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Mechanisms of oral tolerance to insulin in offspring of rats with experimental gestational diabetes

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Abstract

Background: The aim of our work was to find out the mRNA gene expression level of *Aire, Deaf1, Foxp3, Ctla4* and *IL10* in mesenteric lymph nodes in the offspring of rats with experimental gestational diabetes and in conditions of insulin oral tolerance formation.

Material and Methods: Using molecular genetic and immunofluorescence techniques we investigated the mRNA gene expression level of *Aire, Deaf1, Foxp3, Ctla4 and IL10* in the offspring of rats with experimental gestational diabetes and in conditions of insulin oral tolerance formation. To determine the level of mRNA studied gene RT-PCR was performed in real time thermocycler CFX96 ™ Real-Time PCR Detection Systems.

Results: We observed such violations of immunotolerance: AIRE gene repression, reduced mRNA levels of Deaf1 and the transcription factor Foxp3. This was accompanied by inhibition of gene expression suppressor cytokine IL-10 and negative costimulatory molecules Ctla4.

Conclusion: Oral insulin during the first 2 weeks graded these changes, causing transcriptional activation of genes *AIRE, Deaf1, Foxp3, Ctla4* and *Il-10*.

Key words: *Experimental gestational diabetes, mesenteric lymph nodes, Aire, Deaf1, Foxp3.*

Introduction

Formation of immunological tolerance to autoantigens is an important mechanism that prevents the development of autoimmune diseases. In recent years there was found extrathymic expression a number of peripheral tissue-specific antigens (PTSAs), including such pancreatic antigens as insulin and proinsulin. Ectopic transcription regulator of them is autoimmune regulator (Aire) (1). A lot of extrathymic Aire-expressing cells (eTACs) are found in lymphatic nodes (LN) and represent one of the critical factors of peripheral immunological tolerance (PIT) (2). Stromal mesenteric lymph node cells that include fibroblast reticular cells, follicular dendritic cells and lymphatic endothelial cells express PTSAs (3), but their expression regulates not only eTACs. They control transcription regulator Deaf1 (deformed autoregulatory factor 1) (4). Consequently Aire and Deaf1 are important differentiation regulators of inducible regulatory T-cells (iTreg), other participant that can proceed negative control of AID developing. iTreg can express transcription factor Foxp3 (5), their action realized through production of suppressor cytokines - IL10, IL13, IL35, TGFβ (6), perforin/granzyme-dependent cytolysis of effector cells and depends on the expression of negative costimulatory molecules such as CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) (7).

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Thus stromal Deaf1-expressing cells of mesenteric lymph nodes (MLN) produce retinoic acid that promotes $Foxp3^+$-regulatory T-cells (8). Yang S. et al. (2015) demonstrated ability of Aire generate in the prenatal period (up to 10 days after birth inclusive) special population of $FoxP3^+$Treg-cells, which remains stable in adults and mice (9).

Consequently intranatal hyperglycemia that develops in gestational diabetes (GD) can influence on the morphogenesis of the immune system and leads to violations of PIT formation to pancreatic antigens. Using mucosa is an attractive way to treatment by administering antigens as tolerogen, especially in young and healthy children. In animal models oral or intranasal administration of antigens can induce PIT, and the main places for PIT induction are mesenteric lymph nodes (MLN), where intensive activation of naïve T-cells and their differentiation into subpopulation of effector cells are happened (10). Therefore, **the aim** of our work was to find out the mRNA gene expression level of *Aire, Deaf1, Foxp3,* C*tla4* and *IL10* in mesenteric lymph nodes in the offspring of rats with experimental gestational diabetes and in conditions of insulin oral tolerance formation.

Material and methods

The studied animals were divided into 6 experimental groups each contains 20 rats. Descendants of intact Wistar rats (males) 1 month of age (group 1) and 6 months of age (group 2). Their mothers were admistered 0,5 ml 0,1 M citrate buffer (pH=4,5) on the 15th day of pregnancy once intraperitoneally. Descendants of rats Wistar (males) with experimental gestational diabetes (EGD) 1 month of age (group 3) and 6 months (group 4). Their mothes were admistered 45 mg/kg of streptozotocin once intraperitoneally on the 15th day of pregnancy. Descendants of rats with EGD 1 month of age (group 5) and 6 month of age (group 6) which received short-acting human insulin orally using a pipette for the first 14 days of life (ACTRAPID® HM, NOVO NORDISK, Denmark) at a dose of 30 IU (1050 µg = 1,05 mg, 1 IU corresponds to 35 µg of anhydrous human insulin).

As objects for molecular genetic studies using real-time reverse transcription polymerase chain reaction (rt-pcr) techniques were MLN of experimental animals. They were placed in the Bouin's fluid, dehydrated with graded concentrations of ethanol and embedded in paraffin. Molecular genetic studies performed on archival material 2 years old. RNA extracted from histology sections 15 µm thick. They were dewaxed in xylene and rehydrated with descending concentrations of ethanol (100%, 96%, 70%). Total RNA was procured from samples by use of "Trizol RNA Prep 100" (Isogen Lab LTD, Russia), that contains Trizol reagent (lysis reagent, which includes denaturing agent guanidine thiocyanate and phenol with pH = 4.0) and ExtraGene E (slurry mixture of ion-exchangers). RNA isolated according to set protocol.

For reverse transcription and obtaining cDNA we used RT-1 set «Syntol» (Russia). The reaction mixture is a total volume of 25 µl and contains 1 µl of Random-6 primer, 2 µl total RNA, 8,5 µl deionized and cleaned of nucleases H2O, 12,5 µl 2,5x reaction mixture and 1 µl of reverse transcriptase MMLV-RT. Reverse transcription conducted at 45°C for 45 min, then 92°C for 5 min for inactivation of MMLV-RT.

To determine the level of mRNA Aire (NM_001106379.1), Deaf1 (NM_031801.1), Foxp3 (NM_001108250.1), IL10 (NM_012854.2) and Ctla4 (NM_031674.1) we used thermocycler CFX96™Real-Time PCR Detection Systems («Bio-Rad Laboratories, Inc.», USA) set of reagents Maxima SYBR Green/ROX qPCR MasterMix (2X) (ThermoScientific, USA). The final reaction mixture for amplification includes coloring SYBR Green, Maxima HotStartTaq DNA Polymerase, 0,2 µl of forward and reverse specific primers, 1 µl cDNA. The reaction mixture brought to total volume 25 µl by adding deionized H2O. Specific primer pairs (5'-3') for analysis of target and reference genes were selected by the software PrimerBlast (www.ncbi.nlm.nih.gov/tools/primer-blast) and produced by Metabion (Germany) (Table.1).

After initial denaturation at 95°C for 10 min amplification consisted of 45 cycles and conducted under the following conditions: denaturation - 95°C for 15 sec., annealing at 59-61°C for 30-60 sec., elongation at 72°C for 30 sec. As reference gene to determine the relative value of changes in the expression level of target genes was used glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Normalized relative quantity of cDNA target genes was determined by the method ΔΔCt. Statistical data analysis of PCR were conducted using available software CFX Manager ™(Bio-Rad, USA).

Table 1. Primer list used in the study.

Gene	Primer	Tm,°C	Product length (bp)	Exon junction
Aire	F = GCCTAAAGCCAGTGATCCGA R = TCTCTACCCTGGGTTCCCTTT	59.82 59.85	43	850/ 851
Deaf1	F = GCAGAGAGGAAGGAGCAGTC R = GTGCACTCACTCATGGCCT	59.82 60	59	1605/ 1606
Foxp3	F = CGAGACTTGGAAGTCAGCCAC R = TCTGAGGCAGGCTGGATAACG	60.94 61.91	61	214/ 215
IL10	F=AGTGGAGCAGGTGAAGAATGA R=GACACCTTTGTCTTGGAGCTTATTA	59.02 59.06	49	445/ 446
Ctla4	F = TACAGTTTCCTGGTCACCGC R = AGGACTTCTTTTCTTTAGCGTCCT	59.97 59.96	57	567/ 568
GAPDH	F = GCCTGGAGAAACCTGCCAAG R =GCCTGCTTCACCACCTTCT	61 60	52	825/ 826

Experiment included negative controls: no template controls (cDNA and mRNA) and no reverse transcriptase control. All amplification reactions were performed on individual samples three times.

Results

Investigation of Aire gene expression in MLN showed that in the offspring of EHD rats there is a significant reduction of mRNA of autoimmune regulator 8,1 times ($p < 0,05$) in the 1-month-old rat and 2,3 times ($p < 0,05$) in the 6-month-old animals compared with control groups (Fig. 1 A, B).

mRNA content of transcription regulator Deaf1 in the 1-month-old animals significantly didn't changed, and in the 6-month-old descendants we observed its reduction 9,2 times ($p < 0,05$) (Fig.1 C, D). As for mRNA of transcription factor Foxp3, there was revealed a significant decrease 50 times ($p < 0,05$) in the 1-month-old rats of this group, and 2,5 times ($p < 0,05$) in the 6-month-old animals compared with control (Fig.1 E, F).

Groups of the offspring of EHD rats, that were administered oral insulin for a duration of 14 first days of life showed growth of Aire gene transcriptional induction in the 1-month-old rats. Contents of mRNA autoimmune regulator increased 13,2 times ($p < 0,05$), in the 6-month-old it grows 2 times ($p < 0,05$) (Fig.2 A, B). Transcription regulator Deaf1 in first age group showed a significant increase 11,5 times ($p < 0,05$), and in the next group came back to level of EHD (Fig.2 C, D).

Studies have shown that expression of the transcription factor Foxp3 in the 6-month-old rats was increase of Foxp3 mRNA 5,2 times ($p < 0,05$), in the 6-month-old animals rise was 3,3 times ($p < 0,05$) (Fig.2 E, F).

In experimental groups that received oral insulin were also investigated mRNA expression of costimulatory molecules Ctla4 and Treg-dependent suppressor cytokine IL-10. There were obtained such results: the relative quantity of Ctla4 mRNA gene increased 12,2 times ($p < 0,05$) at 1 month of age, at 6 months of age it significantly not changed (Fig.3 A, B). Contents of mRNA IL10, on the contrary, in first age group was unaltered, but in the 6-month-old rats it increased 15 times ($p < 0,05$) (Fig.3 C, D).

Discussion

Peripheral immunological tolerance could take place systemically after antigen feeding, there has been no reason to doubt that it takes place largely within the local mucosal immune system. MLN are the main site for oral tolerance (OT) induction between other lymphoid tissues. Presentation of fed antigens occurs preferentially in the MLN, rather than the Peyer's patches (PP) (11). OT cannot be induced in mice lacking MLN, but it is unaffected in mice, that lack PP (12). Furthermore, it has been shown that DCs constitutively traffic from the intestinal epithelium and Peyer's patches to the MLN, so there is a clear mechanism whereby antigen can be picked up at the intestinal epithelial surface and taken to the MLN, where T cell tolerization can occur. (13).

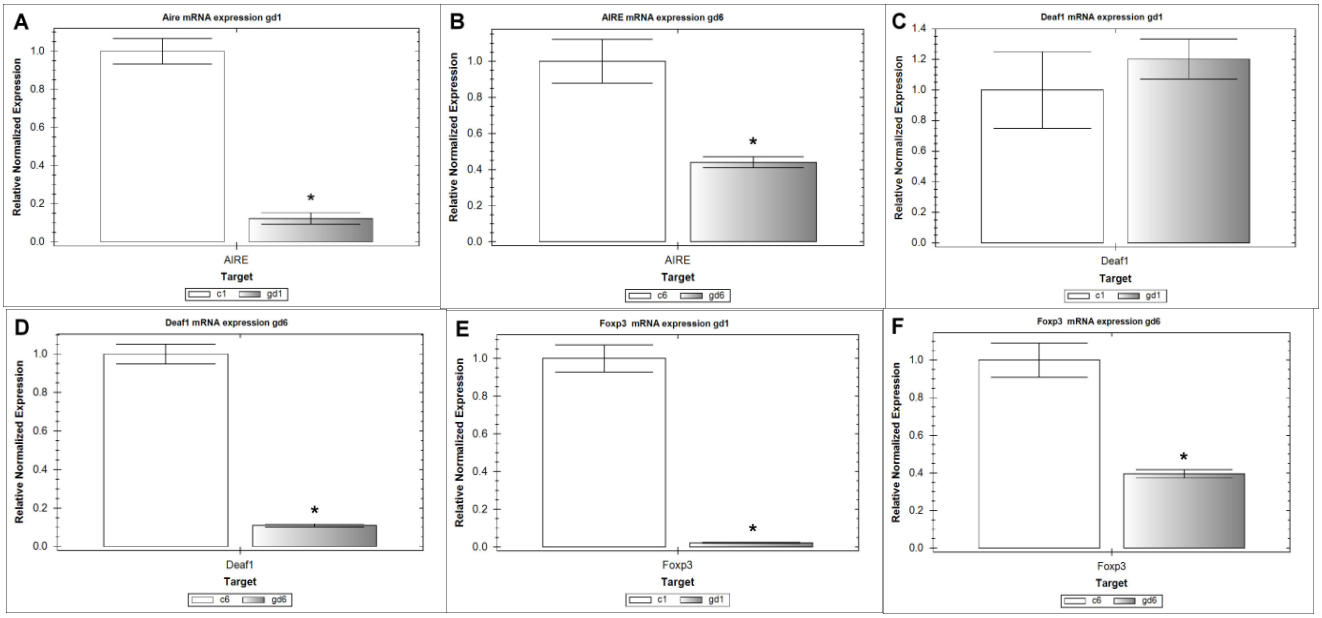

Figure 1. Normalized relative quantity of mRNA Aire (A, B), Deaf1 (C, D) and Foxp3 (E, F) genes in MLN cells. Normalized to reference gene GAPDH by the method ΔΔCt. c1, c6 – control 1 and 6 months; gd1, gd6 – offspring of EHD rats.

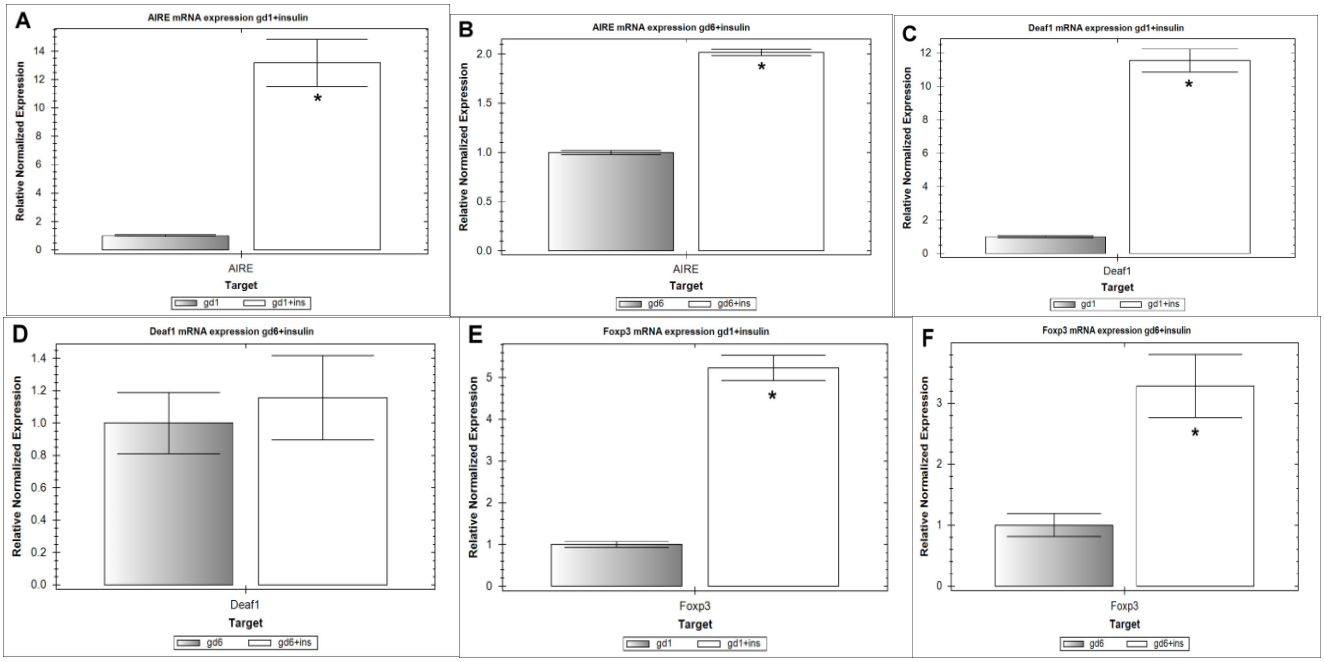

Figure 2. Normalized relative quantity of mRNA Aire (A,B), Deaf1 (C,D) and Foxp3 (E,F) genes in MLN cells. Normalized to reference gene GAPDH by the method ΔΔCt. gd1, gd6 – offspring of EHD rats; gd1+ins, gd6+ins – after insulin administrations.

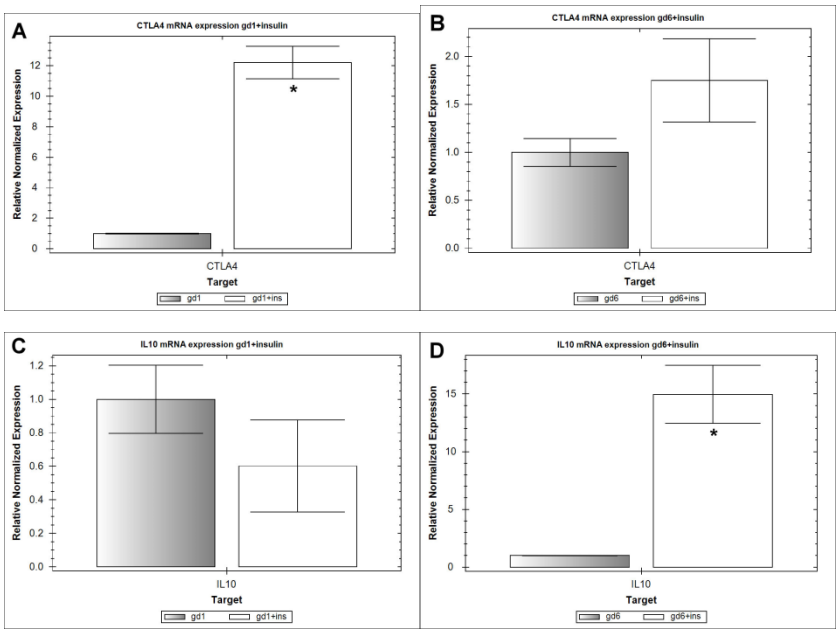

Figure 3. Normalized relative quantity of mRNA Ctla4 (A,B) and IL10 (C,D) genes in MLN cells. Normalized to reference gene GAPDH by the method ΔΔCt. gd1, gd6 – the offspring of EHD rats; gd1+ins, gd6+ins – after insulin administration.

Worbs et al. (2006) evidently shows that in mice the MLN are an obligatory and exclusive site of oral tolerance induction (14). TCR transgenic T cells were seen to proliferate at day 2 after antigen feeding in the MLN, but not until day 4 in peripheral lymph nodes. Using the drug FTY720 (blocker of lymphocyte migration from LN) or surgical excision of the MLN violated the process. The authors then used two experimental systems to show that antigen-laden DCs migrating from the intestinal wall to the MLN can tolerize MLN-resident T cells. First, in an animal with an intestinal transplant in which the vascular supply is anastomosed to the host, but the lymphatic system is separate, an oral protein challenge caused T cells only in the host and not the graft MLN to be stimulated. Second, in mice deficient for the chemokine receptor CCR7, which have impaired DC migration to MLN. In this group T cell stimulation and functional oral tolerance were abrogated. These experiments allowed them to conclude that oral tolerance is exclusively generated in the MLN with antigen transported from the intestinal surface by DCs through the afferent lymphatics.

On the other hand, clinical manifestation of T1DM is preceded by the development of autoantibodies to different islet autoantigens, marking the loss of immunological tolerance to β cells. Development of T1DM in individuals with multiple islet autoantibodies is almost inevitable, and increases the urgency to intervene in the disease process before hyperglycaemia develops. Most trials attempting immune intervention have been conducted in patients with recent onset T1DM (usually within 6 weeks of diagnosis), and have had varying but only limited success. (15). This outcome might partly result from the stage of disease and progressive loss of β cells, in addition to the burden of poor glycaemic control and metabolic β-cell stress over and above the inflammatory insult. Unfortunately, the few attempts to prevent T1DM using immunotherapy in seropositive individuals at risk of the disease were unsuccessful. (16). Although several explanations for this setback exist — including the type of drug or islet autoantigen, dosing and method of administration. Primary prevention before seroconversion might conceivably be an attractive and simple solution, that is why we have chosen this path. The correctness of this approach is confirmed by the preliminary results of the Bonifacio E. et al. (2015) study.

They performed a double-blind, placebo-controlled, doseescalation, clinical pilot study to assess the immune responses and adverse events associated with orally administered insulin in healthy, autoantibody-negative children, but who were genetically at-risk of developing T1DM (17). This treatment approach proved safe, with no serious adverse events related to treatment. No episode of hypoglycaemia (in spite of daily insulin doses up to 67.5mg), allergic reactions or induction of unwanted islet autoimmunity were observed. Instead, moderate changes in immunity to insulin were noted CD4+-T-cell proliferative responses to insulin regulatory type, which was confirmed by transcriptome analysis of clonal CD4 T-cells reactive to insulin or proinsulin and finding Foxp3 (18). Exploiting the mucosa is an attractive choice of treatment to administer antigens as tolerogen, especially in young and healthy children. In animal models, oral or intranasal administration of an antigen can induce specific immune regulation of the tolerogen of choice. (19). Our results also confirmed by the fact that the decrease of Deaf1 mRNA in conditions of streptozotocin-induced diabetes inhibits Foxp3+-regulatory T-cells differentiation in pancreatic lymph nodes of rats (20) .

Conclusion

There is a transcription repression of Aire gene, decrease level of Deaf1 mRNA in the offspring of rats with EGD that violates ectopic transcription of pancreatic antigens in MLN. Reduction of mRNA Foxp3 level leads to a deficiency of suppressor signaling that also confirmed inhibition of gene expression of supressor cytokine IL10 and negative costimulatory molecules Ctla4.

Oral administration of insulin during the first 2 weeks graded these changes, causing transcription activation of AIRE, Deaf1, Foxp3, Ctla4 and IL10 genes.

Ethics Committee Approval: Ethics Committee approval was received for this study from the ethics committee.

Informed Consent: NA

Peer-review: Externally peer-reviewed.

Conflict of Interest: No conflict of interest was declared by the author.

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