TONECA
Coordination Action
on the
Aetiology, Pathology and Prediction of Type 1 Diabetes in Europe

Final
Symposium
04 - 06 May 2008
Siena, Italy

Organizers:
Prof Francesco Dotta, University of Siena, Siena, Italy
Prof Piero Marchetti, University of Pisa, Pisa, Italy

Symposium Venue:
Hotel degli Ulivi, Via Antonio Lombardi 41 - 53100 Siena, Italy
SCIENTIFIC PROGRAMME

Sunday, 4 May 2008

14:00 Arrival and Registration

Posters set up

Scientific Session I: 15:00 – 18:00
Topic: Gene-environment interactions in T1DM (WP 2)

15.00 – 16.00 State of the art lecture:
M. Roivanen: Enterovirus infections and type 1 diabetes

16.00 – 17.00 Oral session:
Holmberg et al.: Antiviral treatment delays onset of diabetes in Ljungan virus infected BB rats
Al-Hello et al.: Genetic determinant of Coxsackievirus B5 responsible for pancreatic islets infection in mouse
Gallo et al.: Coxsackievirus B4 “Tuscany strain” infection determines beta-cell dysfunction and upregulates chedmkine expression in human pancreatic islets
Smura et al.: EV-94: A novel potentially diabetogenic enterovirus serotype

17.00 – 17.30 Coffee break

17.30 – 18.30 Posters viewing and Discussion:

19:30 Wine tasting and buffet dinner

Monday, 5 May 2008

Scientific Session II: 9:00 – 13:00
Topic: Mechanisms of beta cell destruction and death leading to insulin deficiency in human and rodent endocrine pancreas in T1DM (WP 1)

9.00 – 10.00 State of the art lecture:
D. Eizirik: Mechanisms of beta cell death in diabetes mellitus

10.00 – 10.30 Coffee break

10.30 – 11.30 Oral session:
Lenzen: Oxidative stress: The vulnerable beta cell
Mokhtari et al.: Overexpression of NFkB subunit c-Rel protects against human islet cell death in vitro
Nelson-Holte et al.: Improving survival and function of pancreatic islets using adenoviral-driven super-repressor of NFkB
Ortis et al.: TNF-alpha and IL1B induction of NF-kB and its downstream genes has a pro-apoptotic role in pancreatic beta-cells

11.30 – 13.00 Posters viewing and Discussion

13:00 Lunch Buffet

Scientific Session III: 14:00 – 18:00
Topic: New and established T1DM animal models (WP 3)

14.00 – 15.00 State of the art lecture:
C. Mathieu: Vitamin D and diabetes: what can the animal models teach us?

15.00 – 16.00 Oral session:
Hakonen et al.: Role of EGF-receptor signaling in physiological beta-cell mass expansion
Callewaert et al.: Protective role of IRF-1 in innate-mediated beta cell destruction
Joanny et al.: GLP-1 analogue treatment increases beta cell replication and improves metabolic outcome in streptozotocin-diabetic rats transplanted with an insufficient beta cell mass
Maillard et al.: Perfluorocarbons, a new tool for the preservation of pancreatic islets in vitro

16.00 – 16.30 Coffee break

16.30 – 17.30 State of the art lecture:
A. Jorns: The TONECA animal models catalogue

17.30 – 18.30 Posters viewing and Discussion

19:30 Dinner

Tuesday, 6 May 2008

Scientific Session IV: 9:00 – 13:00
Topic: Immunology of T1DM (WP 4)

9.00 – 10.00 State of the art lecture:
C. Cilio: Immunological mechanisms in T1D pathogenesis from early protection to late disease progression

10.00 – 10.30 Coffee break

10.30 – 11.30 Oral session:
Bugliani et al: The direct effects of immunosuppressive drugs on isolated human islets
Ferreira et al.: The 1,25-dihydroxyvitamin D, TX527, as a modulator of human dendritic cells
Willcox et al.: An immunohistochemical study of insulitis in early onset type 1 diabetes in man
Bonato et al.: Innate immunity and human insulitis during beta cell Coxsackie B4 infection
11.30 – 12.30 State of the art lecture:
BO. Roep: T-cell autoimmunity in human autoimmune diabetes

12.30 – 13.15
S. Lenzen: Review of the TONECA Coordination Action

End of Symposium and lunch
POSTERS

GENETIC DETERMINANT OF COXSACKIEVIRUS B5 RESPONSIBLE FOR PANCREATIC ISLETS INFECTION IN MOUSE
Al-Hello H, Ylipaasto P, Smura T, Rieder E, Hovi T, and Roivainen M

IMPORTANCE OF OXIDATIVE STRESS IN DIABETES AND CARDIOVASCULAR COMPLICATIONS LINKED TO DIABETES, HOW CAN WE PREVENT THEM?
Auberval N, Dal S, Vodouhe C, Seyfritz E, Schini-Kerth V, Sigrist S

1,25-DIHYDROXYVITAMIN D₃ AND ITS LESS CALCEMIC ANALOGUE, TX527, AS REGULATORS OF TH17-DIFFERENTIATION.
Baeke F, van Etten E, Overbergh L, Mathieu C

TX527, A LESS CALCEMIC VITAMIN D ANALOGUE, DIRECTS HUMAN T CELLS TO SITES OF INFLAMMATION.
Baeke F, van Etten E, Overbergh L, Mathieu C

INNATE IMMUNITY AND HUMAN INSULITIS DURING BETA-CELL COXSACKIE B4 INFECTION.

THE DIRECT EFFECTS OF IMMUNOSUPPRESSIVE DRUGS ON ISOLATED HUMAN ISLETS.

PROTECTIVE ROLE OF IRF-1 IN IMMUNE-MEDIATED BETA-CELL DESTRUCTION.

EFFECTS OF DONOR AND PREPARATION CHARACTERISTICS ON INSULIN SECRETION AND SURVIVAL OF HUMAN ISLETS.

ISLET INFLAMMATION AND T-CELL AUTOREACTIVITY IN PANCREAS-DRAINING LYMPH NODES DURING RECENT ONSET TYPE 1 DIABETES.
Dotta F, Bonato V, Vendrame F, Sebastiani G, Torricelli V, Marchetti P and Roep BO

THE 1,25-DIHYDROXYVITAMIN D₃ ANALOG, TX527, AS A MODULATOR OF HUMAN DENDRITIC CELLS.
Ferreira GB, Overbergh L, van Etten E, Waelkens E, Mathieu C

EFFECTS OF NITRIC OXIDE ON CAP-DEPENDENT AND CAP-INDEPENDENT INSULIN BIOSYNTHESIS IN HUMAN ISLETS.
Fred R, Sandberg M, Welsh N

HEDGEHOG SIGNALLING DURING EXPANSION AND BETA-CELL DIFFERENTIATION OF HUMAN PANCREATIC ISLET-DERIVED PRECURSORS.
COXSACKIE B4 “TUSCANY STRAIN” INFECTION DETERMINES BETA-CELL DYSFUNCTION AND UPREGULATES CHEMOKINE EXPRESSION IN HUMAN PANCREATIC ISLETS.
Gallo R, Bonato V, Grieco FA, Cataldo D, Masini M, Marchetti P and Dotta F

ROLE OF EGF-RECEPTOR SIGNALING IN PHYSIOLOGICAL BETA-CELL MASS EXPANSION
Hakonen E, Ustinov J, Ormio P, De Medts N, Bouwens L, Miettinen PJ and Otonkoski T

ANTIVIRAL TREATMENTS DELAY ONSET OF DIABETES IN LJUNGAN VIRUS INFECTED BB RATS
R. Holmberg, W. Klitz, P.O. Berggren L. Juntti-Berggren, B. Niklasson

GLP-1 ANALOGUE TREATMENT INCREASES BETA CELL REPLICATION AND IMPROVES METABOLIC OUTCOME IN STREPTOZOTOCIN-DIABETIC RATS TRANSPLANTED WITH AN INSUFFICIENT BETA CELL MASS
Joanny G., Téllez N., Escoriza J., Soler J. and Montanya E.

INTERLEUKIN-4 ACTIVATES THE PI-3-KINASE AND JAK/STAT PATHWAYS TO MEDIATE CYTOPROTECTION IN PANCREATIC BETA-CELLS

TLR4 AGONIST INDUCED CHANGES IN INSULIN SECRETION AND SIGNALLING IN A PANCREATIC B-CELL LINE
A. Kiely and P. Newsholme

OXIDATIVE STRESS: THE VULNERABLE BETA CELL
Lenzen S Hannover Medical School, Hannover, Germany

PERFLUOROCABONS EMULSIONS ENHANCE PSEUDOISLETS FORMATION AND PREVENT HYPOXIA.
E. Maillard; M. Sanchez’ C. Kleiss; MC. Sencier; A. Langlois; W. Beitiger; C. Vodouhe; M.P. Krafft; M. Pinget; A. Belcourt; S. Sigrist

PERFLUOROCARBONS, NEW TOOL FOR THE PRESERVATION OF PANCREATIC ISLETS IN VITRO
E. Maillard; M. Sanchez’ C. Kleiss; MC. Sencier; A. Langlois; W. Beitiger; C. Vodouhe; M.P. Krafft; M. Pinget; A. Belcourt; S. Sigrist

PROTEOMIC PROFILE OF INSULIN PRODUCING INS-1E CELLS UPON EXPOSURE TO HIGH GLUCOSE
Maris M, Overbergh L, D’Hertog W, Waelkens E and Mathieu C

ITERATIVE EXPOSURE OF CLONAL BRIN-BD11 CELLS TO NINHYDRIN ENABLES SELECTION OF ROBUST TOXIN-RESISTANT CELLS BUT WITH DECREASED GENE EXPRESSION OF INSULIN SECRETORY FUNCTION
McCluskey JT, Hui-Kang L, McClenaghan NH, Flatt PR.

THE EFFECTS OF INHIBITORS OF ROS OR NO GENERATION OR ANTIOXIDANTS IN BETA CELL FUNCTION AND INTEGRITY
OVEREXPRESSION OF THE NF-KB SUBUNIT C-REL PROTECTS AGAINST HUMAN ISLET CELL DEATH IN VITRO
D. Mokhtari, A. Barbu, I. Mehmeti, C. Vercamer, N. Welsh

ASSESSMENT OF A GLUCOKINASE ACTIVATOR ON PANCREATIC BETA CELL FUNCTION AND VIABILITY IN CLONAL BETA CELL LINE BRIN-BD11.
Mullooly N, Newsholme P and Smith D

IMPROVING SURVIVAL AND FUNCTION OF PANCREATIC ISLETS USING ADENOVIRAL-DRIVEN SUPER-REPRESSOR OF NF-κB

PREFERENTIAL STIMULATION OF GLUCOSE UTILISATION VERSUS OXIDATION AND PROLIFERATION OF PANCREATIC B-CELLS OF NCX1 PARTIAL KNOCKOUT MICE
Nguidjoe E, Pachera N, Van Praet A, Sokolow S, Schurmans S, Herchuelz A

TNF-α AND IL-1β INDUCTION OF NF-κB AND ITS DOWNSTREAM GENES HAS A PRO-APOPTOTIC ROLE IN PANCREATIC BETA CELLS

ENTEROVIRAL VP1 EXPRESSION IN HUMAN TYPE I DIABETES
Richardson SJ, Willcox A, Bone AJ, Foulis AK and Morgan NG

EV-94, A NOVEL POTENTIALLY DIABETOGENIC ENTEROVIRUS SEROTYPE
Smura T, Ylipaasto P and Roivainen M

AN IMMUNOHISTOCHEMICAL STUDY OF INSULITIS IN EARLY ONSET TYPE 1 DIABETES IN MAN
Willcox A, Richardson S, Bone A J, Foulis A K and Morgan N G.
GENETIC DETERMINANT OF COXSACKIEVIRUS B5 RESPONSIBLE FOR PANCREATIC ISLETS INFECTION IN MOUSE

Al-Hello H¹, Ylipaasto P¹, Smura T¹, Rieder E², Hovi T¹, and Roivainen M¹

¹Enterovirus Laboratory, National Public Health Institute (KTL), Helsinki, Finland
²Plum Island Animal Disease Center, United States Department of Agriculture, Agricultural Research Service, North Atlantic Area, Greenport, New York

Background and aims: Recently, we showed that fifteen successive passages of a laboratory strain of CBV-5 in mouse pancreas (CBV-5-MPP) resulted in apparent changes in the virus phenotype resulting in a capacity to induce diabetes like syndrome in mice. For further characterisation of the passaged virus strain, a murine insulinoma cell line, MIN-6, was selected as an experimental model.

Results: CBV-5-MPP virus strain was not able to replicate in MIN-6 cells in vitro but required a few days’ adaptation for proper progeny production and generation of cytopathic effect. In order to find out genetic characteristics required for virus growth in MIN-6 cells the whole genome of MIN-6 adapted variant was sequenced and critical amino acids were identified by comparing the sequence to that of the mouse pancreas passaged virus strain.

Conclusion: Results of site directed mutagenesis demonstrated that only one residue, 94 of VP1 is a major determinant for virus adaptation to MIN-6 cells.

Acknowledgement: The work was supported by grants from the Academy of Finland, the Finnish Cultural Foundation and Jalmari and Rauha Ahokas foundation. We thank prof Miyazaki, Kumamoto University Medical School, Japan for providing us with MIN-6 cells.
IMPORTANCE OF OXIDATIVE STRESS IN DIABETES AND CARDIOVASCULAR COMPLICATIONS LINKED TO DIABETES, HOW CAN WE PREVENT THEM?

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Oxidative Stress (OS) corresponds to an imbalance between oxygen radicals and antioxidant systems in favour of stress. There are different mediators of OS such as superoxide, hydrogen peroxide or radical species. OS has been involved in many diseases linked metabolic or vascular disorders. β-cells from islets in pancreas are very particular cells, they are extremely vulnerable to OS because of poor quantities of SOD, CAT, GPX (Ammon et al., 1983) and reduced GSH (Tiedge et al., 1999). Some studies show that hyperglycemia cause glucose auto-oxidation (Wolff et al., 1989), proteins glycation, notably SOD what returns it inactivates, and formation of Advanced Glycation End products (AGEs). Once AGEs formed, they bind to different AGE receptors called RAGE leading to production of Reactive Oxygen Species (ROS) (Kennedy and Lyons, 1997; Yim et al., 2001). Furthermore ROS could favour diabetes development unsettling insulin secretion or increasing insulin resistance. In blood vessels, OS is highly present. Various enzymes (cyclooxygenase COX1, lipoxygenases, NADPH oxidase, cyt P450 or eNOS) lead to ROS generation. For example, NADPH oxidase produces ROS in activated neutrophils or macrophages during inflammatory processes (Babior et al., 2002; Segal and Abo, 1993). Furthermore the interaction between AGE and RAGE causes intracellular modifications that may be involved in the development of vascular diseases such as atherosclerosis (Chappey et al., 1997). Other studies showed that elevated antioxidant consumptions improved some markers of glycemia, could decrease cardiovascular risks (Jain et al., 1996) and moderate consumption of red wine, which contents polyphenolic antioxidants, was linked to cardiovascular reduction mortality, called “French Paradox” (Saint Leger et al., 1979; Renaud et al., 1998). Diabetes, OS and cardiovascular diseases are tightly involved. The aim of this thesis is to determine the importance of OS in diabetes apparition and his cardiovascular complications, but also to prevent theses complications. The study concern on the one hand, in vitro experimentations on different models of stress (H2O2, hyperglycemia) using RINm5F cell line and islets, and preventive studies will be realised thanks to diverse antioxidants such as polyphenolic extracts of grape juice or other molecules as resveratrol®. In the other hand, in vivo experimentations will realise on streptozotocin induced diabetes rats (T1DM model) or spontaneous diabetes models (BB rats for T1DM and GK or ZDF for T2DM).

OS can’t be identified and measured with only one marker, that’s why, after adjusting models, oxidative entities and biological markers like insulin or malondialdehyde (MDA), a lipid peroxidation product, antioxidant enzymes (SOD, CAT) will be quantified with ELISA kits, western blotting and enzymatic activities will be determined by spectrophotometric assays. Furthermore mRNA quantities will be studied by RT-PCR. These studies will enable to determine the role of OS in different models of diabetes and his cardiovascular complications, and to know the influence of antioxidants for diabetes preventions. Some of our preliminary results tend to show a diminution of CAT and SOD at the same time an increase of MDA quantity by western blot after H2O2 treatment on RINm5F but we haven’t proceeded to enzymatic assays and other tests yet.
1,25-DIHYDROXYVITAMIN D\textsubscript{3} AND ITS LESS CALCEMIC ANALOGUE, TX527, AS REGULATORS OF TH17-DIFFERENTIATION.

Baeke F, van Etten E, Overbergh L, Mathieu C.
LEGENDO, Catholic University of Leuven, Leuven, Belgium.

Background and aims: 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}) and its less calcemic analogue TX527 are important immunomodulators, showing very promising results in the prevention and treatment of various animal models of autoimmune diseases. Interfering with T-helper (Th) cells, by skewing inflammatory Th1 cell responses to a more protective Th2 phenotype and by inducing regulatory T cells, has been recognized as an important mechanism by which these molecules exert their immunomodulatory properties. More recently, a new proinflammatory subset of Th cells, distinct from Th1 and Th2 cells, has been identified: Th17 cells. Th17 cells, typically expressing IL-17 and the orphan nuclear receptor ROR\textgamma, have been recognized as central players in many autoimmune disorders in mice and men. The aim of this study was to evaluate the effects of 1,25(OH)\textsubscript{2}D\textsubscript{3} and the analogue TX527 on the development of Th17 cells.

Materials and methods: CD8-depleted splenocytes from 6 to 10 weeks old female BALB/c mice were activated (anti-CD3 and anti-CD28) and cultured for 3 days under Th17-driving conditions (IL-6, TGF-\textbeta, anti-IL-4, anti-IFN-\gamma) in the absence or presence of 10\textsuperscript{-8}M 1,25(OH)\textsubscript{2}D\textsubscript{3} or TX527. IL-17 expression by CD4\textsuperscript{+} T cells was analyzed by flow cytometry, using surface and intracellular staining. mRNA levels of IL-17 and ROR\textgamma were determined by quantitative real-time RT-PCR.

Results: Splenocytes cultured under Th17-driving conditions showed a significant induction of IL-17 and ROR\textgamma at the mRNA level. Accordingly, a clear IL-17-expressing cell population could be detected in these cultures by flow cytometry. Addition of 1,25(OH)\textsubscript{2}D\textsubscript{3} to the cell cultures resulted in reduced numbers of IL-17-producing CD4\textsuperscript{+} T cells, concomitant with decreased IL-17 and ROR\textgamma mRNA expression levels. Similar to more pronounced effects were obtained with the 1,25(OH)\textsubscript{2}D\textsubscript{3} analogue TX527. Conclusion: The results of this study provide evidence that 1,25(OH)\textsubscript{2}D\textsubscript{3} and less calcemic analogues not only affect Th1 and Th2 responses, but also interfere with the development and/or function of Th17 cells. These findings reveal a new mechanism by which these molecules exert their immunomodulatory effects. Considering the central role of Th17 cells in various autoimmune diseases, the use of 1,25(OH)\textsubscript{2}D\textsubscript{3} or less calcemic analogues might be an important strategy to control autoimmunity.
TX527, A LESS CALCEMIC VITAMIN D ANALOGUE, DIRECTS HUMAN T CELLS TO SITES OF INFLAMMATION.

Baeke F, van Etten E, Overbergh L, Mathieu C.
LEGENDO, Catholic University of Leuven, Leuven, Belgium.

Background and aims: 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) and less calcemic analogues such as TX527 exert important immunomodulatory effects in vitro as well as in vivo in various animal models of autoimmune diseases. Lymphocyte trafficking, guided by a complex network of chemokine- and other homing receptors, represents a crucial phenomenon during inflammation and organ-specific autoimmune responses. The role of 1,25(OH)2D3 and analogues in lymphocyte trafficking has not been fully explored yet and was therefore the aim of this study. Materials and methods: Human CD3+ T cells, obtained from peripheral blood of healthy volunteers (n=4) were activated by anti-CD3 and anti-CD28. From day 2 onwards, cells were treated with TX527 (10^-8M) or vehicle (ethanol) every 2 days. At day 10, expression of various chemokine receptors and other homing receptors was analyzed by flow cytometry. Results: Skin-homing receptors CCR4 and CCR10 were significantly upregulated upon TX527-treatment whereas expression of cutaneous lymphocyte-associated antigen (CLA) was severely decreased. Gut homing integrin β7 was slightly suppressed by TX527, while CCR9 expression remained unaffected. Importantly, TX527-treated CD3+ T cells showed reduced expression of homeostatic lymph node homing receptors (CD62L,CCR6,CCR7,CXCR4) compared to vehicle-treated controls. On the other hand, chemokine receptors involved in acute inflammation (CCR4, CCR5, CXCR3) were significantly upregulated by the treatment. Conclusion: In conclusion, this study confirms the capacity of 1,25(OH)2D3 and structural analogues to induce the expression of skin-homing receptors (CCR10), while suppressing receptors guiding T cells to the gut (integrin β7). Interestingly, the results also provide evidence that TX527 creates a T cell phenotype with decreased homing capacities for secondary lymphoid organs, while increasing their ability to migrate to sites of inflammation. Looking closer into the phenotype of these migrating T cells will give more information as to the role that they will perform in the different target tissues. These findings reveal a new mechanism by which TX527 might modulate immune responses during autoimmunity. Unraveling the mechanisms of action of 1,25(OH)2D3 and analogues might contribute to a better understanding of the immunomodulatory effects of these molecules, opening new possibilities for therapeutic interventions.
INNATE IMMUNITY AND HUMAN INSULITIS DURING BETA-CELL COXSACKIE B4 INFECTION.

Bonato V\textsuperscript{1, 2}, Gambelunghe G\textsuperscript{3}, Gallo R\textsuperscript{1, 2}, Tondi C\textsuperscript{1}, Sebastiani G\textsuperscript{1, 2}, Calabrese M\textsuperscript{1}, Marchetti P\textsuperscript{4}, Falorni A\textsuperscript{3} and Dotta F\textsuperscript{1, 2}

\textsuperscript{1}University of Siena, Italy; \textsuperscript{2}Fondazione Umberto Di Mario-Toscana Life Science, Siena, Italy; \textsuperscript{3}University of Perugia, Italy; \textsuperscript{4}University of Pisa, Italy.

Background and Aims: Type 1 diabetes mellitus (T1DM) is a multifactorial disease in which multiple genes interplay with environmental factors leading to autoimmunity against self-antigens in β-cells. Viral infections are associated epidemiologically with T1DM. Recently we reported that in a subset of T1DM patients signs of enteroviral infection were detected. Infecting virus was identified as a novel Coxsackie B4 strain, named “Tuscany Strain”. Viral infection, was β-cell specific and caused β-cell dysfunction as shown by impaired glucose-stimulated insulin secretion. Several studies demonstrated that during stress, such as viral infections, cells synthesize factors, such as FAS, IFN-α, MICA, which interact with innate immunity. In particular MICA (MHC class I chain-related) molecules are stress induced, and recognized by NKG2D receptors on natural killer (NK) and CD8\textsuperscript{T} T-cells. We than aimed at characterizing the occurrence of innate immunity-related phenomena in human insulitis during CVB4 infection.

Materials and Methods: To this end, pancreas and purified islets were obtained from organ donors, (2 recently diagnosed T1DM, and 5 controls). Pancreatic specimens were formalin-fixed and paraffin-embedded for immunohistochemical studies, while pancreatic islets were employed for mRNA expression studies.

Results: In pancreas of CVB4 positive T1DM patients, insulitis was present and was characterized mainly by NK cells (CD94+) and by a small amount of CD3 expressing T-cells. Strong FAS was detected in the majority of pancreatic positivity for IFN-α islets as shown by immunohistochemistry. This result was paralleled by real time PCR analyses on isolated T1DM islets. Both in islet and endothelial cells we observed an over-expression of class I MHC molecules. Interestingly, MICA-positive cells were detected in most of the islets and in scattered -cells in exocrine cells. MICA expression co-localized with insulin producing the islets as shown by confocal microscopy experiments.

Conclusion: We have demonstrated that: during infection, innate immunity is indeed involved in human T1DM, thus suggesting that strategies aimed at modulating NK cell function may interfere with the -cell destruction. natural history of
THE DIRECT EFFECTS OF IMMUNOSUPPRESSIVE DRUGS ON ISOLATED HUMAN ISLETS.

Bugliani M, Masini M, Lupi R, Del Guerra S, Galli M, D’Aleo V, Filipponi F, Del Chiara M, Boggi U, Marchetti P. Department of Endocrinology and Metabolism, Metabolic Unit, University of Pisa, Italy.

Background and Aims: Pancreas or islet transplantation can restore normoglycaemia in type 1 diabetes, but the use of immunosuppressive drugs can have a direct drug-induced toxic effect. To better understand the mechanisms responsible for this, we investigated the effects on human pancreatic beta cells of prolonged exposure to therapeutical levels of tacrolimus (Tac) or cyclosporin A (CsA).

Material and Methods: Islets were isolated from the pancreas of 16 multiorgan donors (age: 63±14 yrs; gender: 10M/6F; BMI: 25.1±3.7 kg/m²) by enzymatic digestion and density gradient centrifugation. Functional, survival and molecular studies were then performed after 96hr incubation with 10 ng/ml Tac or 150 ng/ml CsA.

Results: Glucose-induced insulin secretion was significantly decreased in Tac (p<0.05), but not in CsA exposed islets, (stimulation index, i.e. the ratio of insulin release at 16.7 vs 3.3 mM glucose: control islets, Ctrl, 2.3±0.5; Tac 1.6±0.2; CsA, 2.0±0.4). Electron microscopy (EM) showed a significant (p<0.05) decrease of insulin granules in Tac exposed beta-cells (volume density, ml%: 0.63±0.39), but not in the CsA group (1.54±0.09). The percentage of apoptotic beta-cells was higher in Tac (11.7±4.7) than Ctrl (2.7±1.2) (the respective value in CsA was 6.3±1.2). By microarray analysis (Affymetrix) we observed that of the 22,215 probe sets present on the HG U133A chip, 94 and 60 were significantly (p<0.05) altered in Tac and CsA, respectively. In the Tac group, 23 genes were upregulated and 71 downregulated; among them, 5 were associated with apoptosis, 4 with cell proliferation, and 6 with transcription pathways. In the CsA group, 23 genes were upregulated and 37 were downregulated, with 2 regarding apoptosis and 7 transcription phenomena. Comparison of Tac and CsA treated islets showed 129 differently expressed genes, of which 6 regarded apoptosis, 8 cell proliferation and 12 transcription pathways. Quantitative RT-PCR showed that FKBP 12.6, a key gene involved in calcineurin inhibition, was significantly less expressed with CsA than Tac.

Conclusions: In conclusion, CsA had less deleterious effects on isolated human islets, possibly due to a lower influence of gene expression.
PROTECTIVE ROLE OF IRF-1 IN IMMUNE-MEDIATED BETA-CELL DESTRUCTION

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This work was supported by grants of the Flemish Research Foundation [Fonds Voor Wetenschappelijk Onderzoek (FWO) Vlaanderen], the Belgium Program on Interuniversity Poles of Attraction initiated by the Belgian State, the Katholieke Universiteit Leuven, the European Community concerted Sixth Framework Program with acronym SAVEBETA, TONECA (Coordination action on the aetiology, pathology and prediction of Type 1 Diabetes in Europe) and the JDRF Center for Prevention of Beta Cell Therapy in Diabetes.

Background and Aims: IFN-γ, together with other cytokines such as IL-1β or TNF-α, is an important player in cytokine-induced β-cell death causing type 1 diabetes. We previously showed that signal transducer and activator of transcription-1 (STAT-1) is a key player in immune-mediated early β-cell death. Our objective in this study was to investigate the contribution of the transcription factor interferon regulatory factor 1 (IRF)-1 to immune-mediated β-cell destruction, as IRF-1 is induced in pancreatic islets by IFN-γ downstream of STAT-1 and to a lesser degree by IL-1β and TNF-α.

Materials and Methods: To study the role of IRF-1 in immune-mediated β-cell death in vitro and in vivo, we used knock-out mice lacking IRF-1 (IRF-1−/−). IP-10, MCP-1, MIP-3α and iNOS gene expression in whole islets was studied by real-time RT-PCR analysis after exposure to IL-1β plus IFN-γ. Next, NO production and MCP-1 secretion by cytokine-treated IRF-1−/− islets was studied with Griess reagent assay and ELISA. Cell migration towards cytokine-exposed IRF-1−/− islets was studied in vitro by chemotaxis assay and in vivo after islet transplantation. Finally, islets were transplanted in spontaneously non-obese diabetic (NOD) mice and additionally treated with a blocker of IL-1β (IL-1 receptor antagonist [IL-1ra]).

Results: We demonstrated that islets from IRF-1−/− mice showed increased gene expression of IP-10, MCP-1, MIP-3α and iNOS and elevated production of MCP-1 and NO when exposed to IL-1β plus IFN-γ compared to wild-type counterparts. Furthermore, IRF-1−/− islets displayed higher potential to attract migratory cells both in vitro and in vivo after transplantation in spontaneously-diabetic NOD mice. IRF-1 deficiency in the islet graft also resulted in more primary islet graft non-function (PNF) (64% PNF versus 25% PNF, respectively, p<0.05) and a shorter graft survival of the functioning islets grafts (7.0 ± 1.4 days versus 10.4 ± 4.8 days, respectively, p=0.087) compared to wild-type islets. Interestingly, PNF was prevented in IL-1ra-treated NOD mice transplanted with IRF-1−/− islets. However, this treatment did not prolong the graft survival time. The observation of faster rejection of IRF-1−/− islets compared to wild-type islets after transplantation in NOD mice was in contrast with the similar graft survival when transplanting skin of IRF-1−/− or wild-type mice onto NOD mice (15.7 ± 1.2 days versus 14.5 ± 0.6 days, respectively, p=NS).

Conclusions: These data indicate that IRF-1 has a protective role specifically in β-cells. These data, together with previous studies on STAT-1, point towards a dual role of IFN-γ in immune-mediated β-cell destruction, with STAT-1 as the ‘bad guy’ and IRF-1 as the ‘good guy’.
EFFECTS OF DONOR AND PREPARATION CHARACTERISTICS ON INSULIN SECRETION AND SURVIVAL OF HUMAN ISLETS.

D'Aléo V., Bugiani M., Galli M., Lupi R., Del Guerra S., Torri S., Lanza M., Boggi U., Filipponi F., Marchetti P.

Introduction: An adequate insulin secretion is crucial for successful islet transplantation, but this feature can be affected by many variables linked to the clinical characteristics of the donors and the isolation process.

Materials and Methods: Human islets were prepared from 167 donors (age: 57±17 yrs; body mass index, BMI: 25.0±3.2 Kg/m²; gender: 58%M/42%F) by collagenase digestion followed by density gradient purification. Insulin secretion stimulated by glucose, arginine and glibenclamide, evaluated by static incubation, and apoptosis, evaluated by an Elisa method (Cell Death Detection Elisa plus), were studied in relation to donor (age, BMI, C-peptide and glucose plasma levels), and isolation (cold ischemia time, yield, purity, culture days from isolation to functional study) variables.

Results: Basal insulin release was 0.039 ± 0.016 µU/islet/min and increased to 0.095 ± 0.066 µU/islet/min with 16.7 mM glucose (Stimulation Index: 2.4 ± 1.4), 0.087 ± 0.056 µU/islet/min with 20 mmol/l arginine (Stimulation Index: 2.2 ± 1.0) and 0.092 ± 0.067 µU/islet/min with 100 µmol/l glibenclamide (Stimulation Index: 2.4 ± 1.3). Prolonging the period of culture (days) from isolation to stimulus determined increased basal release (p=0.04) and reduced stimulation index in response to high glucose (p=0.003) or gibenclamide (p=0.0002). Better insulin release in response to glucose (p<0.0001), glibenclamide (p=0.04) and arginine (p=0.05) was observed with more pure preparations. No correlation was found between insulin secretion and the other variables considered. Apoptotic rate was 0.497±0.559 arbitrary units of optical density (a.u.), and was significantly affected by the degree of purity (≤ 50%: 0.848 ± 0.758 a.u.; > 50% 0.479 ± 0.492 a.u., p < 0.05). In addition, by linear regression analysis, it was shown that apoptosis inversely correlated with donor age (p=0.01) and positively correlated with days from isolation to apoptosis assessment (p=0.0009). In turn, the higher the apoptosis values the lower resulted the amount of insulin released from the islets in response to glucose (p=0.05).

Conclusions: Islets of adequate purity should be transplanted shortly after isolation due to better function and survival.
ISLET INFLAMMATION AND T-CELL AUTOREACTIVITY IN PANCREAS-DRAINING LYMPH NODES DURING RECENT ONSET TYPE 1 DIABETES.

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Background and Aims: Type 1 diabetes results from a T-cell mediated destruction of insulin-producing pancreatic β-cells. We here present the case where whole pancreas and pancreas-draining lymph nodes were obtained from a recent onset type 1 diabetes patient who died accidentally during clinical remission of the disease. We examined these tissues for the presence and function of autoreactive T-cells and for islet-specific expression of proinflammatory molecules involved in lymphocyte recruitment.

Materials and Methods: Immunohistochemical investigations revealed the presence of insulitis as well as of intact islets containing insulin-positive beta cells.

Results: Insulitis was present in 44% of islets studied (n=75), with insulin-containing β-cells could still present in the vast majority of pancreatic islets analyzed (86%, n=150). Insulitic lesions were characterized by presence of elevated levels of the chemokine CXCL10 and infiltration of lymphocytes expressing the corresponding chemokine receptor CXCR3. In addition, T-cells isolated from pancreatic draining lymph nodes reacted with multiple islet autoantigens (GAD65, insulin peptide B9-23 and IA-2). T-cell autoreactivity was accompanied by production of interferon-γ (GAD65) or IL-10 (IA-2) or both (insulin B9-23), possibly reflecting pathogenic as well as regulatory immune autoreactivity to islets.

Conclusions: We demonstrated that pro- and anti-inflammatory islet autoreactivity, indicating that different adaptive and innate immune responses may contribute to insulitis and β-cell destruction. In addition, our study suggests a relevance of the CXCL10-CXCR3 pathway in facilitating human leucocyte migration to inflamed pancreatic islets, which may lay the basis for the development of a novel therapeutic target.
THE 1,25-DIHYDROXYVITAMIN D₃ ANALOG, TX527, AS A MODULATOR OF HUMAN DENDRITIC CELLS.
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The use of vitamin D and its derivatives is being considered for immune intervention in type 1 diabetes. The active form of vitamin D, 1,25-dihydroxyvitamin D₃, (1,25(OH)₂D₃) and its immunopotent analogues (eg TX527), have important immune effects, mainly mediated by their actions on dendritic cells. Previous studies have shown that (1,25(OH)₂D₃) induces a tolerogenic DC phenotype, with a reduced capacity to fully activate T-cells. The aim of this study was to investigate global protein changes in this tolerogenic DC phenotype induced by TX527, using bi-dimensional difference gel electrophoresis (2D-DIGE), and to analyze if these changes affected functional features of DCs that make them potent antigen presenting cells. For this purpose, human CD14+ monocytes, isolated from peripheral blood mononuclear cells, were differentiated towards immature DCs (IM-DC) (6 days in IL-4/GM-CSF) or to mature DCs (M-DC) (2 additional days in IFNγ/LPS/GM-CSF), with/without TX527 (10⁻⁸M) (n=4). Protein profiles were analyzed by 2D-DIGE, separating protein samples in 2 different pH ranges (pH4-7 and 6-9) and differentially expressed spots (p<0.01) were identified by MALDI-TOF/TOF (118 differentially expressed spots from which 76,3% are identified in IM-DC and 140 differentially expressed spots from which 73,6% are identified in M-DCs). The identified proteins were used to build two human protein interaction networks representing IM and M-DCs (p<0.01). Based on these protein identifications and protein interaction networks, three groups of proteins appeared to be mainly affected by TX527 treatment, namely cytoskeleton, protein biosynthesis/proteolysis and metabolism. To further analyze the functional impact of these molecular changes, we analyzed the surface phenotype, morphology, endocytosis and migratory capacity of the TX527-treated DCs. These functional experiments were shown to be in line with the altered phenotype brought by TX527 treatment as seen by our proteomic approach, inducing a more tolerogenic phenotype in DCs. These results indicate that major changes are taking place in antigen presenting capacity and in actin cytoskeleton rearrangement of DCs. In conclusion, these findings reinforce the capacity of TX527, to silence some important features of DCs that make them potent antigen presenting cells, inducing a more tolerogenic state. This opens many possibilities for the use of this compound in new therapeutic approaches, on the treatment of autoimmune disorders such as type 1 diabetes, neoplasia and prevention of graft rejection.
EFFECTS OF NITRIC OXIDE ON CAP-DEPENDENT AND CAP-INDEPENDENT INSULIN BIOSYNTHESIS IN HUMAN ISLETS
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**Background and aims:** About 10% of all mRNAs are thought to be translated by a cap-independent mechanism, a pathway that ensures protein synthesis of critical proteins during conditions of cellular stress. The polypyrimidine tract binding protein (PTB) is known to promote cap-independent translation when binding to pyrimidine rich sequences of 5'-UTRs. The aim of this study was to investigate whether the PTB binds to the 5'-UTR of insulin mRNA and whether such a putative binding correlates with cap-independent insulin mRNA translation in human islets. Given that cap-independent translation can be activated in response to cellular stress we also wanted to investigate whether nitric oxide affects the putative binding of PTB to insulin mRNA and the rates of insulin biosynthesis.

**Materials and methods:** To measure the affinity of PTB to the insulin mRNA 5'-UTR human islets were incubated in either 1.67 or 16.7 mM glucose for 2 hours w/wo 2.5 mM DETA/NO. Cytoplasmic extracts were prepared and incubated with biotinylated RNA oligonucleotides corresponding to the insulin mRNA 5'-UTR. The RNA-binding proteins were then eluted and separated on SDS-PAGE for immunoblotting. Insulin and total protein biosynthesis was estimated by measuring the incorporation of L-[3,4,5-3H] leucine in 20 human islets incubated for 2 h at 1.67 or 16.7 mM glucose. The islets were incubated with 1 mM DETA/NO (a nitric oxide donor) or 0.1, 1.0, 10 mM hippuristanol (an eIF4A inhibitor that specifically inhibits cap-dependent translation) during the incubation period. Labeled islet insulin was immunoprecipitated using guinea pig anti-insulin antibodies + protein A-sepharose and total protein was precipitated using trichloroacetic acid.

**Results:** The affinity binding assay clearly shows that PTB binds specifically to the insulin mRNA 5'-UTR and preliminary results indicate that the affinity decreases in the presence of DETA/NO.

Insulin biosynthesis data show that, in the presence of 16.7 mM glucose, most of the insulin mRNA translation (approximately 90%) is cap-dependent and that DETA/NO treatment significantly decreases the translation of insulin by 85%. The total protein biosynthesis was decreased by only 70% in response to DETA/NO. Consequently, the percentage insulin biosynthesis was lower in DETA/NO treated islets than in control islets. However, inhibition of cap-dependent insulin mRNA translation was less pronounced in DETA/NO treated islet as compared to control islets. This was manifested as an increased percentage insulin biosynthesis in DETA/NO- and hippuristanol-treated islets as compared to DETA/NO-treated islets. At 1.67 mM glucose the effects of DETA/NO and hippuristanol were less pronounced than at 16.7 mM glucose.

**Conclusion:** Our studies show that PTB binds to the insulin mRNA 5'-UTR and that the affinity seems to decrease in the presence of DETA/NO, indicating a stimulatory role of PTB in the control of insulin biosynthesis rates. We also demonstrate that insulin biosynthesis is mainly cap-dependent at a high glucose concentration, but that the cap-independent biosynthesis of insulin can contribute with up to 10% at a high glucose concentration and 50% at low glucose.
HEDGEHOG SIGNALLING DURING EXPANSION AND BETA-CELL DIFFERENTIATION OF HUMAN PANCREATIC ISLET-DERIVED PRECURSORS.

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Backgrounds and Aims: Hedgehog (Hh) signalling is a key regulator of cell fate and differentiation, as well as of stem/progenitor cell maintenance in many organs. In vitro generation of insulin-producing cells from stem/progenitor cells is crucial to develop a proper cell-therapy for type 1 diabetes. Since Hh pathway plays an important role both in endocrine pancreas development and in the regulation of insulin secretion, we studied the expression of Hh components during expansion and beta-cell differentiation of precursors derived from human pancreatic islets.

Materials and Methods: Human Pancreatic Islet-Derived Mesenchymal (hPIDM) cells were obtained by plating human pancreatic islets in tissue culture dishes in RPMI 1640 medium containing 10 % FBS. hPIDM cells, cultured in serum-free RPMI, differentiated into a pancreatic endocrine phenotype. Origin and phenotype of proliferating and differentiated hPIDM were studied by immunofluorescence, electronmicroscopy, FACS, and Real-Time-PCR analysis. Hh signalling was evaluated by Real-Time-PCR expression of target genes Gli1 and Gli2, activators of Hh pathway, and of repressor Gli3, in human islets and in the proliferating as well as differentiated hPIDM cells. The effect of Hedgehog signalling on proliferation of hPIDM cells was evaluated using Hh inhibitor cyclopamine.

Results: Human islets attached within 24 hrs in in vitro culture, showing a central “core” of aggregated cells surrounded at the periphery by a migrating population of flattened epithelial cells. Double immunofluorescence for Ki-67, nestin, C-peptide and PDX-1, after 3 and 6 days of culture, showed a subset of proliferating cells. These cells were maintained in culture for up to 20 passages and approximately $10^{10}$ cells were obtained from each original islet seeded. FACS, immunofluorescence and Real-Time-PCR revealed a transition from endocrine to mesenchymal phenotype of such proliferating cells (hPIDM), which, in serum free RPMI, generated “islet-like” clusters showing C-peptide, glucagon and somatostatin induction by immunofluorescence and beta-cell like secretory granules by electron microscopy analysis. Real-Time-PCR analysis confirmed an acquired endocrine pancreatic phenotype. In addition, Gli1 and Gli2 mRNA levels were highly upregulated, paralleled by down-regulated Gli3, in hPIDM cells vs human islets, in accordance with an activation of Hh signalling in hPIDM cells. Interestingly, hPIDM-derived islets-like aggregates, an inhibition of the Hh pathway was confirmed by reduction of Gli1 and Gli2 and induction of Gli3 expression. Cyclopamine administration decreased the proliferating rate of hPIDM cells in a dose-dependent manner.

Conclusion: Taken together, these results indicate a direct involvement of Hh signalling in the generation and expansion of mesenchymal cells from culture human islets. Based on these data Hh signalling could contribute either to stimulate proliferation, or to maintain a “precursor” phenotype in the hPIDM cells.
COXSACKIE B4 “TUSCANY STRAIN” INFECTION DETERMINES BETA-CELL DYSFUNCTION AND UPREGULATES CHEMOKINE EXPRESSION IN HUMAN PANCREATIC ISLETS.

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Backgrounds and Aims: Epidemiological evidence suggests that enteroviral infections may contribute to type 1 diabetes (T1DM) pathogenesis, although the effect(s) of enteroviral infection on the β-cell remain to be elucidated. Following the observation of beta-cell specific Coxsackie B4 “Tuscany Strain” viral infection accompanied by non-destructive insulitis in a subset of T1DM patients and to explore the hypothesis that such infection can by itself compromise beta-cell function, we studied insulin secretion in islets from 10 non-diabetic donors which were in vitro infected by the enterovirus isolated from a T1DM patient.

Materials and Methods: Virus was extracted from islets and viral replication occurred in KB cells. Islet beta-cells obtained from non-diabetic multiorgan donors were infected with the replicated virus, and were evaluated by electron microscopy. In addition insulin secretion in response to glucose (3.3 mM and 16.7 mM) was assessed by the static incubation method and the perifusion procedure. We analyzed by real time PCR the expression of molecules associated to the insulitis process such as FAS, IL-1, TNFa, MCP-1, IP-10, IFN and IFN in in vitro Coxsackie B4 “Tuscany Strain”-infected islets.

Results: Replicated virus was demonstrated to specifically infect beta-cells islets from all non-diabetic multiorgan donors by electron microscopy, with viral inclusions detected in the cytoplasm of 17±7% and 33±14% of beta-cells after 4 and 7 days of co-culture respectively, associated with several signs of cellular suffering, including pyknotic nuclei. Insulin secretion (expressed as % of insulin content) in control and infected islets was respectively 1.7±0.3 and 1.5±0.3 at 3.3 mM glucose (N.S.), 3.8±0.7 and 1.8±0.5 at 16.7 mM glucose (p=0.02). A significantly (p<0.001) increased expression of FAS, IFN, IP10 and MCP1 was observed in virus-infected islets.

Conclusion: Our results demonstrate that: a) a Coxsackie B4 virus isolated from islets of a T1DM patients is able to in vitro infect human beta-cells; b) such infection impairs glucose-stimulated insulin secretion and induces morphological signs of cell suffering; c) in vitro enterovirally infected human islets hyperexpress FAS and chemokines that can promote islet inflammation and damage.
ROLE OF EGF-RECEPTOR SIGNALING IN PHYSIOLOGICAL BETA-CELL MASS EXPANSION

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Background and aims: Better understanding of beta-cell regeneration mechanisms is important for the generation of new therapeutic approaches for diabetes. Beta-cell mass is known to increase in response to insulin demand and during pancreatic regeneration induced by injury. Epidermal growth factor receptor (EGF-R) signaling is required for normal pancreatic beta-cell development and proliferation. In this study we investigated the role of EGF-R signaling in physiological situations of beta-cell mass expansion.

Material and methods: E1-DN mice, which have a kinase negative EGF-receptor under the Pdx-1 promoter, and wild type (WT) mice were subjected to high fat diet (HF-diet) or partial pancreatic duct ligation (PDL). The mice were kept on HF-diet or standard chow for 8 weeks. BrdU was added to the drinking water (1 mg/ml) for long-term labeling of proliferating beta-cells. Intraperitoneal glucose tolerance test (IPGTT) was done on weeks 4 and 8. Morphometrical analysis of the pancreata was performed at 8 weeks. In the PDL model the pancreatic duct of 8 weeks old E1 and WT mice was partially ligated. After one week the tail and head parts of the pancreata were collected. Morphometrical analysis of the pancreata was performed. Beta-cell mass, islet size and distribution were analyzed.

Results: After 8 weeks on HF-diet E1-DN mice (n=6) were clearly diabetic in IPGTT (2h b-gluc=26 mmol/L) compared to E1-DN mice (n =5) on standard chow (2h b-gluc=15 mmol/L, p<0.01). WT mice on HF-diet (n=6) had mildly impaired glucose tolerance in IPGTT (2h b-gluc=14 mmol/L) compared to WT mice (n=6) on standard chow (2h b-gluc=10 mmol/L, p<0.01). HF-fed WT mice could increase their beta-cell surface area by 67 % (n=3, p<0.05) compared with those on standard chow, while the response in HF-fed E1-DN mice was only 19 % (n=3). WT mice had 30% more proliferating beta-cells compared to E1-DN mice (p<0.05). Surprisingly, we did not detect any significant difference in beta-cell proliferation between WT mice on standard chow vs. WT mice on HF-diet or between the two E1-DN groups. There was no difference in beta-cell apoptosis (immunohistochemistry for activated caspase 3) between the four groups. After partial duct ligation beta-cell surface area in the tail part of pancreas was increased by 300 % compared to the head part of pancreas in WT mice (n=3) and by 389% in E1-DN mice (n=3). However the total beta-cell surface area, islet number and size in the tail part remained smaller in E1-DN mice (islet size 2.6 vs. 3.7 μm², islet number 3.7 vs. 5.9 islets/mm², n=3 in each group).

Conclusion: When fed on HF-diet, WT mice showed marked beta-cell hyperplasia, whereas the hyperplastic response of E1-DN mice was insufficient to maintain normoglycaemia. Partial duct ligation induced islet neogenesis in both WT and E1-DN mice. However, the number and size of the regenerating islets remained smaller in the E1-DN mice. These results suggest that EGF-R signaling is required for beta-cell mass expansion both in HF-diet induced insulin resistance and in pancreatic regeneration after partial duct ligation.
ANTIVIRAL TREATMENTS DELAY ONSET OF DIABETES IN LJUNGAN VIRUS INFECTED BB RATS
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Background and aims: It has been proposed that infectious agents carried by rodents may be involved in the development of type 1 diabetes in humans. This has been based on observations of an association between rodent density and disease incidence. These observations initiated a search for an etiologic agent in small rodents and it resulted in the isolation of a novel picornavirus from the bank vole (Myodes glareolus). The virus was named “Ljungan virus” (LV). Diabetes-prone (DP) BioBreeding (BB) rats become diabetic at around 60 days of age, which gives an excellent opportunity to study the impact of different prediabetic interventions on the onset of the disease. As in humans, there have been speculations that different viruses may play a role in the pathogenesis of type 1 diabetes in these rats. We therefore wanted to investigate if LV was present in the BB rats and if so, whether treatment of the infection could influence the onset of diabetes.

Material and methods: We stained formalin fixed paraffin embedded pancreas tissue using immunohistochemistry (IHC). We also used real-time RT-PCR assays to identify and quantify LV infection. The influence of LV on the development of diabetes in BB rat was examined. A group of 12, 38 days old, DP rats were given a single dose i.p. of LV- specific murine antiserum (group I) and 13 control DP rats, of the same age, were given an injection of saline (group II). A group of six, 38 days old, DP rats were treated with a combination of the two antiviral compounds, Pleconaril and Ribavirin, orally with a tube connected to a syringe two times daily for nine consecutive days (group III). Five control DP rats of the same age were given equal volume of water for the same period of time (group IV).

Results: A total of 16 BB rats from a laboratory in Sweden and 10 BB rats from the USA were all LV positive by IHC. Positive staining was found in several organs indicating a systemic infection. In the pancreas the staining was located to the islets. IHC positive BB rats from both continents were confirmed by PCR. Regarding the anti LV treatments, the two control groups (group II and IV) were combined after they were found not to differ in days to diabetes onset (F = 0.0001, P = 0.99). Single classification analysis of variance performed on the control group, control + antiserum and control + antiviral treatment groups testing for differences in days-to-diabetes was significant (F = 6.48, P = 0.0042). These differences were accounted for by additional days to diabetes onset for each of the two treated groups (64.3 days for the antiserum treated and 67.0 days for the antiviral treated) compared to the control group (60.6 days).

Conclusion: This demonstrates that each of the treatments were effective in delaying diabetes onset and that the presence of LV can play a role in the development of diabetes.
GLP-1 ANALOGUE TREATMENT INCREASES BETA CELL REPLICATION AND IMPROVES METABOLIC OUTCOME IN STREPTOZOTOCIN-DIABETIC RATS TRANSPLANTED WITH AN INSUFFICIENT BETA CELL MASS

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Background and aims:
We have previously shown that a new DPPIV stabilized GLP-1 analogue enhanced beta cell regeneration in partially pancreatectomized rats. The treatment increased beta cell replication and neogenesis, and decreased beta cell apoptosis resulting in an increased beta cell mass. Shortage of islets is a major limitation in human islet transplantation and usually determines the transplantation of an insufficient islet mass, and the requirement of two or more transplants to restore normoglycemia. Strategies to increase graft beta mass could alleviate the shortage of islets and improve the outcome of islet transplantation. The aim of this study was to determine the effects of this new DPPIV stabilized GLP-1 in rat transplanted islets. In this model, the contribution of neogenesis is negligible due to the almost complete absence of ductal or exocrine tissue in the grafted cells, allowing a direct analysis of the role of enhanced beta cell replication in the outcome of the graft.

Material and methods: We used a DPPIV stabilized GLP-1 analogue which has been derivatised with a chemical moiety that binds to albumin in a reversible manner thereby extending plasma half-life to at least one day. Streptozotocin-diabetic Lewis rats were syngeneically transplanted with an insufficient beta cell mass of 800 islets and were treated with the GLP-1 analogue (100 μg/kg . day, SC once daily) or with vehicle from the day of transplantation to day 14. An additional control group of non-transplanted STZ-diabetic rats treated with the GLP-1 analogue was studied. Blood glucose and body weight were monitored on days 0, 3, 7, 14 and after graft removal. Beta cell mass was determined by morphometry, beta cell replication by BrdU incorporation and beta cell apoptosis by the TUNEL technique in insulin stained sections of harvested grafts.

Results: Blood glucose levels decreased in transplanted GLP-1-treated animals compared to control non-transplanted GLP-1-treated group at all time points (day14: 285±44 vs 515±52 mg/dl; p<0.05), but were not significantly reduced in transplanted vehicle-treated animals. Furthermore, 30% (3 of 10) of transplanted rats treated with GLP-1 achieved and maintained normoglycemia until the end of the study. In contrast, only 11% (1 of 9) of vehicle treated and none of non-transplanted GLP-1-treated animals achieved normoglycemia. All normoglycemic animals reverted to clear hyperglycemia after graft removal. Beta cell replication in islet grafts of GLP-1-treated group was higher than in grafts of vehicle-treated animals (1.31±0.35% vs 0.57±0.10%; p<0.01), and than in normal pancreas (0.26±0.02%; p<0.01). Beta cell apoptosis was similar in grafts of animals treated with GLP-1 or vehicle, and higher than in control pancreas. Beta cell mass in islet grafts of GLP-1-treated rats was 44% higher than in vehicle-treated animals (1.24±0.32 vs 0.86±0.22 mg) although the difference did not reach statistical significance.

Conclusions: Treatment with this new GLP-1 analogue increased beta cell replication and improved metabolic evolution in diabetic rats transplanted with an insufficient islet mass. These results suggest that the effect of the GLP-1 analogue on replication of transplanted beta cells improved the outcome of the graft.

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INTERLEUKIN-4 ACTIVATES THE PI-3-KINASE AND JAK/STAT PATHWAYS TO MEDIATE CYTOPROTECTION IN PANCREATIC BETA-CELLS
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Introduction: IL-4 is a multifunctional cytokine that plays a critical role in the regulation of immune responses. Previous work has shown that treatment of animals with IL-4 can reduce the incidence of type 1 diabetes suggesting that IL-4 is protective against β-cell death. The effects of IL-4 depend upon binding to and signalling through a receptor complex consisting of the IL-4 receptor alpha (IL-4Ra) chain and either the cytokine receptor common gamma chain (γc) (in type I IL-4 receptors) or the α1-chain of IL-13 receptors (in type II IL-4 receptors), resulting in a series of phosphorylation events mediated by receptor-associated kinases.

In a previous study we confirmed, at both the RNA and protein level, the presence of each component of the Type 1 and Type II IL-4 receptors in rat pancreatic β-cells and that these receptors are functionally competent. However it was not established which signaling pathways are activated in response to IL-4 in β-cells. Therefore, the aim of the present study was to confirm that IL-4R is also found on human pancreatic β-cells and to investigate the signaling pathways by which IL-4 exerts its effects in β-cells.

Methods: Expression of IL-4 receptors was detected in sections of adult human pancreas by immunocytochemistry. Clonal BRIN-BD11 and INS-1 cells were cultured in RPMI-1640 and viability was measured using flow cytometry after staining with propidium iodide. Western blotting analysis was used to detect the phosphorylation of Akt and STAT6. INS-1 cells conditionally expressing PTP-BL were generated using Flp recombinase and were grown in the presence of hygromycin to maintain selection pressure.

Results: Expression of IL-4 receptors was detected on the majority of cells within human islets by immunohistochemistry, confirming that expression of these molecules on β-cells is not restricted to rodents. The signalling pathways regulated by IL-4 receptors were then studied in the clonal β-cell lines BRIN-BD11 and INS-1. IL-4 dose-dependently protected these cells against the loss of viability caused by pro-inflammatory cytokines (IL-1β and IFNγ) and this effect was abrogated by the PI-3-kinase inhibitor, wortmannin. Western blotting revealed that IL-4 increased the phosphorylation of the PI-3-kinase substrate, Akt in the cells, confirming that activation of the PI-3-kinase pathway occurs in response to IL-4. Involvement of the JAK3/STAT6 pathway in the actions of IL-4 was also suggested, since a selective inhibitor of Jak 3 inhibitor significantly reduced the cytoprotective response of IL-4 in cells exposed to pro-inflammatory cytokines (IL-1β/IFNγ : 15±1% cell death; IL-1β/IFNγ/IL-4: 10.5±0.5% (p<0.01); IL-1β/IFNγ plus Jak3 inhibitor: 17±1.5%; IL-1β/IFNγ/IL-4 plus Jak3 inhibitor: 18.5±2.5%; p<0.001 vs IL-4 in the absence of Jak3 inhibitor). In confirmation of this, IL-4 increased the phosphorylation of the transcription factor, STAT6, in β-cells. Both effects were prevented by over-expression of the non-receptor tyrosine phosphatase, PTP-BL, which is known to utilise STAT6 as a substrate.

Conclusion: The results demonstrate that IL-4 receptors are functionally competent in pancreatic β-cells and that they signal via PI-3-kinase and Jak/STAT pathways to mediate a cytoprotective response.
TLR4 AGONIST INDUCED CHANGES IN INSULIN SECRETION AND SIGNALLING IN A PANCREATIC B-CELL LINE.

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Background and Aims: Changes in the insulin receptor coupled signal transduction pathways of muscle, adipose tissue and liver have been associated with obesity and Type 2 diabetes. Insulin signalling in these and tissues can also be altered by pro-inflammatory cytokines (such as TNF-α), resulting in resistance to the action of insulin. In addition, Toll IL-1 like receptors have recently been implicated in insulin resistance. Specifically TLR4 and TLR2, whose agonists include lipopolysaccharides and saturated fatty acids, are implicated in altered patterns of insulin signalling in animal models of insulin resistance and obesity. Beneficial effects on the insulin signalling pathway and improved insulin action were demonstrated in loss of function mutations in the TLR4 receptor of adipose, muscle and liver tissues in mice.

Materials and Methods: Clonal BRIN BD11 -cells were treated for 24 hours with increasing concentrations of LPS. Chronic (24 h) insulin secretion and nutrient consumption and subsequent acute (20 min) stimulated insulin secretion were determined. Glucose and glutamine concentrations were determined by enzymatic assays. Protein was extracted using RIPA buffer, subjected to SDS-PAGE and electrophoretically transferred onto a nitrocellulose sheet and probed with anti IRS-1, p-IRS-1, Akt, p-Akt or IR (Isis), and visualised.

Results: Incubation of BRIN-BD11 -cells for 24 hours in the presence of increasing concentrations of the TLR4 ligand LPS significantly decreased chronic (24 hour) insulin secretion from 1.09 ± 0.19 to 0.76 ± 0.18 μg insulin / mg protein in the presence of 100 ng/ml LPS (p < 0.05). There was no change in acute (20 min) stimulated insulin secretion, or insulin content. The reduction in chronic insulin secretion was not accompanied by changes in nutrient (glucose or glutamine) consumption. IR expression levels were increased significantly from 1 ± 0.52 to 8.6 ± 1.83 units (p < 0.01), and Akt phosphorylation was slightly reduced. There was no change in IRS-1 protein expression or phosphorylation after 24 hours.

Conclusions: It is known that glucose and insulin induce expression of IR in pancreatic -cells. We have now demonstrated that 24hr exposure to the TLR4 agonist LPS increased IR expression and reduced insulin secretion. Insulin content was not altered. Levels of activated Akt were reduced after exposure to LPS, which could potentially impair the viability of the -cell after longer periods of incubation. However, the concentrations of LPS used in this study did not impair cell viability during the period of our experiments.
OXIDATIVE STRESS: THE VULNERABLE BETA CELL

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Damage to and death of the pancreatic beta cell is the cause of type 1 diabetes. The pancreatic beta cell has a very low antioxidative defence status and is therefore considered to be a particularly vulnerable cell type. Oxidative (ROS mediated) and nitrosative (NO mediated) stress contribute significantly to beta cell death mediated by proinflammatory cytokines, even though the relative contribution of the two forms of stress to beta cell damage and destruction has to be clarified. Both oxidative and nitrosative stress can cause damage to beta cells through affecting the function of the mitochondria, the endoplasmic reticulum (ER stress) and the plasma membrane. Since the beta cell uses its oxidative metabolism not only for ATP generation for energy supply, needed for example for biosynthesis and ion transport, but also for generation of the signal for glucose-induced insulin secretion, the question must be considered whether this particular vulnerability is the price which the beta cell has to pay in order to make use of the metabolism for both purposes.
PERFLUOROCABONS EMULSIONS ENHANCE PSEUDOISLETS FORMATION AND PREVENT HYPOXIA.

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Aim & Background: One of the obstacles to the success of islets transplantation is the loss of islets viability during culture. Indeed hypoxia affected cells in the inner core of islets bigger than 1 mm³. To prevent hypoxia of the inner islets cell mass during in vitro culture, our interest have been focused on perfluorocarbons, already used in organ conservation. Perfluorocarbons have high oxygen solubility and are used as oxygen carrying moieties in preservation solution. In a previous study, we reported that perfluorocarbons emulsions are able to prevent adhesion and enhance detachment of β-cell lines (MIN-6 or RINm5F) onto tissue culture plastic. In this study, we compared β cells clusters formed in the presence of PFCs (PFCsPI) to classical pseudoislets (CcPI) and we analyzed whether PFCs emulsion could decrease oxygen deprivation on β-cells.

Material and method: RINm5F have been incubated for 1 week in the presence of PFCs emulsion in media (3.5% w/v), our positive control was classical pseudoislets formed on untreated Petri dish and our negative control was adherent cells gown on treated Petri dish. After 1, 3, 5 and 7 days, RT-PCR, western blot, stimulation test were performed on cells. The study of expression of insulin, cadherin and Cdk4 allowed us to characterize cluster enhance by the presence of PFCs emulsion in medium and marker of hypoxia were studied as the expression of HIF-1α; VEGF; and apoptosis (Bax and Bcl-2).

Results: Comparison between clusters formed in the presence of PFCs and classical pseudoislets showed the same rate of expression of specific protein such as E-cadherin insulin, Cdk4 indicating that cluster look like classical pseudoislets. For example, Immunoblot measurement of E-cadherin expression during cluster formation (from the 3rd day) demonstrated increased of E-cadherin content (1.73±0.2) as during CcPI formation (1.33±0.2) in contrast to adherent cells (1.01±0.1). To achieved this hypothesis, a stimulation test showed an increase of insulin secretion in both pseudoislets and clusters as compare to adherent cells either in basal condition (3 days: PFCsPI 14±3 μg insulin/ mg total protein, CcPI 15±3 μg insulin/ mg total protein, adherent cells 4±2 μg insulin/ mg total protein; n=3; p<0.05) or in stimulated condition (3 days: PFCsPI 27±4 μg insulin/ mg total protein, CcPI 34±2 μg insulin/ mg total protein, adherent cells 6±2 μg insulin/ mg total protein; n=3; p<0.001). So PFCs are able to enhance formation of pseudoislets (PI-PFC). Moreover study of apoptosis, showed a decrease of Bax mRNA in PI-PFCs (0.7±0.2) in comparison to adherent cells (1.3±0.3) (n=3; p<0.01) and an increase of Bcl-2 mRNA expression (0.8±0.2 vs 0.4±0.1; n=3; p<0.01), leading to a preservation of cells. Finally the study of hypoxia marker as HIF-1α showed a decrease of mRNA (PFCs-PIC: 0.8±0.1 vs CcPI: 1.5±0.2 and adherent cells : 1.3±0.2; n=3; p<0.05) or protein levels in PI-PFCs as compared to CcPI or adherent cells ( 0.3±0.1 vs 0.6±0.2 or 0.7±0.1 respectively; n=3; p<0.001), leading to a decrease of VEGF transcription in PFCs-PIC (0.3±0.1 vs CcPI: 0.7±0.2 or adherent cells: 0.8±0.1 respectively; n=3; p<0.05).

Conclusion: PFCs emulsion enhanced pseudoislets formation and protected cell inside cluster form apoptosis and hypoxia. PFCs could be a new tool in islets preservation in vitro.

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PERFLUOROCARBONS, NEW TOOL FOR THE PRESERVATION OF PANCREATIC ISLETS IN VITRO

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Introduction: Pancreatic islets transplanted to treat type 1 diabetes often fail to function likely because of islets hypoxia. The use of oxygen carriers as perfluorocarbons could reduce islets loss. The aim of this study was to correlate decrease of hypoxia, islets fibrosis and islets viability and functionality in the presence or not of perfluorocarbons (PFCs) emulsions.

Materials and methods: Primary culture rat islets had been incubated for 1, 3 and 5 days in the presence or not of 10% (v/v) of emulsion of PFCs in culture media charged in oxygen during 1 hour. Viability (FDA/PI), apoptosis (Hoechst and Bax, BCL2 mRNA) stimulation index linked to insulin secretion (ELISA) during glucose stimulation tests was analysed. Expression of VEGF, Matrix Metalloproteinase-2 and Matrix Metalloproteinase-9 was studied by RT-PCR, western blot and immunohistochemistry, ELISA.

Results: Islets viability was similar in presence or not of PFCs (80% of viability inside islet) throughout the experiment, whereas there was a significant increase of BCL-2 mRNA expression in islets cultured in the presence of PFCs emulsions in function of control (1.28±0.3 vs 0.36±0.1 p<0.001) and a significant decrease of Bax mRNA expression (0.5±0.2 vs 0.9±0.07; p<0.05) after 24 hours of culture. While, stimulation index of islets in the presence of PFCs was maintained during the whole study (index around 3), index of islets control decreased (2.78±0.54; 1.46±0.07 and 1.15±0.56 (p<0.01) after 1, 3 and 5 days in the presence of PFCs respectively). Moreover, study of VEGF showed a decrease of mRNA expression the first day after isolation in the presence of oxygenated PFCs (0.49±0.15) as compare to control (0.75± 0.19; p<0.05; n=5). Concerning protein secretion, a significant decrease of VEGF secretion was observed in the presence of oxygenated PFCs in comparison to control after 2 and 3 days of culture (0.05±0.03µg of VEGF/mg of protein vs 0.44±0.12 µg of VEGF/mg of protein for control; p<0.001; n=3). Expression of MMP-2, enzyme secreted to permit angiogenesis, is linked to hypoxia. In islets incubated in the presence of PFCs, there was a decrease of MMP-2 secretion in comparison to control (0.05±0.03 versus 0.13±0.06pg of MMP-2/mL) after 1 and 3 days of incubation. This decrease of degradation enzyme was linked with a conservation of islets structure when there were cultured in the presence of PFCs. In islets control, a Masson’s Trichrome staining showed a invasion of collagen fibers which could be due to fibrosis enhance by hypoxia.

Conclusion: PFCs are very useful to fight against hypoxia and consequently decrease fibrosis. PFCs emulsions bring oxygen to islets and islets were capable to use this oxygen to decrease hypoxia which permitted to grow up the pool of islets before transplantation.

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PROTEOMIC PROFILE OF INSULIN PRODUCING INS-1E CELLS UPON EXPOSURE TO HIGH GLUCOSE
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Background and aims: Type 2 Diabetes is a dual disease that develops when the pancreatic β-cells can no longer compensate for insulin resistance in the target organs, mainly skeletal muscles, but also liver and adipocytes. This relative insulin deficiency is the consequence of β-cell dysfunction and/or β-cell death. The cause of this progressive β-cell failure remains elusive, but it has repeatedly been demonstrated in vivo that hyperglycemia is a prerequisite for β-cell dysfunction. It has been observed, in whole islets, as well as in different β-cell lines, such as INS-1E and MIN6, that insulin gene expression, insulin content and glucose stimulated insulin secretion, were drastically compromised over time upon exposure to high glucose concentrations. To date, the exact mechanisms and pathways involved in β-cell dysfunction by high glucose treatment have not been unraveled. The aim of the present study was to analyze protein changes in insulin-producing INS-1E cells exposed to high glucose.

Materials and methods: INS-1E cells were exposed to 25 mM glucose for 24 hrs (as compared to 11 mM for control cells) and alterations in protein profile were analyzed by two-dimensional difference gel electrophoresis (2D-DIGE), covering 2 different pH ranges (pH 4-7 and pH 6-9). Proteins altered in expression (n=4, p<0.05) were identified by MALDI-TOF/TOF. The proportion of apoptotic and necrotic cells was evaluated by microscopic counting after Hoechst/Propidium Iodide staining.

Results: Exposure of the cells to high glucose, increased the proportion of apoptotic cells (14.40 ± 1.71 versus 2.87 ± 0.19 for control (p<0.001)), as well as the necrotic rate (6.94 ± 2.2 versus 1.15 ± 0.15 for control (p<0.05)). 2D-DIGE analysis revealed within two different pH ranges a total of 2189 ± 16 (pH 4–7) and 1467 ± 97 (pH 6–9) spots. Of these, 124 protein spots were altered in expression (n=4, p<0.05), of which 102 spots (82.3 %) could unambiguously be identified by MALDI-TOF/TOF. The identified proteins were involved in different pathways of which the glycolysis/TCA cycle, endoplasmic reticulum (ER) stress response/protein folding, cellular transport, protein synthesis and degradation were most abundant. More specific, many chaperone proteins were downregulated by high glucose treatment. As these proteins have previously been associated with protective and anti-apoptotic functions, we conclude that downregulation of these proteins may hamper β-cell defenses, making them more vulnerable to apoptosis. In addition, also proteins involved in intracellular transport were downregulated by high glucose. These included proteins involved in nucleo-cytoplasmatic transport, trafficking to the ER, transport between ER and Golgi apparatus and exocytosis-related proteins. We conclude that perturbations in this intracellular trafficking may hamper correct protein synthesis and formation of post-translational modifications, leading to β-cell dysfunction and death.

Conclusion: In this study we identified new molecular pathways that were not described before as being involved in high glucose-induced β-cell dysfunction and death. In addition, the present findings may provide new key target proteins for the prevention of β-cell dysfunction and death in type 2 diabetes.
ITERATIVE EXPOSURE OF CLONAL BRIN-BD11 CELLS TO NINHYDRIN ENABLES SELECTION OF ROBUST TOXIN-RESISTANT CELLS BUT WITH DECREASED GENE EXPRESSION OF INSULIN SECRETORY FUNCTION

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Background -cell destruction combined with and aims: Prevention of pancreatic preservation of insulin-secretory function is an important goal for cell-based diabetes therapy. This study describes the generation and characteristics of -cells. toxin-resistant

Materials and methods: By using iterative exposures to ninhydrin, a new class of robust ninhydrin-tolerant insulin-secreting BRIN-BD11 cells (BRINnt) was generated. Low -cell function and tolerance and high passage BRINnt were used to evaluate against toxins in comparison with native BRIN-BD11 cells. Differences in viability, gene expression, insulin secretory function, anti oxidant enzyme activity, DNA damage and DNA repair efficiency were compared.

Results: BRIN-BD11 ninhydrin-tolerant cells exhibited resistance towards ninhydrin and hydrogen peroxide but not streptozotocin (STZ). Both total superoxide dismutase (SOD) and catalase enzyme activities of BRINnt cells were significantly enhanced, and ninhydrin-induced DNA damage was decreased. BRIN-BD11 ninhydrin-tolerant cells also exhibited enhanced DNA repair efficiency. However, this was accompanied by loss of secretagogue-induced insulin release, decreased cellular insulin content and deficits in insulin and glucose transporter 2 gene expression. Prolonged culture of BRINnt cells in the absence of ninhydrin, reversed the degenerated function of BRINnt cells but restored ninhydrin susceptibility.

Conclusions: -cell toxin resistance and These date illustrate dissociation between secretory function, indicating difficulties in the generation of robust and well-functioning cells using this approach.
THE EFFECTS OF INHIBITORS OF ROS OR NO GENERATION OR ANTIOXIDANTS IN BETA CELL FUNCTION AND INTEGRITY

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Background and aims: Oxygen metabolism is essential for sustaining aerobic life, and normal cellular homeostasis works on a fine balance between the formation and elimination of reactive oxygen species (ROS). Oxidative and nitrosative stress, a cytopathic consequence of excessive production of ROS and NO and the suppression of ROS removal by antioxidant defense systems, is implicated in the development of diabetes. The possible sources of oxidative stress in diabetes might include autooxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase. ROS generated by high glucose are considered as a causal link between elevated glucose and the other metabolic abnormalities important in the development of diabetes mellitus. However, the exact mechanism by which oxidative stress could contribute to the development of diabetic complications and glucolipotoxic beta cell dysfunction still remains to be clarified. The aim of this study was to determine the effectiveness of inhibitors of oxidant generation (ROS and NO) and antioxidants to protection from dysfunction and death of beta cells.

Materials and methods: Stably transfected rat insulinoma cells (RINm5F, exhibiting over or reduced expression of Prx III) or clonal BRIN BD 11 beta cells or mouse islets were treated with various stressors (cytokines 0.3125U/ml IL-1β, 31.25U/ml TNF-α, 15,625U/ml IFN-γ), hydrogen peroxide 0.3%, high glucose 25mM, or palmitic acid 0.1mM) in the presence or absence of antioxidants such as superoxide dismutase (20U/ml), catalase (100U/ml), NAC (0.2mM), the NADPH oxidase inhibitor Apocyanin (0.2mM), or iNOS inhibitor 1400W (0.2mM). Disruption of the mitochondrial transmembrane potential was determined by DiOC6(3) absorption and fluorescence emission spectroscopy. Insulin secretion was determined from viable cells by ELISA. Cell viability was determined by MTS.

Results: Treatment with cytokines or hydrogen peroxide, but not palmitic acid or high glucose reduced insulin secretion in the BRIN BD11 cell line. Moreover, in RINm5F cells exhibiting reduced expression of Prx III, addition of cytokines, hydrogen peroxide, or streptozotocin or alloxan caused cell death, which was associated with elevated levels of iNOS and cleavage of PARP. Furthermore, apocyanin and 1400W protected mouse islets from cytokine and palmitic acid induced dysfunction while antioxidants such as catalase, superoxide dismutase and N-acetylcysteine protected against cytokines, hydrogen peroxide and high glucose in the cell lines. Cells overexpressing Prx III were protected due to elevated mitochondrial antioxidant activity.

Conclusion: Cellular antioxidants play a crucial role in protecting from cytokine or chemical stimulated ROS production, thus maintaining insulin secretion and preventing β-cell death.
OVEREXPRESSION OF THE NF-KB SUBUNIT C-REL PROTECTS AGAINST HUMAN ISLET CELL DEATH IN VITRO

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Background and aims:
The transcription factor NF-kB has been proposed to exert both beneficial and deleterious effect on the insulin producing b-cell. The aim of the present investigation was to study expression and cytokine-induced translocation of NF-kB subunits in human islet cells, and whether a genetic gain of NF-kB function affects islet cell survival in response to the toxins hydrogen peroxide (H₂O₂), the nitric oxide donor DETA/NONOate (DETA/NO) or streptozotocin (STZ).

Materials and methods:
Human islets were exposed to the cytokines IL-1β, TNF-α and IFN-γ and the nuclear and cytosolic fractions were prepared for immunoblot analysis of Rel-A/p65, Rel-B, c-Rel, p50/p105, p52 and the ribosomal protein of the small subunit 3 (rpS3). Cytokine-exposed human islets were used for confocal analysis of NF-kB subunit localization. Human islet cells were also dispersed by trypsin treatment and transduced with an adenoviral vector expressing c-Rel for cell viability analysis. The c-Rel adenoviral vector was also used for in situ transduction of rat pancreas to reach the core of intact rat islets. The islets were then isolated and used for cell viability analysis.

Results:
We detected expression of p65, Rel-B, p50, p105, p52 and rpS3 in human islet cells. Only p65 and rpS3 were translocated from the cytosolic to the nuclear fraction in response to cytokines. c-Rel adenoviral transduction of dispersed human islet cells resulted in detectable expression of c-Rel, increased kappaB-activity in nuclear fractions and an augmented expression of Bcl-XL. c-Rel expression protected against STZ- and H₂O₂-induced cell death, both in human and rat islet cells. Control islets with no adenoviral expression displayed increased cell death in response to STZ, H₂O₂ and DETA/NO, whereas no significant increase was observed in islets transduced with the GFP + c-Rel viruses.

Conclusion:
A genetic increase in the activity of NF-kB results in protection against cell death in human islets. Thus, strategies aiming at preventing b-cell death in diabetes should probably not involve inhibition of NF-kB.
ASSESSMENT OF A GLUCOKINASE ACTIVATOR ON PANCREATIC BETA CELL FUNCTION AND VIABILITY IN CLONAL BETA CELL LINE BRIN-BD11.

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Background and aims: The enzyme glucokinase has been designated the “glucose sensor” of the pancreatic beta cell due to its concentration dependent control over glucose metabolism and thus insulin secretion. This enzyme catalyses the rate limiting step of glycolysis - the phosphorylation of glucose to glucose-6-phosphate. The importance of this enzyme and its tissue selectivity, have led to the development of molecular activators of GK. One such drug is GKA50, the AstraZeneca produced potent activator of GK activity. Genetic analysis and structural studies reveal the enzyme possesses an allosteric activation site. Molecular compounds which bind to this site stabilize glucokinase in its closed confirmation increasing its affinity for glucose and should enhance glucose stimulated insulin secretion in the pancreatic beta cell. We have tested the glucokinase activator GKA50 with respect to clonal beta cell (BRIN-BD11) metabolism, insulin secretion and cellular integrity. This bioengineered cell line has a high glucokinase-hexokinase ratio compared to other beta cell lines, and exhibits bi-phasic glucose stimulated insulin secretion, consistent with normal beta cells.

Materials and Method: The metabolic and insulin secretory action of GKA50 was determined using basal (1.1mM) and stimulatory (16.7mM glucose or 16.7mM glucose + 10mM alanine) static incubation conditions. Insulin secretion was determined by ELISA. The effects of GKA50 on cell growth and viability was assessed using the WST-1 cell viability assay.

Results: Results have shown that GKA50 has an insulinotropic action and that secretion is glucose dependant. Micromolar concentrations of GKA50 are potent activators of glucokinase and increase glucose stimulated insulin secretion in BRIN-BD11 cells in a dose dependent manner. GKA50 stimulated glucose dependent insulin secretion was further enhanced by the presence of 10mM alanine. No significant difference in viability was found between cells incubated in the absence or presence of GKA50, even at concentrations of 100μM.

Conclusion: Increased activation of glucokinase increases cellular glucose metabolism and insulin secretion. Our results highlight the importance of glucokinase in metabolic regulation and stimulus secretion coupling in pancreatic beta cells. Glucokinase activator drugs offer the possibility of effective pharmacological control over glycaemia and may be a novel therapeutic approach to the treatment of defective glucose stimulated insulin secretion.
IMPROVING SURVIVAL AND FUNCTION OF PANCREATIC ISLETS USING ADENOVIRAL-DRIVEN SUPER-REPRESSOR OF NF-κB

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Background and Aims: Islets transplanted in patients with Type 1 Diabetes are faced with an immune assault, with NF-κB playing a central role in immune-mediated b-cell destruction. The aim of this study was to provide proof-of-concept that interfering with NF-κB using adenoviral vectors can protect β-cells against inflammation-induced cell death.

Materials and Methods: To determine transduction efficiency with adenoviral vectors, whole mouse islets were transfected with Ad-pCMV-EGFP expressing the reporter gene EGFP. Next, mutant, non-phosphorable IκBα(SA)2, able to restrain NF-κB in the cytosol, was introduced into whole islets with an adenoviral construct (Ad-pCMV-HA- IκBα(SA)2). Transduction efficiency was evaluated by confocal microscopy after staining for the hemaglutinin (HA)-tag on IκBα(SA)2. Finally, the Ad-pCMV-HA-IκBα(SA)2-transduced islets were exposed to IL-1β+IFN-γ and cell death, NO production and glucose-stimulated insulin release were studied.

Results: We obtained high EGFP reporter gene expression with preserved viability after transduction of whole islets with Ad-pCMV-EGFP. Moreover, confocal microscopy analysis of Ad-pCMV-HA-IκBα(SA)2-transduced islets showed that the HA-tag colocalized with both insulin- and glucagon-positive cells within the islets without detrimental effects on islet viability and function. Interestingly, islets transduced with Ad-pCMV-HA-IκBα(SA)2 showed less cell death and NO production compared to non-transduced islets when exposed to IL-1β+IFN-γ. Even more, the inability to secrete insulin after an acute rise in glucose in non-transduced islets exposed to inflammatory cytokines was prevented in Ad-pCMV-HA-IκBα(SA)2-transduced islets. Conclusions: These data suggest that adenoviral vectors are efficient gene therapy tools to introduce a gene of interest into whole pancreatic islets without detrimental effects on viability and function. Moreover, by blocking NF-κB with adenoviral vectors expressing the non-degradable blocker IκBα(SA)2, we showed that this transcription factor is a "master switch" -cell death and dysfunction and of cytokine-induced islet transplantation.
PREFERENTIAL STIMULATION OF GLUCOSE UTILISATION VERSUS OXIDATION AND PROLIFERATION OF PANCREATIC B-CELLS OF NCX1 PARTIAL KNOCKOUT MICE

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Background and aims: The Na/Ca Exchanger (NCX) is a protein mediating cellular calcium (Ca2+) outflow (forward mode) or Ca2+ inflow (reverse mode), and controlling intracellular Ca2+ homeostasis. In a previous study, we observed that NCX overexpression reduced β-cell proliferation. In the present study, we analyzed mice displaying heterozygous null mutation for Na/Ca exchanger 1 (NCX1), with respect to glucose metabolism and β-cell proliferation.

Material and methods: Mice pancreatic islets were prepared using the collagenase method. Glucose metabolism was evaluated by the measurement of 14CO2 output and 3H2O production by islets incubated in the presence of labelled glucose. Briefly, the islets were incubated in 3H and 14C labelled glucose for 90 minutes in 50 bicarbonate-buffered medium containing bovine serum albumin (5 mg/ml) and D-glucose (2.8-16.7 mM), mixed with tracer amounts of D-[5-3H]glucose and D-[U-14C]glucose (NEN Life Science Products, Boston, MA, USA) as previously described (Hutton J.C., et al. Biochem. J. 184:291-301, 1979). For PNCA (Proliferating Cell Nuclear Antigen) staining, mouse monoclonal anti-PNCA antibody (Dako, Glostrup, Denmark) was diluted to 1:600 and was applied to tissue sections overnight at 4 °C. After incubation with primary antibody, pancreas sections were washed in PBS and treated using the Avidin-Biotin Complex method (Vectastain ABC kit standard, Vector laboratories, Burlingame, CA, USA). The peroxidase activity was visualized using diaminobenzidine, generating a brown reaction product. Tissue sections were then counterstained with toluidin blue. For negative control, IgG mouse was applied to substitute for the primary antibody (Laura L. et al. J. Vet. Diagn. Invest. 10:237-246, 1998). All results are expressed as mean values (number of individual determinations (n). The statistical significance of differences between mean values was assessed by use of Student’s t-test.

Results: Islets from control (NCX1+/+) and NCX1 partial knockout (NCX1+/-) mice showed a nice increase in both glucose utilization (3H2O production) and oxidation (14CO2 output) in response to a rise in glucose concentration from 2.8 to 16.7 mM. In NCX1+/- islets there was a slight tendency towards an increase in glucose utilisation and a significant decrease in glucose oxidation (P<0.01), compared to NCX1+/+ islet, resulting in a drastically decreased 14CO2/3H2O ratio at 16.7 mM (0.15 ± 0.02 vs 0.08 ± 0.01, n= 5, P<0.01). PNCA staining showed a significant increase in β-cell proliferation.

Conclusions: Compared to control islets, NCX1+/- islets showed a preferential stimulation of glucose utilisation vs oxidation, a behaviour comparable to that seen in tumoral cells that preferentially use the anaerobic glycolysis to generate ATP. Simultaneously, NCX1+/- islets showed a higher proliferation rate compared to control islets. Our data indicate that changes in intracellular Ca2+ homeostasis may represent a new tool to increase β-cell proliferation or neogenesis.
TNF-α AND IL-1B INDUCTION OF NF-κB AND ITS DOWNSTREAM GENES HAS A PRO-APOTOTIC ROLE IN PANCREATIC BETA CELLS

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Background and Aims. IL-1β and TNF-α contribute to pancreatic beta cell death in type 1 diabetes. Both cytokines activate the transcription factor NF-κB, but recent observations suggest that NF-κB blocking prevents IL-1β+IFN-α- but not TNF-α+IFN-γ-induced beta cell apoptosis. We presently compared cell death and the pattern of NF-κB activation and global gene expression induced by IL-1β and TNF-α in beta cells.

Material and Methods: Cell viability was measured after exposure to either IL-1β or TNF-α alone or in combination with IFN-γ and blocking of NF-κB activation or protein synthesis. INS-1E cells exposed to IL-1β or TNF-α in time course experiments were used for IKK activation assay, immunocytochemistry for p65 NF-κB, real-time RT-PCR and microarray analysis.

Results. Blocking NF-κB activation protected beta cells against IL-1β+IFN-γ- or TNFα+IFN-γ-induced apoptosis. Blocking de novo protein synthesis did not increase TNF-α- or IL-1β-induced beta cell death, in agreement with similar cytokine-induced expression of the anti-apoptotic genes A20, IAP-2 and XIAP. Microarray analysis of INS-1E cells treated with IL-1β or TNF-α showed similar patterns of gene expression. IL-1β, however, induced a higher expression of NF-κB target genes putatively involved in beta cell dysfunction and death and a stronger activation of the IKK complex leading to an earlier translocation of NF-κB to the nucleus.

Conclusions. NF-κB activation in beta cells has a pro-apoptotic role following exposure not only to IL-1β but also to TNF-α. The more marked IL-1β-induced beta cell death is at least in part explained by higher intensity of NF-κB activation, leading to increased transcription of key target genes.
ENTEROVIRAL VP1 EXPRESSION IN HUMAN TYPE I DIABETES

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Background and aims: Enteroviral infections have long been implicated in the initiation of beta-cell destruction in human Type 1 diabetes (T1D) and a recent study reported the expression of the enteroviral capsid protein, vp1, in the beta-cells of 3/6 (50%) T1D patients. vp1 was not detected in any of 26 controls. However, it is not known whether such a high prevalence of beta-cell VP1 expression is observed in a larger cohort of patients. The aims of this work were to:

1) Examine the prevalence of enteroviral vp1 expression in a larger cohort of recent onset patients with T1D and to compare this with appropriate controls
2) Correlate additional markers of viral infection (such as induction of protein kinase R (PKR) and MHC class 1 hyper-expression) with vp1 expression in the islets of patients with T1D.

Materials and methods: Insulin, glucagon, vp1, PKR and MHC1 expression were examined by immunohistochemistry in serial sections of fixed, paraffin-embedded, pancreas samples removed post mortem from 72 recent-onset T1D patients and 86 relevant controls (including non-diabetic neonatal, paediatric and adult pancreases and adult Type 2 diabetic pancreases).

Results: Highly localised and intense vp1 staining was observed in the islets of 44/72 (61%) of the recent-onset T1D patients. This staining was confined to insulin-containing islets and was usually restricted to only 1-5 cells per islet section. Equivalent vp1-positivity was observed only very rarely in normal paediatric control pancreas (3/50 (6%)) suggesting that persistent vp1 expression is uncommon in the islets of non-diabetic children. Intense islet vp1 staining was present in 3/19 (16%) normal adult pancreases but, surprisingly, was observed in the islets of 9/12 (75%) pancreases from adults with type 2 diabetes (T2D). In adult pancreas, vp1 staining was not confined to islets but was observed frequently in centro-acinar and smooth muscle cells. This pattern was much less common in paediatric samples. A second antibody raised against vp1 also selectively stained islet cells in some recent onset T1D patients, consistent with the presence of the viral antigen. Moreover, a strong correlation was established between vp1 immunopositivity and additional markers of viral infection, including PKR expression and hyper-expression of MHC class 1, in the insulin-containing islets of T1D patients.

Conclusions: These studies confirm that the expression of enteroviral vp1 is common in the islets of recent-onset T1D patients but not in normal paediatric controls. vp1 expression was also strongly correlated with PKR induction and MHC1 hyper-expression in the insulin-containing islets of T1D patients, consistent with a persistent viral infection. However, vp1 immunostaining was also observed frequently in the islets of adult T2D patients, suggesting that the phenomenon is not restricted solely to T1D.
EV-94, A NOVEL POTENTIALLY DIABETOGENIC ENTEROVIRUS SEROTYPE
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Human enteroviruses are classified into four species, Human enterovirus A (HEV-A) to HEV-D, comprised of almost 100 currently known serotypes, many of which have been associated with type 1 diabetes. Enterovirus 94 (EV-94) is a recently described enterovirus serotype that is, together with EV-68 and EV-70, a member of Human enterovirus D species. The seroprevalence studies in the Finnish population have revealed a high prevalence of EV-94 over past two decades.

In this work, we studied the ability of EV-94 to infect, replicate and inflict damage in cellular models of tissues that are considered to play essential role in the pathogenesis of enterovirus induced chronic diseases e.g. type 1 diabetes. EV-94 was able to infect and form infectious progeny in leukocyte cell lines with granulocytic, monocytic, T-cell or B-cell characteristics and in primary human umbilical vein endothelial cells. Moreover EV-94 was able to damage pancreatic islet -cells and infect, replicate and cause necrosis in primary human pancreatic islets. These results provide in vitro evidence that EV-94 might be a potent pathogen and should be considered as a potentially diabetogenic enterovirus type.
AN IMMUNOHISTOCHEMICAL STUDY OF INSULITIS IN EARLY ONSET TYPE 1 DIABETES IN MAN

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Background and Aims: Type 1 diabetes (T1D) in man is caused by immune-mediated destruction of pancreatic β-cells. However, the precise sequence of immune cell infiltration; the composition of the inflammatory infiltrate and the mode of β-cell death have not been well documented. The present study aimed to investigate these parameters in fixed, paraffin embedded, pancreases recovered post-mortem from recent onset T1D patients.

Materials and Methods: Up to 26 recent-onset T1D cases were stained by immunohistochemical methods to reveal the presence of insulin, glucagon and a range of specific immune cell markers, including CD4, CD8 and FoxP3 positive cells (to define helper, cytotoxic and regulatory T cells (Treg) respectively); CD20 (B-cells); CD68 (macrophages); CD138 (plasma cells) and Pen5 (natural killer (NK)) positive cells.

Results: A total of 2642 islets were examined. Insulitis was defined as a minimum of 5 immune cells per islet section and was verified by reference to non-diabetic control pancreas. Early insulitis was defined as being present in an inflamed islet if more than 50% of the area of the islet was composed of insulin secreting beta cells. 609 islets contained insulin and, of these, 200 had insulitis (32.8%). 116 of 2033 insulin-deficient islets had insulitis (5.7%). Individual immune cell sub-types were evaluated in 245 islets. CD8+ T cells were the most abundant population in those islets with early insulitis and the CD8+ cell number increased ~4-fold (p<0.02) during the later stage of β-cell destruction. CD4+ T cells were the least abundant cell type present in the infiltrate at all stages of β-cell loss. The presence of FoxP3+ cells (Tregs) was detected in only one islet in one T1D patient, indicating that these are not an abundant cell type within the inflammatory infiltrate. CD68+ cells were detected at all stages of insulitis and increased modestly in parallel with CD8+ cells but declined after total β-cell destruction. Large numbers of CD20+ cells were recruited to islets during the progression of insulitis but their numbers decreased significantly (p<0.02) in islets with no residual β-cells. CD138+ plasma cells were detected in the islets of 6 T1D cases and their presence correlated with the extent of insulin positivity. Pen5+ cells were detected in low numbers in 19 of the patients suggesting that NK cells are present among the inflammatory cell population but that these cells do not comprise a major component.

Conclusions: This study provides the first detailed analysis of the progression of insulitis in human T1D. It reveals a defined temporal sequence of immune cell recruitment and implies that CD8+ cytotoxic cells and macrophages may both contribute to early β-cell death. CD20+ B-cells could also be involved although their role remains to be defined. NK cells appear to play a more minor role and no significant numbers of Tregs were seen.