The immune background of type-1 diabetes mellitus patomechanism and the cardiovascular complications

Ph.D. Doctoral Thesis

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INTRODUCTION

The main topic of my thesis was the examination of the type-1 diabetes mellitus (T1DM) and the involvement of heat shock proteins as possible target antigens in the autoimmune diabetic process. Besides the immunogenic patomechanism I examined the effect of immunization with presumedly patomechanism-specific antigens as a potential novel treatment mode in T1DM.
Furthermore, in addition to the immunological aspects of T1DM, the cardiovascular complications caused by diabetes were also analyzed.
In the experiments carried out in T-lymphocytes prepared from freshly diagnosed T1DM patients and healthy controls I examined the possible factors of pathomechanism underlying the diabetic autoimmune processes. The aim was to determine the shift in Th1/Th2 immune response characterized in terms of T cell-specific cytokine production upon stimulation with presumed target antigens (p277, LAK). In case of in vivo animal experiments I examined the effect of immunization with heat shock proteins on Streptozotocin (STZ) induced diabetic wild type (WT) and histidine decarboxylase knockout (HDC-KO) mice and rats.
The third part of my work encompassed electrophysiological measurements. In these experiments I compared the cardiac electrophysiological parameters of WT and HDC-KO mice and the STZ diabetes-induced alterations in cardiovascular electrophysiological functions in both.

The patomechanism of T1DM

It is generally accepted that auto-reactive T-cells mediate the destruction of the β-ells in T1DM.
The β-cell destruction can be induced by both direct and indirect mechanisms:
Direct destruction of β- cells: The auto-antigens presented by MHCI molecules on the β-cells are recognized by antigen specific $CD8^+$ cytotoxic T cells. These effector cells may cause the apoptosis of islet β-cells by direct cell contact.
Indirect destruction of beta cells: The auto-antigens presented by MHCII molecules on the APCs (macrophages, dendritic cells) are recognized by $CD4^+$ helper T-cells. These cells can induce the expression of co-stimulatory molecules (pl. CD28/CD80), and can enhance the release different cytokines (pl. IFN-γ, TNF-α) and nitrogen monoxide (NO) from $CD8^+$ cells which can cause death of the β-cells.

It can be concluded that β-cell destruction is a cell-mediated disease and both $CD8^+$ cytotoxic and $CD4^+$ helper cells might be involved in the diabetogenic process.
$CD4^+$ T helper cells are differentiated by distinct cytokine profiles and effector functions.
The Th1 cells and the pro-inflammatory Th1 cytokines (IL-12, IFN-γ, IL-2, TNF-α and TNF-β) may activate the macrophages, cytotoxic T cells and the natural killer cells therefore they are capable to activate the cellular immune response. In contrast, the Th2 cells and the anti-inflammatory Th2 (IL-4, IL-10 and IL-13) cytokines play a major role in the regulation of the humoral immune response.
Th1 and Th2 cells negatively cross-regulate each other's function through their respective cytokines. Th1 cytokines (e.g. IFN-γ) induce Th1 activity and suppress Th2 activity, whereas Th2 cytokines (IL-4 and IL-10) promote Th2 cells while inhibiting Th1 activity and cytokine release.
$CD4^+$ Th2 cells play a prominent role in the maintenance of the immune tolerance to β-cells therefore these cells can prevent the autoimmune process by inhibition of auto-reactive Th1 cells.
According to a recent hypothesis, a shift in the physiological Th1/Th2 immune balance can lead to pathologically increased immune response and consequently, a T-cell mediated autoimmune destruction of β-cells.
In case of this Th1/Th2 imbalance the auto-reactive Th1 cells will loose the Th2 cell regulation and can activate the cytotoxic T-cells.
Based on current results, decreased activity of the regulatory cell (nTreg, Tr1, Th3) may also play an important role in the development of the autoimmune process.
Taken together, the decreased activity of the regulatory and Th2 type immune cells is crucial for the development of autoimmune diabetes.

Possible target-antigens of the diabetogenic autoimmune process

The possible target-antigens of the autoimmune diabetogenic process are as follows: insulin, glutamate decarboxylase (GAD65), tyrosine phosphatase-like protein (IA-2) and heat shock proteins (hsp).
Among the possible heat shock protein targets in the autoimmune process the most remarkable are the 60 kD heat shock proteins (hsp60) and hsp epitopes p277 and LAK peptide.
Antibodies and T-cells reactive to hsp60, hsp65 have been detected in mice with autoimmune diabetes induced by the multiple low doses of β-cell toxin STZ. T-cells reactive to the p277 peptide have also been found in STZ susceptible C57BL/6KsJ mouse strain. Moreover, both hsp 60 and p277

reactive T-cells and antibodies have been detected in NOD/Lt mice in the course of the spontaneous autoimmune insulitis.
The multiple low-dose STZ induced diabetic animal model can demonstrate well the role of the hsp60 autoimmunity in the patomechanism of both toxin induced and spontaneous autoimmune diabetes. The multiple low doses of STZ may serve as a stress factor and can induce hsp60 expression on the β-cells rendering these cells more susceptible to the auto-reactive immune response against hsp60.
Another target-antigen candidate is the LAK peptide which shares homology with GAD. Elevated anti-LAK antibody level was detected in the serum of T1 diabetic children.
The role of heat shock proteins is also supported by the fact that single administration of the heat shock proteins (hsp60, hsp65) or peptides (p277) could induce diabetes in NOD or C57BL/6KsJ mice.

Immunization with heath shock proteins, a novel prospect in the T1DM therapy

The classic therapy of diabetes is insulin which is a substitutive type of medication.
The most recent attempts tend to prevent or arrest the diabetogenic autoimmune process by immune therapy.
The easiest procedure is offered by immune suppression but because of the general effects and the serious toxicity this application is fairly limited. Another option might be the antibody-based immunotherapy trying to inactivate the immune component of the autoimmune process by different monoclonal antibodies.
However, possibly the most adequate therapy could be the immunization/vaccination with the target-antigens of the autoimmune process.
The therapeutic effect might be the suppression of Th1 driven pathogenic/destructive autoimmune process by induction of a protective, hsp60 specific Th2 response. The immunization can presumably activate further regulatory T-cells to prevent β-beta cell destruction caused by Th1 cells.
The effect of immunization was examined both in humans an animal models.
Vaccination with different hsps (hsp60, hsp65) and hsp peptide (p277) has prevented the development of multiple low-dose STZ induced diabetes in C57BL/6KsJ mice and spontaneous diabetes in NOD mice.

In NOD mice due to immunization there was a shift from Th1-driven pathogenic autoimmune process to a protective Th2 antibody response and the Th2 cytokine level had enhanced.
In phase II human clinical trials treatment with a vaccine containing peptide p277 (DiaPep277) was reported to be effective by preserving endogenous insulin production. Patients treated with Diapep277 produced less IFN-γ and more IL-10/IL-13 in response to the peptide. T-cell reactivity to p277 showed an enhanced Th2 cytokine phenotype.
It has been concluded that the shift induced in the immune response had protective effect and was able to maintain the insulin production and slow down the progression of the disease.
The favorable effect of the vaccination can be the suppression of pro-inflammatory, destructive Th1 cells and immune responses. This effect can be due to p277 activated IL-10 secretory cells that could be both Th2 type immune cells and $CD4^+CD25^+$ nTreg cells. With high probability it can be concluded that all these cells may contribute to the therapeutical effect of the vaccination.

The cardiovascular complications of diabetes mellitus

Diabetes mellitus is associated with increased risk of sudden cardiac death which cannot easily be attributed to general pathophysiological causes - such as atherosclerosis, hyperlipidemia, hypertension or heart failure. Patients with diabetes mellitus exhibit a high incidence of diabetic cardiomyopathy, characterized by complex changes in the mechanical and electrical properties of the heart. In diabetic patients, the most prominent electrical alteration is the prolongation of the QT interval and the accompanying arrhytmia.
The majority of earlier studies aimed to elucidate the cellular mechanism of diabetes-induced repolarization abnormalities were performed in rats. In STZ induced diabetic rats the APD has been found significantly prolonged and the V_{max} significantly decreased, whereas no differences were observed in action potential amplitude and resting membrane potential.
The maximum rate of depolarization (V_{max}) is Na^+ channel dependent therefore the decreased amplitude of Na^+ current and the reduced expression of Na^+ channels could be responsible for the V_{max} depression.
Furthermore these studies suggested that the decreased amplitude of the transient outward K^+ current (I_{to}) and the reduced expression of Kv4.3/Kv4.2 channel proteins were responsible for the elongation of repolarization in diabetic rats.
KK/Ta mouse is an animal model of non-insulin dependent diabetes mellitus. In this animal model the significant decrease of V_{max} and

prolongation of APD was detected as well. A blocker of transient outward current, 4-aminopyridine failed to cause further prolongation in the KK mice, suggesting that similarly to diabetic rats there is an I_{to} malfunction in diabetic mice.
These findings partly correlate with the results obtained from alloxan-induced diabetic canine heart. The prolongation of the QT was accompanied by significant reduction in the density of the transient outward K^+ current (I_{to}) and the slow delayed rectifier K^+ current (I_{Ks}).
It can be concluded that the prolonged APD might be due to diabetes-induced decreased activity of transient outward K^+ and slow delayed rectifier K^+ channels.

AIMS

Human in vitro experiments

To determine the shift in Th1/Th2 immune response based on the spectrum of cytokine release after stimulation with the supposed target-antigens (p277, LAK)

In vivo experiments on diabetic mice (WT/HDC-KO) and rat models
Trial of immunization

To establish the multiple low-dose STZ induced autoimmune diabetic model in WT and HDC-KO mice
To establish the multiple low-dose STZ induced autoimmune diabetic model in Wistar rats
To examine the effect of immunization with p277 and hsp65 on diabetic WT and HDC-KO mice
To examine the effect of immunization with hsp65 on diabetic rats

Electrophysiological experiments

To characterize and compare the cardiac electrophysiological parameters of WT and HDC-KO mice.
To examine the effect of STZ induced diabetes on these electrophysiological parameters in WT and HDC-KO mice.

MATHERIALS AND METHODS

Subjects

Diabetic and healthy controls

A total of 11 T1 diabetic patients (4–11 days after insulin initiation) were included into this study. Patients were eligible if the time period between the first adequate treatment with insulin and blood sampling was less than 2 weeks. Nine healthy volunteers were included in the study as controls.

Animals

In mice experiments wild type (WT) male BALB/c and histidine decarboxylase-knockout mice on BALB/c background (25–29 g, age: 6 month) were used. In rat experiment we used Wistar (180-200g) rats.

Methods

In vivo protocols and methods

Induction of diabetes

Diabetes was induced using a low intraperitoneal dose (40 mg/kg) of STZ dissolved in 0.1 mol/l citrate buffer, given for 3 consecutive days. Control animals received equivalent volume of vehicle.

Blood glucose measurement

Blood glucose level was measured immediately before the first treatment ("0 week") and every second week after STZ administration. Blood samples were obtained by puncture of the retro orbital venous plexus and glucose concentration was measured by a commercially available colorimetric kit. Changes were followed for 6 weeks and hyperglycemia was defined as a plasma glucose level>11mmol/l.

Treatment regimes

The experimental mice (both WT and HDC-KO mice) were assigned to 4 groups, 10 mice in each. Two groups received only vehicle, next two STZ treatment alone and finally two-two the same in combination with peptide p277 (1 × 100 μg) i.p. or hsp65 (recombinant *Mycobacterium bovis* hsp65) (1 × 100 μg) i.p.; hsps, supplemented by Freund complete adjuvant, were given seven days before the STZ treatment.

The rats were divided into four groups. The first, control group (n=5) received only vehicle. The second group (n=4) received only STZ (3x). The rest of the animals were also treated with STZ (3x) but they were further split into two groups. We used hsp65 for i.p. immunization. The members of the third group (n=6) got hsp65 once (1x100 μg), seven days before the STZ (3x) treatment. Subjects of the fourth group (n=8) were treated twice

with hsp65 (100 µg) seven days before and after the STZ (3x) treatment.

In vitro protocols and methods

T-cell investigation: ELISPOT assay

The Enzyme-linked immunosorbent spot (ELISPOT) assay is a common method to differentiate the Th1 and Th2 immune responses based on the cytokine profile.

PBMC were prepared by density gradient centrifugation of heparinised blood from healthy and T1 diabetic blood donors on a Ficoll gradient. After three washes in phosphate-buffered saline (PBS), PBMC were diluted in RPMI 1640 supplemented with 10 % of human donor AB serum, Penicillin (100 units/ml), Streptomycin (100 µg/ml), L-glutamine (2 mM). The cell concentration was adjusted to 1.8 × 106 cells/ml. Then the cells were exposed for 18 hours to target antigens. Following stimulants were used: a combination of 10 ng/ml phorbol 12- myristate 13-acetate and 10 mmol/ml Ionomycin (PI), 10 µg/ml Tetanus toxoid (as positive controls); and the examined test peptides: 15 µg/LAK peptide, 10 µg/mlp277 peptide.

Capture anti-cytokine antibodies (for IFN-γ and for IL-13) were diluted 1 : 100 in sterile PBS and coated to plastic ELISA plates, 50 µl/well and left at +2 °C to +8 °C overnight. After overnight incubation each well was rinsed 5 times with sterile PBS supplemented with 0.05 % Tween- 20. The plates were blocked with 1 % BSA in PBS overnight. After blocking, triplicates of 100 µl cell suspension per well were added and the plates were incubated at 37 °C with 5 % CO2 for 5 hours to capture secreted cytokines. Biotinylated antibodies (for IFN-γ and for IL-13) were diluted 1:100 in PBS supplemented with 1 % bovine serum albumine (BSA).

The cell suspension was removed, the plates were washed several times with PBS and 100 µl of diluted biotinylated detector antibody was added to each well. The plates were left for 1 hour at 37 °C at 5 % CO2- humidified atmosphere. After further washing, 50 µl of diluted gold-labeled anti-biotin antibodies (GABA) (1:50 in PBS-BSA 1 %) was transferred per well. Finally, 30 µl of activator solution was added and cytokines appeared as separate spots. The spots were counted with a Bioreader-3000.

PMA is a non-antigen specific positive control causing cytokine release by activation of Protein kinase C (PKC). In mammals cells ionomycin acts as a potent and selective Ca2+ionophore. The enhancement of Ca2+ influx by Ionomycin can cause non-antigen specific cytokine release. Due to this the combination of the two positive controls is susceptible to induce maximal, non-antigen specific cytokine release.

Analyzing the IFN-γ and IL-13 responses in the control and patient groups, we compared the stimulated responses to the background (BG). P277 and LAK stimulated spots were determined as (mean number of spots in the

presence of antigen) divided with (mean number of spots without stimulation). A response was scored positive when the spot number exceeded 1.25 times the spontaneous cytokine secretion.

Measurement of antibody levels: ELISA assay

We assessed the hsp60-, hsp65- and p277-specific antibody levels by ELISA (Enzyme-linked Immunosorbent Assay) at the end of the experiment. Briefly, polystyrene plates were coated with 0.1 μg/well of hsp60, p277 or Mycobacterium bovis hsp65. After washing and blocking (PBS, 0.5 % gelatine) wells were incubated with 100 μl of serum samples diluted 1:500 in PBS containing 0.5 % gelatine and 0.05 % Tween 20. Binding of anti-hsp antibodies was determined using γ-chain-specifi c HRP-labeled anti-IgG and o-phenylene-diamine. The optical density was measured at 490 nm (reference at 620 nm) and means of duplicate wells were calculated. A serial dilution of a control anti-hsp60 rabbit polyclonal antiserum reacting with all hsps tested was used as standard. Data obtained as optical density values were transformed to arbitrary units/ml related to this standard.

Recording of action potentials from ventricular myocardium

After opening the chest the heart was quickly removed. Thin papillary muscles were excised from the right ventricle and immersed in oxygenated modified Tyrode solution (in mmol/l: NaCl 120, KCl 5.4, CaCl 2.7, MgCl2 1.1, NaHCO3 24, glucose 6). The pH of this solution was set to 7.4±0.05 when saturated with the mixture of 95% O2 and 5% CO2 at 37 °C. Transmembrane action potentials were recorded using the conventional microelectrode technique. The preparations were stimulated at a frequency of 1-2 Hz using rectangular constant current pulses, of 1ms duration delivered through a bipolar platinum electrode. At least 1 h was allowed for each preparation to equilibrate before beginning of the measurements. Transmembrane action potentials were recorded and digitalized by glass capillary microelectrodes (5-20 MΩ) filled with 3M KCl using an assembly of an amplifier coupled to an INTRASYS computer analyzing system (Experimetria).

Data analysis

In case of human experiments Mann-Whitney test was used to compare the diabetic and healthy subjects.

In case immunization experiments ANOVA (with Bonferroni correction) was used. The survival curves were analyzed by Mantel-Hansel test.

In the electrophysiological experiment I used ANOVA (with Bonferroni correction).

RESULTS AND DISCUSSION

Th1 and Th2 cell responses of type 1 diabetes patients and healthy controls.

My thesis can be divided into three parts. In the first part, the aim of my experiments in T-lymphocyte preparations obtained from healthy subjects and freshly diagnosed T1DM population was to analyze the nature of immune regulation disorder. Therefore I determined the shift in Th1/Th2 immune response characterized in terms of T cell-specific cytokine production upon stimulation with presumed target antigens (p277, LAK).

The first peptide used was the p277, which previously was used for human immunisation; the second one was the LAK peptide. Earlier studies have shown higher anti-LAK peptide antibody level in T1DM which peptide shows high homology with the GAD antigen.

Analyzing the INF-γ (Th1) and IL-13 (Th2) responses - according to the previous findings, namely in newly diagnosed T1-diabetic patients the Th2 cytokines were less inducible - we detected significantly lower absolute Th2 responses compared to the healthy controls.

In case of Th1 responses we could not detect any significant difference between the control and patient group. Analyzing the Th1 and Th2 responses to LAK peptide no significant difference has been between the investigated groups, therefore the role of LAK peptide in the patomechanism of diabetes could not be confirmed.

Polarization of p277 and LAK specific immune response was investigated by calculating the Th1/Th2 ratios.

Stimulating with p277 we detected a significant shift toward Th1 response in type 1 diabetic patients compared to the control group. There was no significant shift towards Th1 or Th2 responses in case of LAK peptide.

Analyzing the data using a novel method/regime of reference we managed to discover the distinct tendencies in the Th1 and Th2 immune responses between the healthy and diabetic groups.

The p277- and LAK-induced specific Th1 and Th2 immune responses were compared to the PI (positive control) induced immune responses/cytokine release and this held us to determine the polarization of the immune responses.

The p277 induced Th1 type immune response (IFN-γ release) comparing to the PI was significantly higher in the diabetic patients; meanwhile the compared Th2 type immune response (IL-13 release) was significantly lower. In case of LAK peptide there were no significant differences between the investigated groups.

This new method/regime of reference helped us to demonstrate not only the significantly decreased Th2 immune response characteristic for T1DM, but also the significantly enhanced Th1 type immune response as well.
Regarding the T1DM autoimmune patomechanism, we got closer to answering two other questions as well. These answers were related to the possible target-antigens on the one hand and to the possible immune regulatory disorder on the other hand.
The significantly higher Th1 response induced by p277 in the diabetic patients refers that this peptide may be considered as a target-antigen of the autoimmune diabetic process.
The significantly increased Th1 and significantly degreased Th2 type immune responses detected in the T1 diabetic patients are further evidences that T1DM is an autoimmune disease caused by the disorder of immune regulation.

The different technical characteristics of STZ-diabetic model in different experimental animal groups

In the second part, my first aim was to establish the multiple low-dose STZ induced autoimmune diabetic models in WT/HDC-KO mice and Wistar rats.
The β-cell toxin STZ - in single high doe - because of the direct toxic effect can induce diabetes. In contrary the multiple low-dose of STZ (5x30mg/tkg) as a stress factor can induce hsp60 expression on the β-cells that makes these cells more susceptible to the auto-reactive immune response against hsp60, therefore the multiple low-dose STZ can induce autoimmune diabetes.
In both animal models at the beginning we applied the classic multiple low-dose of STZ (5x30mg/tkg).
During the treatment the lethality was high therefore we have diverted from the conventional protocol and we have determined the proper STZ dose in pilot experiments.
In the rat experiments the adequate dose was 30mg/tkg STZ for three consecutive days. In this case we detected significant blood glucose level increase without high lethality.
In case of WT and HDC-KO mice experiments we used the same STZ dose. Our first aim was to examine/determine the STZ sensitivity of wild type BALB/c and HDC-KO mice comparing to the widely examined C57BL6/KsJ mice.

In the HDC-KO mice we have detected a significant increase of blood glucose level only by the 4th week, compared to the C57BL6/KsJ animals where the blood glucose level increase developed already by the 2nd week.
Based on these results we can conclude that in spite of the fact that these WT/HDC-KO animals presumably are less sensitive to STZ than the C57BL6/KsJ mice, the multiple low-dose of STZ can induce autoimmune diabetes in these animals, as well.
The decreased STZ sensitivity of the HDC-KO animals might be partially due to the unexpected fact that the blood glucose level has increased in the control (non STZ treated) animals as well. This can be explained by the increased anti-GAD antibody level detected is these animals, which might reveal an increased susceptibility to autoimmune diabetes
Further explanation/reason of the increased sensitivity could be the lack of the histidine decarboxylase enzyme; this might be evaluated with further experiments.

Effects of vaccination with heat shock proteins on STZ induced autoimmune diabetes in WT/HDC-KO mice and rats.

The aim of our in vivo experiment was to examine the effects of immunisation with heat shock proteins on WT/HDC-KO mice and rats.
The novelty of these experiments was that these HDC-KO animals haven't been earlier examined in such immunisation experiments.
As only a handful of similar rat experimental data exists today, our analysis significantly contributes to the enlargement of this database.
We carried out our experiments on STZ induced autoimmune diabetic mice and rat models.
The lack of functional endogenous histamine in the tissues of HDC-KO mice drew our attention on this animal mode.
Among other functions, histamine is involved also in the regulation of several aspects of the immune response, including antibody production following immunization. Histamine has effects on the Th1/Th2 balance as well. It down-regulates the production of IFN-γ, TNF-α, IL-12 meanwhile it has the capability of enhancing IL-4, IL-13 and IL-10 production by Th2 cells, so it can induce a shift towards Th2 cytokines.
Previous experiments have demonstrated higher antibody levels against the GAD antigen in these animals, which may suggest a higher susceptibility to autoimmune diabetes. This finding was the second reason why we have chosen this animal model.
We performed the immunisation with two different heat shock proteins, with the Mycobacterial hsp65 and the hsp60 epitope the p277.

First of all we detected the effect of hsp65 and p277 on control HDC-KO and WT animals. In both groups the heat shock proteins caused a significant transient blood glucose elevation by the 2nd week, which has decreased by the 6th week in both animal groups. The same temporary blood glucose level elevation has developed by immunisation of diabetic animals as well.
The transient blood glucose level increase induced by p277 and hsp65 is further evidence mice is further evidence that heat shock proteins might be target-antigens in the patomechanism of T1DM.
Than we examined the effect of vaccination on STZ induced autoimmune diabetes in WT and HDC-KO mice.
In both p277 and hsp65 treated HDC-KO animals the blood glucose level has decreased although the effect of hsp65 wasn't significant any more by the 6th week.
We have detected similar effects in case of WT animals as well; both hsp caused significant blood glucose level decrease.
Comparing the efficacy of the heat shock proteins we didn't find significant difference, but on tendency levels the rate of the blood glucose level decrease was higher in case of p277.
In this study we examined the anti-p277, anti-hsp60 and anti-hsp65 antibody levels changes due to the vaccinations and STZ treatment.
Examining the WT and HDC-KO groups we detected significantly increased anti-p277 antibody levels only in the p277-treated WT animals. The p277 treatment did not cause any increase of the antibody levels in the HDC-KO mice.
Analyzing the anti-hsp65 antibody levels we detected similar results - the antibody level has increased only in hsp65-treated WT animals. In case of hsp60 antibody levels, there were no significant differences.
The differences detected in the efficacy of heat shock proteins has manifested on antibody levels as well. The immune response (antibody elevatory effect) caused by hsp65 treatment was less pronounced than the immune response caused by the p277.
Based on previous animal immunisation data, there is an antibody level increase due to the immunisation and this increase can be related to the efficacy of vaccination.
Although it is theoretically possible that in WT mice the changes in blood glucose levels correlates with the antibody production by vaccination, we could not prove the direct relationship between the therapeutic effect and antibody level increase caused by immunization due to the contradictory result from HDC-KO mice.
The lack of the antibody level increase in HDC-KO mice might be explained differently.

In HDC-KO mice the lack of histamine can cause disturbances in the Th2 response, therefore the failure to obtain increased antibody levels due to immunization could be explained by the decreased humoral immune response due to the impaired Th2 cell activity.
We cannot exclude even the possibility that the immunisation itself serves as a stimulus for the improvement of immune response independently of the antibody levels. It can induce the therapeutic effect by causing a shift in the Th1/Th2 response or by activating other regulatory T cells.
The results of our preliminary rat experiments were different from the results obtained subsequently in mice in several respects.
Although, comparing with the mouse model STZ caused significantly higher blood glucose level increase in the rat model, the immunization with hsp65 failed to prevent the development of the diabetes but prolonged the survival of animals
The survival rate in the twice-treated group was 100% as compared to the 25% in the STZ treated group.
Based on these results it can be concluded that the multiple low-dose of STZ can induce autoimmune diabetes in the HDC-KO mice that can be moderated by immunisation with the presumed target antigens hsp65 and p277.

Electrophysiological characteristics of heart ventricular papillary muscles in diabetic WT and HDC-KO mice

The third part of my work was based on electrophysiological measurements. In this experimental set I examined the cardiovascular complications of diabetes in WT and HDC-KO mice.
Similarly to the previous experiment I used the multiple low-dose STZ induced diabetic model, therefore the detected electrophysiological parameter changes could be attributed with high probability to the developed diabetes.
The novelty of my electrophysiological experiments was the characterization of changes of cardiac action potential in HDC-KO mice as compared to the heart parameters of WT animals.
Comparing the action potentials of HDC-KO and WT mice depressed repolarization and depolarization were detected in the HDC-KO group.
The prolongation of action potential duration (APD) and the decrease in maximum rate of rise of depolarization phase (V_{max}) resemble the alterations characteristic of diabetes. This may confirm our previous results that HDC- KO mice are more susceptible to the development of autoimmune diabetes.

The STZ or alloxan induced diabetes, especially in rats prolongs the APD. The decreased amplitude of the transient outward K^+ current (I_{to}) is supposed to be responsible for the elongation of repolarization especially in the early phase.
In the STZ treated WT mice we detected prolonged action potential duration as well. The difference was that meanwhile in diabetic rats the APD prolongation was proportional at all levels of repolarization (APD25, APD90) in diabetic WT mice we have detected lengthening only in late phase of repolarization. (APD90).
The prolongation of the APD90 can be due to the depression of the delayed outward rectifier K^+ channels, but further ion current investigations are needed to clarify the exact reason.
Besides the APD changes, the detected V_{max} decrease correspond to the existing data and it implies that not only the K^+ channels but also the Na^+ channels can be influenced by diabetes. This also necessitates further ion current investigations.
Confirming the conclusions of our previous experiment that HDC-KO mice are more susceptible to the development of autoimmune diabetes and are less sensitive to the effect of STZ, it was expected, that in the HDC-KO mice the STZ treatment could not cause any further significant changes in the APD and V_{max}.
We can conclude that the alteration of the APD and V_{max} detected in the control HDC-KO mice are deviations characteristics for the diabetic heart and in case of HDC-KO mice they have developed without any STZ treatment.
The alterations of the control HDC-KO mice action potential parameters might be explained differently.
One of the likely explanations may be that - due to the increased susceptibility to diabetes - in HDC-KO mice the alterations of the heart electrophysiological parameters (APD, V_{max}) have been generated by the diabetes-induced shift in the expression/function of certain K^+ channel populations. Further direct measurements are needed to substantiate this assumption.
Thus presumably in control animals the reason behind the parameter changes could be the lack of histamine that can influence K^+ channels responsible for repolarization. We need further direct ion-current experiments to determine the exact mechanisms behind these changes.
Despite we were not able to give an absolute answer to all electrophysiological experiments these results are quite significant as until recently there have been no electrophysiological experiments performed on these HDC-KO animals.

MOST IMPORTANT NOVEL RESULTS

- In type 1 diabetic patients the p277 induced Th1 response was significantly higher; meanwhile Th2 was lower as compared to healthy controls. Therefore, this peptide may be considered as a target-antigen of the autoimmune diabetic process.
- In my human experiment the significant shift detected toward Th1 immune response can be taken as a further support to the theory that T1DM is an autoimmune disease caused by the disorder of immune regulation
- The in vivo experiment's novelty was the establishment of the multiple low-dose of STZ induced autoimmune diabetes for an absolutely new model in HDC-KO mice.
- We have examined the effect of immunisation with target antigens - a novel T1DM therapy - on HDC-KO mice animal model, which have not been examined earlier in this field.
- The transient blood glucose level increase induced by p277 and hsp65 in WT and HDC-KO mice is further evidence that heat shock protein might be target-antigen in the patomechanism of T1DM.
- The results obtained in the experiments with WT/HDC-KO mice immunized with the purported target antigens hsp65 and p277 indicated that immunization can moderate the development of multiple low dose of STZ induced autoimmune diabetes. Therefore vaccination may be regarded as a potential novel treatment mode in T1DM
- Measuring the antibody level changes due to immunization, the anti-p277 and anti-hsp65 antibody levels have been increased significantly only in WT animals– in case of HDC-KO mice we could not find a similar effect.
- Based on these results could not be proved a direct relationship between the therapeutic effect and antibody level increase caused by immunization. The vaccination can induce the therapeutic effect independently from antibody levels by activating other regulatory T cells as well.
- In case of rat experiments the STZ was able to cause more pronounced diabetes although the hsp65 failed to prevent the development of the diabetes but prolonged the survival of animals.
- The novelty of my electrophysiological experiments was the characterization of cardiac action potential in HDC-KO mice as compared to the heart parameters of WT animals.

- Based on the electrophysiological measurements it can be concluded that the action potential alterations (prolonged APD, decreased V_{max}) detected in control HDC-KO mice are very similar to the changes found in STZ induced diabetic WT animals. I have also found relevant that the STZ treatment could not cause any further significant changes in the APD and V_{max}.
- The differences in the STZ treatment-related alterations of AP parameters (APD, V_{max}) in HDC-KO and WT animals might be attributed to the increased susceptibility of HDC-KO mice to autoimmune diabetes.

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PUBLICATIONS

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