	24.11	28.20	< 0.0001
Waist circumference [cm]	72	88	< 0.0001
Fasting glucose [mmol/L]	4.34	4.80	0.001
Glucose at 2 h [mmol/L]	5.78	6.21	0.04
Fasting insulin [pmol/L]	54	81.6	0.0448
Postprandial insulin [pmol/L]	132.4	369.6	0.0244
Homoeostatic model assessment (HOMA) index	1.730	3.150	0.0251
Total cholesterol [mmol/L]	4.02	4.26	0.0205
Triglycerides [mmol/L]	0.90	1.35	0.0170
LDL-cholesterol [mmol/L]	2.17	2.33	0.0433
HDL-cholesterol [mmol/L]	1.26	1.23	0.0380
Uric acid [mg/dL]	5.41	5.65	NS
Urea [mg/dL]	25.9	26.5	NS
Creatinine [mg/dL]	0.7	0.72	NS
AspAT [IU]	23	20	NS
AlAT [IU]	22	26.5	NS
TSH [mIU/L]	2.115	2.04	NS
Cortisol (morning) [nmol/L]	424	431	NS
Cortisol (night) [nmol/L]	90	103	< 0.3489
Systolic blood pressure [mm Hg]	115	126	< 0.0001
Diastolic blood pressure [mm Hg]	66	72.5	< 0.0001

dysfunction [16]. Obesity and its consequences are accompanied by specific changes in pro- and anti-inflammatory cytokines and cells. It is currently believed that chronic inflammation leads to the formation of atherosclerotic plaques and vascular complications [17]. Abnormal counts and/or function of regulatory T cells play an important role in destabilisation of the atherosclerotic plaque [18]. Implementation of immunomodulation aimed at suppressing the immune response may therefore become a tool to prevent the complications of obesity, MS and atherosclerosis [19]. In our experiment, we showed a possibility of obtaining regulatory T cells from peripheral blood lymphocytes in children meeting the diagnostic criteria of MS. Our results may be a starting point for designing a therapeutic intervention in children and adolescents at risk of CV disease.

In our study, we used the transcription factor FoxP3 as a Treg marker. Although most authors agree that FoxP3 is a gold standard in determining regulatory T cells, isolated reports indicate that this factor may be expressed during activation of all T cells [20, 21]. In order to transform conventional into regulatory T cells we used the CD3-CD28 combination and interleukin-2. Other authors used TGF-β and interleukin-2 for this purpose, obtaining functional regulatory T cells [22]. Glisic et al. [23] used a similar protocol (CD3/CD28 + IL-2) to generate Treg cells in patients with type 1 diabetes mellitus. The principal measures of culture efficacy was a functional assessment in proliferative tests as well as FoxP3 expression. We observed a different effectiveness of regulatory T cells depending on the timing of blood collection: recently diagnosed vs long-standing disease. Similar problems may well occur in further studies investigating the immunotherapy of obesity and atherosclerosis. In our study, we did not collect data on the duration of the disease. In contrast to type 1 diabetes mellitus, it is difficult to unequivocally determine the onset of the immune abnormalities accompanying obesity in children.

Data on the assessment of regulatory T cells in humans with CV risk factors are very scanty. One of the reports suggests a decreased suppressive activity of the Th$_3$ lymphocyte subpopulation (T cells that produce TGF-β_1) in patients with acute coronary syndrome [24]. Another study, the only one carried out in children, did not confirm any differences in the percentage counts of regulatory T cells between obese and non-obese children, although the sample size was very small in this experiment (12 vs 10 children) [25]. Histopathologic examinations of blood vessels (obtained during surgical procedures or autopsies) revealed low percentage counts of regulatory T cells in atherosclerotic lesions compared to healthy blood vessels [26].

Chronic inflammation accelerates the development of atherosclerotic changes, and the immune response is, without a doubt, involved in this process. It has been known for a long time that this response involves Th_1 lymphocytes, and a considerable body of evidence has been accumulated in the recent years to support the involvement of regulatory T cells in this phenomenon as well [27]. Preliminary data on the treatment of atherosclerotic changes with regulatory T cells are only available for the mouse model. For instance, infusion of Treg cells has been shown to redress the immune balance between pro- and anti-inflammatory cytokines and to suppress atherosclerotic changes in mice with apolipoprotein E deficiency [28]. It seems that one of the underlying mechanisms of this phenomenon may involve the suppressive effect of homocysteine on Treg cells. In another experiment, an atherosclerosis vaccine based on a fragment of the apolipoprotein B peptide also exerts its effects by affecting regulatory T cells [29]. Some authors, however, suggest that induced and natural Treg cells differ between each other and it is not certain whether the induced cells will be fully functional [30]. Other data indicate that infusion of regulatory T cells may only lead to atherosclerotic plaque stabilisation but not regression [31].

CONCLUSIONS

The results of our study show that it is possible to generate regulatory T cells from conventional T cells isolated from peripheral blood of children with MS. These findings should be used to develop future immunotherapy intervention regimens in groups of patients with CV risk factors.

Conflict of interest: none declared

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Przekształcenie konwencjonalnych limfocytów T w komórki T-regulatorowe u dzieci z zespołem metabolicznym

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Streszczenie

Wstęp: W ostatnich latach poszukuje się związków między otyłością i zespołem metabolicznym a układem immunologicznym. Wiele danych wskazuje, że niedobór i/lub dysfunkcja jednej z subpopulacji limfocytów, tj. komórek T-regulatorowych, może przyczyniać się do przewlekłego, minimalnego stanu zapalnego obecnego u pacjentów z otyłością, w konsekwencji prowadzącego do powstania blaszek miażdżycowych.

Cel: Celem pracy była próba generacji limfocytów T-regulatorowych z krwi obwodowej dzieci spełniających kryteria rozpoznania zespołu metabolicznego.

Metody: Do badania włączono 25 dzieci z zespołem metabolicznym i 25 dzieci z grupy kontrolnej. Materiał do badań stanowiła krew obwodowa, z której wyizolowano limfocyty o fenotypie $CD4^+/CD25^-$, aby następnie umieścić je w 4-tygodniowej hodowli z odczynnikiem Treg-expander (CD3/CD28) oraz interleukiną 2. Ekspresję czynnika transkrypcyjnego FoxP3 jako markera limfocytów T-regulatorowych oceniano przed hodowlą i po jej zakończeniu za pomocą łańcuchowej reakcji polimerazy (na poziomie mRNA) oraz cytometrii przepływowej.

Wyniki: Przed hodowlą zanotowano nieznacznie niższe odsetki limfocytów T-regulatorowych u dzieci z zespołem metabolicznym w porównaniu z dziećmi z grupy kontrolnej. Po hodowli wykazano istotny statystycznie wzrost ekspresji mRNA dla FoxP3 i wzrost odsetka komórek FoxP3-dodatnich. Nie obserwowano różnic między hodowlami z krwi dzieci z zespołem metabolicznym a hodowlami limfocytów dzieci z grupy kontrolnej.

Wnioski: Uzyskane wyniki świadczą o możliwości generacji limfocytów T-regulatorowych z krwi dzieci z rozpoznaniem zespołu metabolicznego i powinny być wykorzystane w przyszłości do konstruowania modeli interwencji immunoterapeutycznej w grupie pacjentów z obecnymi czynnikami ryzyka rozwoju chorób układu sercowo-naczyniowego.

Słowa kluczowe: zespół metaboliczny, otyłość, limfocyty T-regulatorowe, dzieci

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