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Distribution of FoxP3$^+$regulatory T-cells in rat's pancreatic lymph nodes under streptozotocin-induced diabetes and metformin administration

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Key words: *Diabetes mellitus, FoxP3, Metformin, Treg.*

Type 1 diabetes mellitus is a T-cell mediated autoimmune disease characterized by the destruction of β-cells of the pancreas. Numerous studies have demonstrated the key role of FoxP3$^+$ regulatory T-cells in the development of type 1 diabetes.

The aim of our study:1) to determine the expression patterns of transcription factor FoxP3 in the pancreatic lymph node cells in animal model of diabetes mellitus and 2) to assay an effect of metformin on these processes.

Methods and results.The study was conducted on 60 male Wistar rats with streptozotocin-induced modeling diabetes with various duration of the diabetes. Within development of type 1 diabetes mellitus the number of Treg pancreatic lymph nodes has been reduced.

Conclusion.These findings demonstrate the ability of metformin to increase the number of Treg.

Розподіл FoxP3$^+$ регуляторних Т-клітин у панкреатичних лімфатичних вузлах у щурів при стрептозотоцин-індукованому діабеті та при корекції метформіном

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Цукровий діабет 1 типу є Т-клітинно-опосередкованим автоімунним захворюванням, що характеризується руйнуванням β-клітин підшлункової залози. Численні дослідження показали ключову роль FoxP3$^+$ регуляторних Т-клітин у розвитку цукрового діабету 1 типу. З метою визначення особливостей експресії транскрипційного фактора FoxP3 у клітинах панкреатичних лімфатичних вузлів при експериментальному цукровому діабеті та після введень метформіну здійснили дослідження на 60 самцях щурів лінії Вістар, у яких моделювали стрептозотоцин-індукований цукровий діабет із різною тривалістю патологічного процесу, а також після введень метформіну. Визначено, що при розвитку цукрового діабету 1 типу кількість Treg у панкреатичних лімфатичних вузлах знижується, а метформін демонструє здатність збільшувати щільність їхньої популяції.

Ключові слова: *цукровий діабет, FoxP3, метформін, Treg.*

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Распределение FoxP3$^+$ регуляторных Т-клеток в панкреатических лимфатических узлах у крыс при стрептозотоцин-индуцированном диабете и при коррекции метформином

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Сахарный диабет 1 типа – Т-клеточно-опосредованное аутоиммунное заболевание, характеризующееся разрушением β-клеток поджелудочной железы. Многочисленные исследования показали ключевую роль FoxP3$^+$ регуляторных Т-клеток в развитии сахарного диабета 1 типа. С целью определить особенности экспрессии транскрипционного фактора Foxp3 в клетках панкреатических лимфатических узлов при экспериментальном сахарном диабете и после введений метформина провели исследования на 60 самцах крыс линии Вистар, у которых моделировали стрептозотоцин-индуцированный сахарный диабет с разной продолжительностью патологического процесса, а также после введения метформина. Установлено, что при развитии сахарного диабета 1 типа количество Treg в панкреатических лимфатических узлах снижается, а метформин демонстрирует способность увеличивать плотность их популяции.

Ключевые слова: *сахарный диабет, FoxP3, метформин, Treg.*

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Type1diabetes mellitus (T1DM) is a T-cell mediated autoimmune disease characterized by the destruction of pancreatic β-cells. Numerous studies have demonstrated the key role of FoxP3$^+$regulatory T-cells (Tregs) in the development of T1DM [16]. Most studies of the role of Tregs in T1DM were performed on peripheral blood rather than pancreas or pancreatic lymph nodes. The autoimmune cascade that culminates in diabetes initiates within pancreatic lymph nodes (PLNs). At present all indications are that the PLN are essential in the initial activation of diabetogenic T-cells, prior to their islet migration [*2*]. The importance of the PLN in the development of diabetes was shown among two experimental settings. In the first setting, surgical excision from NOD mice resulted in the absence of diabetes without apparent priming of T cells [5].In the second setting, offspring of pregnant NOD mothers injected with lymphotoxin-β receptor fused to human Ig Fc lacked lymph nodes and did not develop diabetes [10]. The level of autoreactivity was limited in these mice lacking PLN. In both situations, transfer of activated diabetogenic T-cells resulted in diabetes.

The mammalian target of rapamycin (mTOR) is now appreciated to be a central regulator of immune responses. Emerging evidence suggests that mTOR activity regulates development of CD4 T cell subsets, that include helper subsets (Th1, Th2, Th17, and follicular helper T or Tfh) and Treg [15]. Powell and colleagues have shown that differentiation T-cells into Th1, Th2, and Th17 is inhibited by CD4 T cells with a conditional deletion of mTOR[9]. Sufficient mTOR activity induces effector CD4$^+$ T helper subsets. A complete or strong block of the signaling mTORpathway

prevents the generation of these effector cells, instead promotes Treg differentiation [1]. Metformin as an anti-diabetes drug might have double-edged sword effects (1) by acting on the organism to decrease hyperglycaemia and hyperinsulinemia in diabetic patients and (2) at the cellular level, by inhibiting the mTOR-supporting pathway through AMP-Activated Protein Kinase-dependent (AMPK) and independent mechanisms [13].

The aim of our study:

1) to determine the expression patterns of transcription factor FoxP3 in the pancreatic lymph node cells in animal model of diabetes mellitus and 2) to assay an effect of metformin on these processes.

Materials and methods

Animals and tissue isolation. Sixty six-month-old male Wistar rats were purchased from Veterinary Medicine Association Ltd. «Biomodelservis» (Kiev) and they were kept a 12-h light/dark cycle with controlled humidity (60–80%) and temperature (22±1°C). All experiments on animals were performed according to international principles of «The European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes» (Strasbourg, 1986) and «General ethical principles of animal research» (Ukraine, 2001). Test animals were divided into 5 experimental groups: control group, which were administered once intraperitoneally (i.p.) with 0.5 ml of 0.1 M citrate buffer (pH = 4.5) (group 1); rats with 21-day and 35-day STZ-induced diabetes mellitus (SIDM) (group 2 and 3); rats with 21-day and 35-day SIDM (group 4 and 5), which were respectively treated with metformin during 3 and 5 weeks at a dose of 50 mg/kg from 1-st day of diabetes induction.

Induction of experimental diabetes. Streptozotocin (STZ) (SIGMA Chemical, USA) was injected intraperitoneally at a dose of 50 mg/kg dissolved in 0.5 ml of 0.1 M citrate buffer (pH 4.5). Blood samples were collected from the tail vein. Determination of glucose concentration in peripheral blood was performed by the glucose-oxidase method using the instrument «BIONIME Rightest TM GM 110» (Switzerland) in 12 hours on the 3rd, 7th, 14th, 21st and 35th days after injection of STZ. Measurement of blood glucose levels was performed after 6 hours from the last meal on the 3rd day after injection of STZ. For further studies animals with fasting glucose level > 8.0 mmol/l were selected.

Immunohistochemical staining. Population structure of FoxP3^{+}-cells was studied on the basis of analysis of serial histological sections and their data of morphometric and densitometric characteristics. For this study a rotary microtome MICROM HR-360 (Microm, Germany) did 5 micron serial sections of PLN. They were deparaffinized in xylene, rehydrated in a descending carried concentrations of ethanol (100%, 96%, 70%), washed-up in 0.1 M phosphate buffer (pH = 7.4) and stained with a rabbit polyclonal primary antibodies (PAbs) to the transcription factor FoxP3 (Santa Cruz Biotechnology, USA) for 18 hours in a humid chamber at t = 4°C. After washing of the excess primary antibody in a 0.1 M phosphate buffer, sections were incubated for 60 minutes (t = 37°C) with a secondary antibody molecule to the total rabbit IgG (Santa Cruz Biotechnology, USA), conjugated with FITC. After incubation, sections were washed with 0.1 M phosphate buffer and embedded in a mixture of glycerol and a phosphate buffer (9:1) for the subsequent fluorescence microscopy. Treated histological sections were studied using computer software Image J (NIH, USA). Images which were obtained on the microscope PrimoStar (ZEISS, Germany) in the ultraviolet spectrum of excitation 390 nm (FITC) with using a highly sensitive camera AxioCam 5c (ZEISS, Germany) and the software package for receiving, archiving, and preparing images for publication AxioVision 4.7.2 (ZEISS, Germany) were immediately entered into the computer. In the automatic mode, areas with the statistically significant fluorescence characteristics of cells that express FoxP3 were identified. Morphometric and densitometric characteristics of immunopositive cells were determined. When painting the PAbs FoxP3^{+}-cells in the poracortical zone and medullary cords of PLN was examined.

Statistical analysis. All the experimental data were processed using statistical package of EXCEL (Microsoft Corp., USA), STATISTICA 6.0 (Stat-Soft, 2001). All variables were presented as the mean value (m) and the standard error of the mean (SEM). To identify the significance of differences in the results of studies of experimental and control groups of animals Student's coefficient (t) was determined, after which the sample was determined by the possibility of difference (p) and the average confidence interval. Critical significance level when testing statistical hypotheses assumed was equal to 0.05.

Results and discussion

Development of experimental streptozotocin-induced diabetes mellitus (3-week ESIDM) led to a change in representation of FoxP3^{+}-lymphocytes in paracortical zone and medullary cords of pancreatic lymph nodes (PLN), in which the total density decreased by 25% ($p < 0.05$) and 28% ($p < 0.05$) as compared to control group (*fig.1 A*). Indicators in the group of rats with 5-week ESIDM decreased by 50% ($p < 0.05$) only in medullary cords of PLN. The distribution into individual classes of FoxP3^{+} lymphocytes in the PLN in the group of experimental animals with a 3-week ESIDM showed an increase in medullary cords of PLN percentage of FoxP3^{+}-lymphoblasts by 40% ($p < 0.05$), FoxP3^{+}-medium lymphocytes by 42% ($p < 0.05$), respectively, the percentage of FoxP3^{+}-small lymphocytes decreased by 21% ($p < 0.05$), and their population density by 42% ($p < 0.05$), respectively.

After the administration of metformin in rats with 3-week ESIDM total density of FoxP3^{+}-lymphocytes increased by 96% ($p < 0.05$) in the paracortical area and by 93% ($p < 0.05$) in medullary cords of PLN rats with respect to 3-week ESIDM (*fig.1B*). The distribution into individual classes of FoxP3^{+}-cells in the paracortical zone in the group of animals with 3-week ESIDM: the population density of FoxP3^{+}-lymphoblast increased by 83% ($p < 0.05$), the average FoxP3^{+}-medium lymphocytes increased by 100% ($p < 0.05$), FoxP3^{+}-small lymphocytes increased by 2,2-fold ($p<0.05$). In medullary cords of PLN a change in the form of increasing the number of FoxP3^{+}-lymphoblasts by 46% ($p<0.05$), and small lymphocytes by 2,7-fold ($p < 0.05$) has taken place, too.

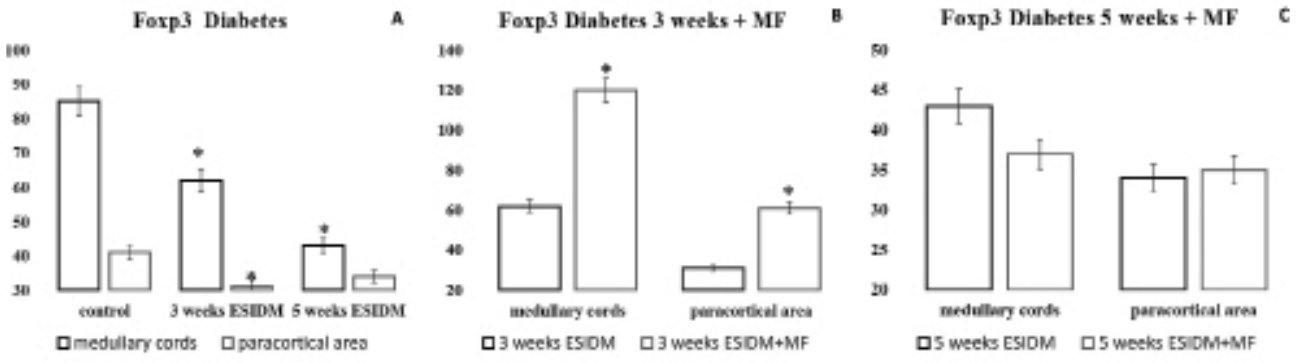

Fig. 1. The number (on 1 mm^2) of FoxP3$^+$ cells in paracortical area and inmedullary cords of PLN. The development of experimental STZ-induced diabetes (ESIDM)(A). The administration of Metformin (MF) (B, C) to experimental animals. *Note:* * – P < 0.05.

The fluorescence intensity of FoxP3$^+$-cells appeared to be significantly increased in medullary cords of PLN as at 3-week ESIDM and at 5-week ESIDM, namely FoxP3$^+$-medium and small lymphocytes. At 5-week ESIDM FoxP3$^+$-lymphoblasts concentration of transcription factor FoxP3 by 10% ($p < 0.05$) increased (*fig.2* A-C). Administration of metformin resulted in a decrease in medullary cords of PLN concentration FoxP3 in FoxP3$^+$-medium and small lymphocytes by 4%-13% ($p < 0.05$) with respect to indicators of a group of animals with a 3-week ESIDM that did not take metformin. A FoxP3 concentration in FoxP3$^+$-lymphoblasts, on the contrary, increased by 9% ($p < 0.05$). In experimental animals with a 5-week ESIDM concentration of transcription factor FoxP3 in FoxP3$^+$-small lymphocytes was significantly increased in the paracortical area of PLN (*fig.2 A-C*).

The obtained results coincide with the results of other investigators. PLN-derived Treg functions were impaired in T1DM subjects. Functional defects in T-regulatory cells are key features of the pancreatic lymph nodes in patients with type 1 diabetes [4]. Willcox et al. (2009) analyzed postmortem pancreatic samples from 29 T1DM patients. FoxP3$^+$Tregs were only found in islets from a single patient, suggesting that the lack of Treg cells may play a pivotal role in autoimmunity among T1DM patients [18].

Compared to the results of studies in human subjects, several animal studies have focused on Tregs in the pancreas and PLNs. Tonkin et al. (2009) generated TGF-β-induced islet-specific Tregs and demonstrated their ability to suppress the transfer of diabetes into NOD scid mice using diabetic spleen cells [17]. Infiltration of both Teffs and Tregs were observed in the pancreas, suggesting the active role that Tregs play in the inflammatory site. By the induction of hemopoietic chimerism, antea-diabetic mice were restored to adequate pancreatic islet function even after they had been rendered hyperglycemic. Compared to the antea-diabetic mice, the numbers of Tregs in the PLNs were significantly decreased in NOD mice, indicating that Tregs in the PLNs had a potential role in ameliorating disease progression in the model [14]. This observation supports conclusions that: (1) the lack of the Treg cells in the PLNs of the NOD mice may play a role in diabetogenesis; and (2) that improved Treg cell accumulation in the PLNs reflects recovery of the peripheral tissue tolerance in the PLNs of the antea-diabetic NOD mice and is part of the mechanisms leading to the resumption of euglycemia in these animals. The accumulation of Treg cells that was observed in the islets and PLNs in mice models likely played a significant role in contr olling anti-islet inflammation [7].

Yaochite J. et al. (2013) have shown the kinetics of Treg and Th17 subsets and disease severity during the streptozotocin (STZ)-induced diabetes mellitus [19]. Susceptible C57BL/6 mice were administrated with multiple low doses of STZ and we evaluated the frequency/absolute number of these T-cell subsets in the PLNs and Th1, Th17, Treg cytokine production in the pancreatic tissue. During the initial phase of diabetes development (day 6), they noted increased numbers of CD4(+) and CD8(+) T-cells in PLNs. The numerous of Th17 cells in PLNs were enhanced too. In addition,

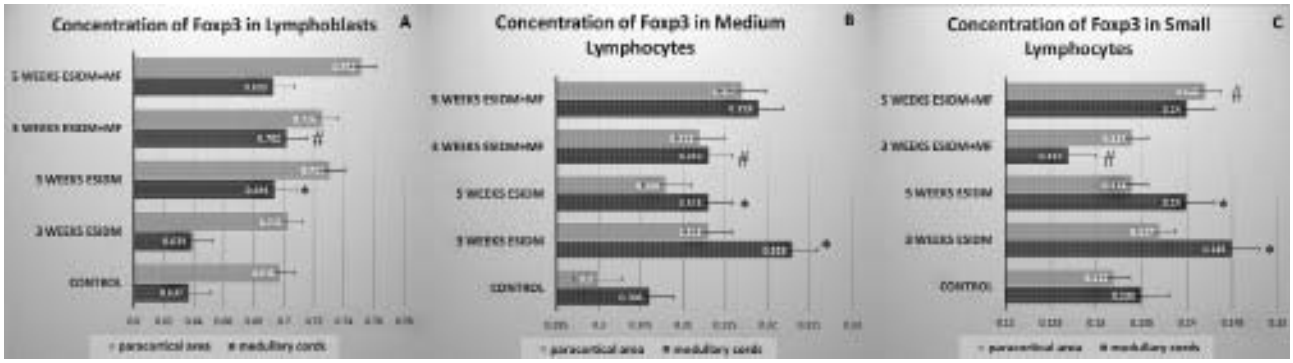

Fig. 2. Concentration of transcription factor FoxP3(fluorescence intensity in arbitrary units, AU) in FoxP3$^+$- Lymphoblast's (A), FoxP3$^+$-Medium Lymphocytes (B) and FoxP3$^+$- Small Lymphocytes (C). *Note:* * – P <0.05 relative to the control, $^{\#}$– P <0.05 relative to diabetes.

the early augment of interferon gamma (IFNγ), tumoral necrosis factor (TNFα), IL-6 and IL-17 levels in pancreatic tissue correlated with pancreatic islet inflammation and mild β-cell damage. Notably, the absolute number of Treg cells increased in PLNs during over time when compared to control group. Interestingly, increased IL-10 levels were associated with control of the inflammatory process during the late phase of the type 1 diabetes (day 25). Ferraro A. et al. (2011) phenotypically and functionally characterized Tregs and Th17 cells residing in the pancreatic-draining lymph nodes (PLNs) of 19 patients with type 1 diabetes and 63 nondiabetic donors and those circulating in the peripheral blood of 14 type 1 diabetic patients and 11 healthy subjects [4]. Autors have shown that the key features of the PLN of diabetic subjects are *1*) an unbalanced Treg/Th17 cell ratio; *2*) increased IL-17–producing $CD4^+$ T-cells in response to diabetes-related antigens; and *3*) the presence of $CD25^{bright}$ T-cells epigenetically imprinted to be Tregs but which overall are reduced in FoxP3 expression and have a defective suppressive activity. Thus, one could speculate that certain Tregs in diabetic patients, for still unknown reasons, turn off FoxP3 expression once they migrate to the pancreas, leading to defective control of Th17 cells, which expand and cause the destruction of the pancreas by releasing IL-17. Zdravkovic N. et al. (2009) used the model of multiple low doses of streptozotocin (MLD-STZ) induced diabetes in susceptible C57BL/6 mice and resistant BALB/c mice to study these regulatory mechanisms [20]. They have shown that low dose cyclophosphamide (CY) sensitive $CD4^+CD25^+FoxP3^+$Treg cell-dependent mechanisms can be demonstrated in C57Bl/6 mice susceptible to MLD-STZ diabetes induction. CY pretreatment decreased $Foxp3^+$ cell count, glycemia, glycosuria and insulitis.

A key role is played by signaling systems acting as metabolic «sensors» linking energy/nutritional status to regulatory T-cell functions. Dynamic activity of intracellular metabolism, through mTOR modulation, might represent a shift in understanding the molecular mechanisms governing Treg cell tolerance [6]. Recent studies revealed that mTOR signaling impacts conventional T-cell homeostasis, activation and differentiation [11]. mTOR integrates nutrient sensing and signaling pathways to match the energy requirements of activated T-cells. Th1, Th2, and Th17 cells require high levels of glycolysis that is mediated by high mTOR activity, whereas Treg differentiation requires low mTOR activity, reduced glycolysis, and lipid oxidation. Consistent with the above findings, Treg display higher levels of AMP kinase activity and preferential lipid oxidation for their energy requirements [12]. The AMP-activated kinase acts as a sensor of the AMP/ATP ratio, which is increased during hypoxia and inhibits mTOR kinase to promote mitochondrial oxidative metabolism rather than glycolysis [8]. Interestingly, activation of AMP kinase via Metformin, a drug used to treat diabetes mellitus, increased the Treg population in the $CD4^+$ T-cell compartment in an *in vivo* murine model of asthma.

Conclusion

Our results demonstrate the metformin is able to increase the Treg number in PLN, by the way these effects manifest on the third week of the diabetes mellitus and they reduce on the fifth week of the pathological process duration.

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