

Juvenile Diabetes Foundation Fellows and Their Research

*2000 Meeting of
Career Development
Awardees and Fellows*

March 24–26, 2000

Lansdowne Resort

Leesburg, VA

Sponsored by:

Juvenile Diabetes Foundation International

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National Institute of Allergy and Infectious Diseases, NIH

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Juvenile Diabetes Foundation International
The Diabetes Research Foundation

Juvenile Diabetes Foundation Fellows and Their Research

Agenda

Friday

March 24, 2000

12:30 pm

Welcome and Introduction

*Sandra Puczynski, PhD
Robert A. Goldstein, MD, PhD*

1:00–3:50 pm

Plenary Session 1

1:00–1:50 pm

The Developmental Biology of Beta Cells

Douglas Melton, PhD

1:50–2:40 pm

Islet Development and Regeneration

Nora Sarvetnick, PhD

2:40–3:00 pm

Break

3:00–3:50 pm

Gene Transfer/Gene Therapy Approaches
in Diabetes

Ronald G. Crystal, MD

4:00–5:30 pm

Concurrent Poster Sessions

Group 1

Gene Therapy and Beta Cell Growth
and Differentiation

*Nora Sarvetnick, PhD
Douglas Melton, PhD*

Group 2

Insulin Action

Kenneth Polonsky, MD, PhD

Group 3

Immunology—I

*Mark Atkinson, PhD
Hugh Auchincloss, Jr., MD*

7:00 pm

Dinner

Guest speakers *Michael Stephens,
House Appropriations Committee Staff,
Ruth Farabee Clark and Judith Terra,
JDF volunteers, kidney donor and recipient*

Agenda

Saturday **March 25, 2000**

8:00–9:00 am **Breakfast**

9:00 am–12:25 pm **Plenary Session 2**

9:00–9:50 am The Causes of Type 1 Diabetes

John Todd, PhD

9:50–10:40 am Delineating Genetic Pathways
of Autoimmunity

Edward K. Wakeland, PhD

10:40–11:00 am Break

11:00–11:50 am Prediction and Prevention of Type 1

Mark Atkinson, PhD

11:50 am–12:25 pm Immune Tolerance Network

Jeffrey Bluestone, PhD

12:25–1:25 pm **Lunch**

1:30–3:00 pm **Concurrent Poster Sessions**

Group 4 Genetics and Beta Cell Biology

John Todd, PhD

Douglas Melton, PhD

Group 5 Metabolism and Complications

Kenneth Polonsky, MD, PhD

Group 6 Immunology—II

Hugh Auchincloss, Jr., MD

Jean Francois Bach, PhD

3:15–4:15 pm How to Write a Grant Workshop

*Robert A. Goldstein, MD, PhD
and Staff*

7:30 pm **Dinner**

Agenda

Sunday

March 26, 2000

8:00-11:50 am

Plenary Session 3

8:00-8:50 am

Effects of Diabetes Susceptibility Genes
on Insulin Secretion and Action

Kenneth Polonsky, MD, PhD

8:50-9:40 am

Translation Efforts to Achieve
Tolerance in the Clinic

Hugh Auchincloss, Jr., MD

9:40-10:00 am

Break

10:00-11:50 am

Type 1 Autoimmune Diabetes:
Beta-Cell Antigen Mediated
T-Cell Activation

Jean-Francois Bach, PhD

11:50 am

Wrapup

Distinguished Faculty

Mark A. Atkinson, PhD

Department of Pathology
University of Florida, Gainesville

Hugh Auchincloss, Jr., MD

Transplantation Unit
Massachusetts General Hospital, Boston

Jean-Francois Bach, PhD

Necker Hospital, Paris

Jeffrey Bluestone, PhD

Ben May Institute for Cancer Research, Chicago

Ronald G. Crystal, MD

Division of Pulmonary & Critical Care
New York Presbyterian Hospital, New York

Douglas Melton, PhD

Department of Cellular and Molecular Biology
Harvard University, Boston

Kenneth Polonsky, MD, PhD

Division of Endocrinology
University of Chicago

Nora Sarvetnick, PhD

Department of Immunology
The Scripps Research Institute, La Jolla

John Andrew Todd, PhD

Department of Medical Genetics
Cambridge Institute for Medical Research,
United Kingdom

Edward K. Wakeland, PhD

Director, Center for Immunology
University of Texas Southwestern Medical Center,
Texas

Abstracts

Group 1

Gene Therapy and Beta Cell Growth and Differentiation

s

Faculty

Nora Sarvetnick, PhD

Douglas Melton, PhD

Human Insulin Expression in Rat Intestinal STC-1 Cells

John McLenithan, Keith Tanner, Jessica Pray, Jesse Roth, Alan Shuldiner

Univ. of Maryland, Dept. of Medicine, Div. of Endocrinology, Diabetes and Nutrition, Baltimore, MD.

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Purpose: We have begun studies to address a novel gene therapy approach for diabetes that would restore regulated insulin secretion and normoglycemia. K-cells in the proximal small intestine normally sense changes in glucose concentrations after a meal and secrete glucose-dependent insulinotropic peptide (GIP), a hormone that augments insulin release from the pancreatic beta-cells.

Methods: The coding region of human proinsulin or luciferase was introduced into a transgene vector under the control of the 517 bp proximal promoter of the rat GIP gene which should target expression of human insulin or luciferase to K-cells. STC-1 cells, a neuroendocrine K-cell tumor line, were used to characterize expression and processing of the proinsulin transgene.

Results: The 517 bp GIP-promoter luciferase construct was transactivated in STC-1 cells thirteen fold over the promoter-less luciferase vector. Transfection of 517 bp GIP-proinsulin in

STC-1 cells resulted in the expression, secretion and processing of mature human insulin and C-peptide in the culture media as determined by human-specific RIA. The lack of GIP-driven insulin expression in transfected CHO and RIN1046 cells indicated cell type-specific expression of the transgene. A replication-defective adenovirus harboring the GIP-proinsulin transgene was created to provide a mechanism of insulin gene transduction that could be utilized for gene therapy. Infected STC-1 cells exhibited high levels of insulin expression by immunofluorescence microscopy and insulin secretion into the culture media.

Conclusions: This preliminary characterization of the GIP-proinsulin transgene and the recombinant adenovirus in STC-1 cells lays the groundwork for future animal studies that will place this novel insulin replacement therapy in a more physiological context. Transgenic mouse lines harboring the GIP-proinsulin transgene are currently being generated to test this novel approach.

Production of helper-dependent (gutless) adenoviral vectors for insulin gene delivery to the liver

Nuria Morral, Marcia Meseck, Savio L.C. Woo

Institute for Gene Therapy, Mount Sinai Medical Center, New York

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Purpose: Hyperglycemia represents one of the major problems in Type I diabetes. Constitutive expression of low level insulin expression has resulted in the prevention of ketoacidosis without significant risk of hypoglycemia under fasting conditions. Helper-dependent (gutless) adenoviral vectors have resulted in negligible toxicity to the liver and expression of transgenes for longer than two years in non-human primates. Our goal is to reach basal levels of insulin expression in spontaneously diabetic BB/Worcester rats. We will use gutless vectors to deliver the engineered rat insulin gene to the liver. The first aim towards the development of insulin gene therapy has been the optimization of gutless adenoviral vector production, which requires a helper adenovirus to provide the viral proteins in *trans*.

Methods: C7Cre8 cells and helper HV-loxP2 were used to propagate gutless vectors. pC29NM12, a plasmid containing the minimal viral sequences to allow DNA replication and packaging (i.e., ITRs and packaging signal), was used to clone expression cassettes containing the rat insulin or *E. coli* b-galactosidase cDNAs and rescued in a gutless vector.

Results: Expression cassettes containing an engineered form of rat insulin, or b-gal were introduced in plasmid pC29NM12. The resulting constructs were transfected in C7Cre8 cells, and after transduction with helper virus, gutless vectors were rescued. Amplification of the vector was achieved through successive passages in C7Cre8 cells, with optimal yields at a helper multiplicity of infection (MOI) of 3. Large-scale production resulted in approximately 2×10^{12} particles of vector, with 2% of helper contamination.

Conclusions: We have been able to produce gutless vectors expressing rat insulin and b-gal at yields sufficient to perform animal studies. Future goals include the administration of these vectors in the liver of diabetic BB/Worcester rats to determine what level of hepatic insulin expression is sufficient to prevent ketoacidosis and reduce the severity of non-fasting hyperglycemia, without the risk of fasting hypoglycemia.

Glucose stimulated and self-limited insulin production

Ruihuan Chen, Marcia Meseck, Robert C. McEvoy, Savio L.C. Woo.

Institute for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine

Group 1

Gene Therapy and Beta Cell Growth and Differentiation

Mentor: Savio L.C. Woo

Purpose: To achieve glucose stimulated and insulin inhibited insulin production in hepatocyte-derived cells.

Methods: Adenoviral vectors, harboring engineered rat insulin gene (erINS) or reporter gene (CAT) driven by a constitutive promoter (RSV) or regulatable promoter (G6Pase) were constructed, rescued and purified. Insulin production and glucose 6-phosphatase promoter activity in response to both insulin and glucose were explored in H4IIE hepatoma cells.

Results: The insulin production driven by G6Pase promoter was shown to be glucose concentration-dependent. Compared to the level of insulin production in the presence of 5.5 mM glucose (100%), the level of insulin production was reduced to

30% in the absence of glucose, increased to 170% in the presence of 27.5 mM glucose.

In insulin-expressing hepatoma cells, the produced insulin can feedback inhibit the G6P promoter activity, leading to the self-limited insulin production. The G6Pase promoter activity was suppressed by 50% in the presence of insulin at 100 pg/ml. Effects of glucose and insulin on G6Pase promoter activity appear to be independent of each other. While insulin strongly inhibited the G6Pase promoter activity, the glucose stimulatory effect dominated, to some extent, over the inhibitory effect of insulin in the presence of high levels of glucose.

Conclusions: The properties of insulin production driven by the G6Pase promoter match the needs for the development of gene therapy for type I diabetes.

Bioengineering of surrogate beta cells for the treatment of diabetes

R. Faradji, E. Havari, Q. Chen, R. Mulligan, Y. Liang & M. Lipes.

Joslin Diabetes Center, Harvard Medical School

Group 1

Gene Therapy and Beta Cell Growth and Differentiation

Mentor: Myra Lipes

Purpose: We have shown, using transgenic mouse techniques, that intermediate lobe (IL) pituitary cells from nonobese diabetic (NOD) mice can be engineered to secrete abundant amounts of fully processed, mature insulin (ins), at sufficient levels to cure diabetes when implanted into diabetic NOD recipients. However, unlike transplanted islets, which were rapidly destroyed by recurrent autoimmunity, insulin producing IL pituitary (IL-ins) grafts remained free of autoimmune attack and destruction. These and other findings suggest that IL-ins cells may represent an excellent starting material for the bioengineering of surrogate beta cells. However, a limitation of this insulin gene delivery system is that insulin secretion is not glucose-regulated. The goal of these studies is to introduce glucose-sensing properties into IL-ins cells.

Methods & Results: In an attempt to introduce glucose sensing properties into IL-ins cells, recombinant adenoviruses were created containing the glucose transporter isotype, GLUT2 (Ad-GLUT2) and the islet isoform of glucokinase

(Ad-GK.B1). While glucokinase activity was not detectable in primary cultures of IL-ins cells, transduction of these cells with Ad-GK.B1 resulted in high levels of functional glucokinase activity; comprising > 90% of the total glucose phosphorylating activity, similar to β cells. Likewise, treatment of IL-ins cells with Ad-GLUT-2 resulted in glucose transport kinetics similar to β cells. Our studies showed that the co-infection of IL-ins cells with Ad-GLUT-2 and Ad-GK.B1, resulted in a marked step up in 5-[3 H] glucose usage at the physiologic glucose range (from 3 to 20 mM glucose). Studies are currently underway to assess whether these impressive glucose-induced metabolic changes are coupled to increases in insulin secretion.

Conclusions: These findings suggest that the co-expression of GLUT-2 and glucokinase confers glucose-sensing capabilities in the physiologic glucose range to IL-ins cells. These studies represent the first important steps towards the creation of immunoresistant surrogate β cells for the treatment of diabetes.

Gene therapy approaches to facilitate islet transplantation

Group 1

Nick Giannoukakis,¹ Massimo Trucco² and Paul D. Robbins.¹

¹Department of Molecular Genetics and Biochemistry.

²Division of Immunogenetics, Department of Pediatrics, University of Pittsburgh School of Medicine.

*Gene Therapy and
Beta Cell Growth
and Differentiation*

Mentor: Paul D. Robbins

Purpose: Engineering islets to express immunoregulatory gene products can potentially protect islet transplants from immune rejection as a therapy for type I diabetes. Interleukin-1 β (IL-1 β) induces beta cell dysfunction and Fas-dependent apoptosis activation, processes believed to be important at the onset of autoimmune diabetes. Blockade of IL-1 β signaling by secreted antagonists as well as by intracellular regulators could facilitate the survival of islet transplants as a potential therapy for type I diabetes.

Methods: Initial studies focused on the ability of adenovirus, herpes simplex virus 1 (HSV-1), HIV-1 and EIAV lentiviral vectors to infect human islets in culture. Beta cell function of infected islets was evaluated by static glucose stimulated insulin release assay. cDNAs encoding IL-1 β antagonists (interleukin-1 receptor antagonist protein; IRAP, soluble type II IL-1 receptor; sIIR, insulin-like growth factor I; IGF-I and I κ B; I κ B) were engineered into adenoviral vectors, for proof-of-principle, and intact human islets in culture were then infected with these recombinant vectors. Infected islets were then challenged with IL-1 β in the presence or absence of an activating Fas antibody

to determine the level of protection achieved by each of the antagonists. Finally, the degree of inflammation of IRAP-expressing islets in syngeneic NOD recipients was also assessed.

Results: HIV-1-based lentiviral vectors are able to infect intact human islets as efficiently as adenoviral vectors. EIAV and HSV-1 vectors can also infect islets, but to a lesser degree. Islets expressing the transgenes from adenoviral and lentiviral vectors responded normally to glucose. IL-1 β antagonist expression (IRAP, sIIR, IGF-I and I κ B) protected beta cells from IL-1 β *in vitro*, as assessed by dynamic insulin response. Furthermore, islets expressing IRAP, IGF-I and I κ B were protected from IL-1 β -stimulated, Fas-triggered apoptosis activation. Finally, the degree of inflammation around islets expressing IRAP in syngeneic NOD recipients was considerably reduced compared to controls.

Conclusions: We conclude that gene transfer of IL-1 β antagonists is a promising approach to prevent beta cell dysfunction and Fas-triggered apoptosis activation and may be a means of facilitating islet transplantation as a potential therapy for type I diabetes.

Crystallographic studies on HNF1 α /DcoH/DNA ternary complex

Group 1

Young-In Chi, Lone Hanson, Daniel A. Frantz, Steven E. Shoelson.

Joslin Diabetes Center

*Gene Therapy and
Beta Cell Growth
and Differentiation*

Mentor: Steven Shoelson

Purpose: We are attempting to solve the structure of the HNF1 α bound to the dimerization cofactor (DcoH) and DNA to map where MODY3-causing point mutations are located and to understand the functional binding mode at the molecular level.

Methods: We employ the crystallographic method to solve the three-dimensional structure of this ternary complex. This method requires several steps including overexpression and purification of proteins, synthesis and purification of DNAs, complex formation and crystallization, heavy atom preparation, data collection, phase calculation, electron density map generation, model building and refinement.

Results: Currently, we successfully produced soluble proteins (HNF1 α containing dimerization domain and DNA binding

domain, and the full length DcoH) and obtained pure DNAs for crystallization. Exhausted screening of different conditions resulted in small crystals that diffract to only 8 angstrom. These crystals are very fragile and need to be improved in terms of stability and diffraction quality. We are in the process of optimizing the harvesting conditions as well as trying different oligos for better crystals. In the meantime, we are also preparing engineered proteins and DNAs for heavy atom derivatives.

Conclusions: We are trying to obtain amenable native and heavy-atom derivative crystals from which the phases can be derived. These phases will be combined with the observed intensity data to generate the electron density map that will serve as a basis for model building. The final structure should shed light on how those mutations found in MODY3 patients affect the regular function of HNF1 α and how certain factors control the islet cell maturation and survival.

Misexpression of the POU homeo-domain transcription factor brain-4 to pancreatic BETA cells in transgenic mice results in ectopic glucagon gene expression

Mehboob A. Hussain, Christopher P. Miller, Joel F. Habener

Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA and Genetics Institute, Cambridge, MA

Group 1

Gene Therapy and Beta Cell Growth and Differentiation

We previously reported that the POU-domain transcription factor brain-4 is highly expressed in glucagon-producing pancreatic beta cells. Furthermore, brain-4 and that it activates the beta-cell-specific G1 element of the glucagon gene promoter. Here we that brain4 immunoreactivity is expressed in the epithelium of the dorsal pancreatic bud as early as day 10 of mouse embryo development. Thus, we hypothesize that in an appropriate context of other transcription factors brain-4 is an early determinant of the beta-cell phenotype. To achieve expression of brain-4 in an environment of transcription factors similar to those found in pancreatic beta cells we expressed brain-4 in pancreatic beta-cells *in vivo*. We expressed the brain-4 in transgenic mice under the control of 4.6 kb of the IDX-1/PDX-1 promoter as well as under control of the rat insulin-II promoter. Pax6, a paired domain homeobox transcription factor, is also known to regulate glucagon, as well as insulin and somatostatin gene expression and pax6 gene knockout mice lack pancreatic beta cells and have a reduced beta-cell mass.

Therefore we also overexpressed pax6 directed by the IDX-1 or the rat insulin-II promoters.

Misexpression of brain-4 under the IDX-1 promoter results in the ectopic expression of the glucagon gene in pancreatic beta cells which express insulin. The parallel experiment with overexpression of pax6 directed by either the IDX-1 or the insulin II promoters resulted in no phenotypic change. Misexpression of brain-4 under the insulin II promoter also failed to yield an altered beta-cell phenotype. Expression of IDX-1 occurs at day 9 of mouse embryonic development, at least 24 hours earlier than the expression of insulin. Our findings suggest that overexpression of brain-4 at an early developmental stage under the control of the IDX-1 promoter is sufficient to direct glucagon gene expression in pancreatic beta cells, whereas expression of brain4 at a later time during development fails to alter the phenotype of the pancreatic beta cell.

Regulation of islet cell growth by the cell adhesion molecule Ep-CAM

Vincenzo Cirulli,¹ Steven Mah,¹ Manon Winter,² Sergey Litvinov.²

¹Whittier Institute for Diabetes, Univ. of California San Diego ²State Univ. of Leiden, Netherlands.

Group 1

Gene Therapy and Beta Cell Growth and Differentiation

Cell-cell adhesion molecules (CAMs) have been recently shown to transduce a variety of signals supporting cellular functions as diverse as proliferation, differentiation, and survival. These cellular functions appear to be regulated by a fine balance between the cell surface levels of CAMs and the interaction with specific intracellular signaling partners.

We have recently observed that the expression of Ep-CAM, a novel epithelial cell adhesion molecule, is up-regulated in developing pancreatic islets during fetal life, and in adult islets induced to proliferate *in vitro*. These observations prompted us to study the signaling events transduced by this adhesion molecule in human islet cells.

In a series of co-immunoprecipitation experiments we observed that induction of islet cell proliferation by culture on a specialized extra-cellular matrix (804G) caused the physical interaction of p85, the regulatory subunit of the PI3K, with Ep-CAM. Conversely, very little, if any, Ep-CAM was co-immunoprecipitated from lysates of cells cultured in the

presence of the PI3K-specific inhibitor Ly294002. These results show that the PI3K regulatory subunit p85 can interact with Ep-CAM, and that such interaction is favored in culture conditions promoting cell growth. To determine whether a functional PI3K complex associate with Ep-CAM, lysates from islet cell monolayers grown on the 804G matrix were immunoprecipitated with an anti-Ep-CAM-specific antibody, and then used for a PI3K assays to detect the generation of PI(3)P, a second messenger lipid product of PI3K activity. In these experiments we observed that a significant PI(3)P-specific signal is co-immunoprecipitated with Ep-CAM, indicating that the PI3K complex associated with this adhesion molecule is functionally active.

These results indicate that the transduction of growth signals by Ep-CAM in islet cells may occur through the recruitment and activation of the PI3K pathway. We propose that further study of Ep-CAM-mediated signaling may lead to the identification of novel molecular targets for the *ex vivo* expansion of b-cells to be used in transplantation.

The expression and role of laminin in early pancreatic development

George K. Gittes

Children's Mercy Hospital

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Purpose: Our goal is to identify key molecules that control differentiation of the developing pancreas. We thus hope to better understand and control endocrine-specific differentiation of potential pancreatic stem cells. Here we focus specifically on laminin isoforms and their role in determining exocrine vs. endocrine pancreatic differentiation.

Methods: We analyzed freshly fixed and sectioned embryonic pancreas at serial ages both by *in situ* hybridization and immunohistochemistry. In addition, we used a collagen gel culture system of embryonic pancreas to assay expression of laminin-1 and the effect of blocking laminin-1 expression through antisense. Antisense was performed using either serum-free media and standard sulphated oligodeoxynucleotides, or else a new strategy using morpholino oligodeoxynucleotides

Results: We found peri-exocrine distribution of expression of laminin after approximately day 12 of gestation. By late gestation, and in the adult, laminin-1 was distributed over the

basement membranes lining the exocrine tissue, with sparing of the islets. Interestingly, there was a small amount of laminin-1 in the islets that followed the basement membrane of blood vessels. Similarly, *in vitro* growth of embryonic pancreas resulted in expression of laminin-1 in the region of the developing exocrine tissue, with absence of laminin in the endocrine region. Antisense against laminin-1 $\alpha 1$ chain using either of the two antisense systems both were able to dramatically block exocrine differentiation with a concomitant high level of insulin expression.

Conclusions: Laminin-1 is expressed in the developing basement membrane of the pancreas as the exocrine tissue begins to differentiate. Also, laminin-1 expression is directly correlated with exocrine differentiation as shown by the *in vitro* studies, and is necessary for exocrine differentiation, as shown by the antisense studies. We feel that these results build on our previous data and further support an important role for laminin-1 and basement membrane in exocrine vs. endocrine pancreatic lineage selection.

NeuroD is required for proper islet development and function

Jacqueline E. Lee, Takaki Miyata, Tomoko Maeda, Edoardo Marcora, Zhi Mao and Melissa Moore

University of Colorado at Boulder, Boulder, Colorado

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Purpose: To determine the function of NeuroD in developing and mature islets.

Methods: We use both the loss-of-function and gain-of-function phenotypes to assess the consequence of having aberrant levels of NeuroD in developing and mature mouse pancreas.

Results: NeuroD is a basic helix-loop-helix transcription factor implicated in development of both the nervous system and pancreas. We have generated mice lacking NeuroD. These mice are born, albeit with some neurological defects, but soon become severely diabetic and die within a few days after birth. We have performed extensive histological and EM analyses on these pancreas. The NeuroD-null pancreas fails to form islets and the endocrine cells are less granulated. Apart from insulin, we have not been able to find any islet specific-marker whose expression level is significantly reduced. The total pancreatic insulin content at birth is approximately 5% of the normal level. We performed a BrdU pulse-labeling experiment at different stages during development to determine the "birthdate" of insulin-positive cells. In NeuroD-null mice, there is anywhere from 19% to 87% reduction in the number of

BrdU-labeled cells depending on the pulsing stage, indicating that either the cells fail to be generated or are eliminated shortly after birth. Consistent with the idea of cell death hypothesis, we detect a high number of TUNEL-positive cells in the NeuroD-null pancreas of e17.5-18.5, a finding previously reported by Naya et al (1997). Although *neuroD* is only expressed in the post-mitotic cells, cell proliferation is also affected in NeuroD-null mice, suggesting that insulin expression might have a local mitotic activity during development.

We have recently rescued NeuroD-null mice from neonatal diabetes by reintroducing NeuroD under the insulin promoter (RIP-1). Surprisingly, the rescued mice remain hyperglycemic, although they survive generally healthy for many months, albeit severe neurological problems due to their CNS phenotype. We will discuss the endogenous and transgenic insulin promoter activities, as well as the degree of islet rescue in these mice.

Conclusions: NeuroD is a factor critical for development of the endocrine cells in the pancreas. NeuroD also seems to have a negative feedback role to keep the homeostasis in mature islets.

Analysis of Function and Tissue-Specific Expression of the Homeodomain Protein PDX-1

Sabire Ozcan

Department of Biochemistry, University of Kentucky College of Medicine, Lexington, Kentucky

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Purpose: The beta-cell specific transcription factor PDX-1 is required for pancreatic development and for glucose-induced expression of insulin, GLUT2 and glucokinase genes in islet cells. Mutations in PDX-1 cause pancreatic agenesis in animals and humans. Our main goal is to study the function of PDX-1 in pancreas development and in beta-cell specific gene expression.

Methods: PDX-1 wild type and a dominant negative version of PDX-1 are overexpressed in beta-cell lines and in isolated rat islet-cells utilizing the efficient adenovirus-mediated gene transfer. Trans-acting factors involved in beta-cell specific expression of the PDX-1 gene are identified and cloned using the yeast one-hybrid screen with cDNA libraries made from pancreatic tissues.

Results: We have obtained the adenoviral constructs for the wild type and the dominant negative version of PDX-1 protein just recently. We are currently in the process of amplifying and purifying these adenoviruses and will use them to infect the

beta-cell lines bTC6-F7, INS-1, MIN6 and isolated rat-islets to study the effects of PDX-1 overexpression on glucose-stimulated gene expression. These experiments will demonstrate whether PDX-1 overexpression is a suitable strategy for gene-therapy purposes.

To identify trans-acting factors responsible for beta-cell specific expression of PDX-1 we started a yeast one-hybrid screen with the PDX-1 promoter region that has been shown to be sufficient for beta-cell specific expression. We are currently screening a cDNA library made from the human pancreatic tissues. In addition, we isolated RNA from rat-islets to construct a cDNA library that will be employed in the yeast one-hybrid screen. The proteins identified in this screen will contribute to our understanding of how pancreas development is regulated.

Conclusions: The data obtained from the above outlined experiments will be presented and discussed at this workshop.

The role of Pdx1 in pancreogenesis and pancreatic function

Andrew M. Holland* and Raymond J. MacDonald

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*Current address: Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Purpose: To study the function of *Pdx1* by creating a model mouse system in which the expression of the *Pdx1* gene can be regulated by the addition or withdrawal of a small molecule drug.

Methods: We have utilized a tetracycline-regulatable gene expression system to generate genetically modified mice in which the endogenous expression of *Pdx1* has been abolished. In these mice, the coding region of the *Pdx1* locus has been replaced with the coding region for transcriptional activator that can be regulated by tetracycline and which is capable of activating the expression of a bigenic transgene encoding PDX1 and a GFP reporter, which acts as a visible marker of transgene expression.

Results: We have successfully generated several lines of genetically modified mice in which the expression of *Pdx1* should be derived entirely from the regulatable transgene. We have recapitulated the earlier knock-out studies in which *Pdx1*-null

mice were found to be apancreatic. In addition we have shown that the bigenic *Pdx1* transgene is activated in mice bearing the modified *Pdx1* locus containing the coding region for the tetracycline-regulatable transcriptional activator. Thus, expression of GFP was detected as a reporter of transgene expression in the pancreata of heterozygous knock-out/hemizygous transgenic neonatal mice.

Conclusions: Preliminary results indicate that the use of a tetracycline-inducible gene expression system to study gene function will be an effective alternative to currently available temporally regulated gene-knockout systems. The trans-activated expression of a bigenic transgene has demonstrated the potential functionality of this system *in vivo*. Breeding and manipulation of these genetically modified animals is currently ongoing in order to study the role of *Pdx1* in the developing, mature and injured pancreas. These studies will help to define the role of *Pdx1* in pancreogenesis, pancreatic neogenesis and the maintenance of pancreatic function.

Control of enteroendocrine differentiation by bHLH components

Jan Jensen, Erna E. Pedersen, Philip Galante, R. Scott Heller, Jakob Hald, Palle Serup and Ole D. Madsen.

Hagedorn Research Institute, Gentofte, Denmark

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Mentor: Ole D. Madsen

Purpose: We have investigated the role of the Notch signaling system in the development of gastro-entero-pancreatic endocrine cells and addressed the cell lineage relationship between the pancreatic α - and β -cells.

Methods: We have analyzed pancreatic and gastro-intestinal development in mice deficient in the gene *Hes1*. *Hes1* encodes a repressor bHLH factor of the Hairy/enhancer-of-split type and is a known downstream component in the Notch-signaling system. These analyses have been performed using a combination of immunohistological, *in situ* hybridization and semi-quantitative PCR analyses.

Results: *Hes1* mutant mice show a strong bias towards endocrine fate selection in both the pancreas, stomach, and gut.¹ In all tissues, the endocrine cells are post-mitotic after completing differentiation. In the pancreas, the increased endocrine differentiation causes depletion of the PDX1-expressing precursor cells, in turn resulting in a strong pancreatic hypoplasia. In both stomach and gut, all types of endocrine cells are forming prematurely, and in excess numbers. On the

transcriptional level, a marked up regulation of several B-class bHLH factor genes (*Atoh5* (encoding *ngn3*), *Neurod* and *Math1*) is observed. We investigated the developmental relationship between pancreatic α - and β -cells and found that these two cell types develop independently of one another,² using a very similar bHLH cascade where *Atoh5* (*ngn3*) marks endocrine precursor cells within the population of pancreatic epithelial cells. Terminal differentiation of these towards either the α - or the β -cell state is conferred by *Neurod* activation, mitotic exit, and subsequent activation of *Isl1* and *Pax6*. Intrinsic differences between the two cell types are conferred by *Brm4* (α -cells), or high level expression of *Pdx1*, *Nkx6-1* with an additional specifying role of *Pax4* (β -cells).

Conclusions: We conclude that a balance of positive and negative bHLH components governs endodermal endocrine cell development. The involvement of *Hes1* suggest that Notch-mediated lateral inhibition may play a pivotal role in refinement of the endocrine fate directly from endodermal progenitors, whether it be pancreas, stomach, or gut. We show that pancreatic α and β cells are independently selected from epithelial pancreatic precursors using a temporal bHLH cascade involving *ngn3* and *NeuroD*.

Identification of Cis- and Trans-acting Factors Involved in Pancreatic Islet Selective Expression of *pdx-1*

S.E. Samaras and R.W. Stein.

Vanderbilt University Medical Center

Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Mentor: Roland W. Stein

Purpose: PDX-1 is a homeodomain transcription factor essential in pancreatic development and islet β cell function. Thus, the absence of PDX-1 in mice and humans results in pancreatic agenesis. Our laboratory has chosen to use *pdx-1* as a model to identify transcription factors involved in β cell selective expression and early islet cell specification. We expect that this analysis will provide functional data for both characterized and novel proteins.

Methods and Results: Towards this end, we first showed that endogenous *pdx-1* expression could be recapitulated using a promoter fragment from -4.5 kilo bases (kb) to the transcription start site. Sequence comparison between mice, humans and chickens revealed that the only region of significant homology occurred over a nuclease hypersensitive site (HSS) spanning 1 kb, termed HSS1. This region was sufficient to direct β cell selective expression of a reporter *in vivo* and in β cell lines *in vitro*. Three areas of high sequence identity (Areas

I, II and III) were found in the HSS1 region, of which Area I or Area II alone mediated β cell selective expression in transient transfection assays. Interestingly, conserved Area II was only found in mice and humans, suggesting a unique functional role in mammals. During the past year, I have shown that Area II can also independently direct β cell selective expression in transgenic mice. As a consequence, we propose that Area II is a key conserved, functional sub-domain of HSS1. To identify the cis-acting factors involved in β cell selective expression of Area II, sequence-saturating mutations spanning the conserved sequences were made and tested in transient transfection assays in β cells. A mutation that resulted in a 50% decrease in activity and was found to bind the Pax6 paired-domain/homeodomain-containing transcription factor, a protein whose expression within the pancreas is restricted to the islet cells (α , β , δ and PP).

Conclusions: Our data indicate that Pax6 acts in conjunction with an as yet unidentified factor(s) to restrict *pdx-1*/Area II activity to β cells.

Expression of Neurogenin3 Reveals an Islet Cell Precursor Population in the Pancreas

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Group 1

Gene Therapy and Beta Cell Growth and Differentiation

Purpose: Differentiation of early gut endoderm cells into the endocrine cells forming the pancreatic islets of Langerhans depends on a cascade of gene activation events controlled by transcription factors including the cell-type specific (class B) basic helix-loop-helix (bHLH) protein BETA2/neuroD1. The finding that BETA2/neuroD1 $-/-$ mice have a marked reduction of the beta-cell mass but no apparent defect in beta-cell formation, prompted us to search for additional bHLH factors involved in beta-cell formation.

Methods: RNA was prepared from rat islets and different pancreatic cell lines. Reverse transcription-PCR was carried out using degenerate primers derived for the helix 1 and the loop of the conserved bHLH encoding region of neuroD1. RNA in situ hybridization, immunohistochemistry and immunofluorescence assays were performed on paraffin sections. Transgenic mice were generated by pronuclear injection of different vectors containing full length mouse neurogenin3, neuroD1/ BETA2 or myoD cDNA downstream of the mouse pdx1 promoter.

Results: We demonstrate that islet cells express a broad group of class B bHLH genes, the most abundant being neurogenin3 (ngn3), neuroD1/BETA2 and neuroD4/math3. Ngn3 immunoreactivity can be detected transiently in scattered ductal and periductal cells in the fetal mouse pancreas, peaking at embryonic day 15.5. Ngn3 co-stains with early islet differentiation factors Nkx6.1 and Nkx2.2, but not with late factors Isl1 and Pax6 or islet hormones. Analysis of transcription factor deficient mice demonstrates that ngn3 expression is not dependent on Nkx2.2, Nkx6.1, neuroD1/BETA2 or mash1. On the other hand, early expression of ngn3 under control of the Pdx1 promoter drives early and ectopic differentiation of islet cells, a capability shared by neuroD1 but not myoD.

Conclusions: These data support a model in which ngn3 acts upstream of neuroD1/BETA2, neuroD4 and other islet differentiation factors, marking islet cell precursors and driving the differentiation of endocrine cells, but switching off prior to final differentiation.

Pax4 acts as a transcriptional repressor in early pancreatic development

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Group 1

Gene Therapy and Beta Cell Growth and Differentiation

Mentor: Michael S. German

Purpose: Pax4 is a paired-homeodomain transcription factor that is essential for the development of pancreatic beta cells. The purpose of this study was to characterize the transcription factor pax4. In terms of DNA binding specificity, transcriptional activity and temporal expression pattern.

Methods: See Smith et al (1999). Mol. and Cell Biol. 19 (12) 8272-8280

Results: Pax4 recognises a bipartite DNA consensus sequence consisting of a paired domain binding site, located 3' of a homeodomain binding site. The spacing between the two sites is apparently variable, but a population of highly selected binding sites exhibited a common spacing of 15 bp. Comparable sequences were found within the promoters of several islet derived hormones (including insulin and glucagon), and were shown to bind pax4. Pax6 can also bind to these sites.

Pax4 acts as a transcriptional repressor through a single binding site located upstream of a ubiquitous promoter, or through the

insulin and glucagon promoters. The repressive effect of pax4 depends upon the cellular context. Repression is not due to competition with the transcriptional activator pax6, as a pax4-GAL4 DNA binding domain fusion protein, can repress transcription through a GAL4 DNA binding site.

During murine development, pax4 mRNA as determined by RT-PCR, is most abundant at E13.5. It is not detectable in adult islets.

Conclusions: The temporal expression pattern of pax4, suggests that pax4 is primarily involved in beta cell development, rather than in maintenance of the mature phenotype. It is noteworthy that the peak of pax4 expression (E13.5), corresponds to a developmental time point associated with the onset of an expansion in the beta cell population.

Our data is consistent with a model in which, pax4 represses the alpha cell gene expression programme and direct precursor cells towards a beta cell fate.

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Purpose: In the developing pancreas, homeodomain transcription factor Nkx6.1 is required for the normal differentiation of the beta-cells. In the mature pancreas, Nkx6.1 is unique among transcription factors in its absolute restriction to the beta-cells. The purpose of this study is to investigate the mechanism that regulate Nkx6.1 expression,

Methods: We isolated a clone containing Nkx6.1 gene from a mouse genomic library, and mapped the transcription start site by 5' RACE and RNase protection assay. To map regions important for specific expression in beta-cells, we performed reporter gene assays using firefly luciferase as a reporter. Then to identify protein that bind to the mapped region, we performed electromobility shift assays. To investigate the activity of the 5' untranslated region (5'UTR) and test for a possible internal ribosomal entry site (IRES), we used bicistronic assays with the 5'UTR between two reporter genes in a single construct.

Results: 1. The Nkx6.1 promoter lacks a classic TATAA sequence. It has a long GC-rich 5' untranslated region (5'UTR)

downstream of a cluster of transcription start sites adjacent to a CCAAT box. 2. Analysis with the firefly luciferase reporter gene shows that Nkx6.1 gene sequences from -5.6 kb to +1.0 kb (relative to the transcription start site) have specific promoter activity in beta-cell lines but not in NIH3T3 cells. This activity is dependent on sequences between -850 bp and -780 bp and the 5'UTR. 3. Electromobility shift assays demonstrate that homeodomain transcription factors PDX1 and Nkx2.2 bind to sites within the upstream enhancer element. 4. Analysis of the 5' UTR reveals that it can function with a heterologous promoter, but the full sequence is required and it must be downstream of the transcription start site. Further, we demonstrate that that it functions as a potent internal ribosomal entry site (IRES), providing cell-type specific regulation of translation.

Conclusions: These data support a model in which PDX-1 and Nkx2-2 are located directly upstream of Nkx6.1 in the hierarchy of beta-cell transcription factors. In addition, post-transcriptional regulation of Nkx6.1 expression through the IRES in the 5'UTR provide additional mechanisms that further restrict its expression to beta-cells

Aquisition of mature α cell identity is disrupted in correlation with compromised β cell production in the developing mouse pancreas

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Abstract: In the developing pancreas the first endocrine cells to be detected, at e9.5, produce glucagon. Insulin expressing cells are seen shortly afterwards, but remain a minority population until around e14.5 when an expansion of β cells occurs. This event is referred to as the secondary transition.

We show here that the early α cells, prior to this secondary transition, have distinctly different characteristics to mature α cells. They express proteins that are restricted to b and d cells in the adult pancreas, specifically the β cell polypeptide IAPP (Islet amyloid polypeptide) and the convertase PC1. IAPP is thought to require the islet homeodomain transcription factor pdx-1 for its expression, but coexpression of glucagon and pdx-1 is never seen. We show here that the IAPP expressing cells do not express pdx-1.

Transgenic animals made using the pdx-1 promoter driving the bHLH transcription factor ngn3 have premature differentiation of endocrine cells in the developing pancreas at the expense of

exocrine tissue. The majority of these endocrine cells are α cells, suggesting that the secondary transition events driving β cell expansion have not occurred normally in these animals. We show here that many of the α cells in these animals express IAPP and PC-1 at e18.5, whereas in WT animals IAPP and PC1 are excluded from the majority of α cells by e18.5.

Under certain culture conditions e11.5 pancreatic bud explants form endocrine and exocrine cells after 5 days in culture. The majority of the endocrine cells express glucagon, and few insulin expressing cells are present, implying that under these conditions the secondary transition is not occurring. The α cells all express PC-1 suggesting that the signals required to form mature α cells are also absent.

This data indicates that at around the same time as the β cell population start to expand the α cell population begins to take on mature characteristics. In situations where this expansion of β cells is blocked, production of truly mature α cell is impaired also. These processes may therefore require common developmental signals.

Glucose regulates islet amyloid polypeptide (IAPP) gene transcription in a PDX1 and calcium-dependent manner

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Islet amyloid polypeptide (IAPP) is a 37 amino acid peptide hormone co-secreted with insulin from the pancreatic β -cells, and is present in amyloid deposits in islets of Langerhans of patients with NIDDM. Insulin and IAPP mRNA levels are both regulated by glucose, and the promoters of both genes share similar *cis*-acting sequence elements, with both binding the homeodomain transcription factor PDX1, which plays an essential role in the regulation of insulin mRNA levels by glucose. Here we examine the role of PDX1 in the regulation of the human IAPP promoter by glucose. The experiments were facilitated by the availability of a human β -cell line, NES2Y, which lacks PDX1. NES2Y cells also lack operational K_{ATP} channels, resulting in a loss of control of calcium signaling. In the mouse β cell line MIN6, glucose (16 mM) stimulated a 3.5- to 4- fold increase in the activity of a -222 to +450 IAPP promoter construct compared to values observed

in 0.5 mM glucose. In NES2Y cells, glucose failed to stimulate transcriptional activation of the IAPP or the insulin promoter. However, whilst over-expression of PDX1 in NES2Y cells reinstated glucose responsive control of the insulin promoter, it had no effect on the IAPP promoter. Glucose effects on the IAPP promoter were only observed in the presence of PDX1 when normal calcium signaling was restored by over-expression of the two K_{ATP} channel subunits SUR1 and Kir6.2. The importance of calcium was further emphasized by inhibition of these events by the calcium channel blocker verapamil (50 μ M), which had no effect on the insulin promoter. These results demonstrate that, like insulin, glucose regulation of the IAPP promoter is dependent on the activity of PDX1, but unlike insulin it additionally requires the activity of another, as yet uncharacterized factor(s), the activity of which is calcium dependent.

Characterization of the Transgenic Insulin-Deficient Rat

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Purpose: To investigate the genetics and timing of when the pancreas begins to lose its ability to properly secrete insulin in our transgenic rat model of insulin deficient diabetes.

Methods: The changes in hormone levels in plasma and total pancreas are being analyzed from samples collected from rats between days 1 to 35 of life. Total pancreas RNA is being isolated to correlate the timing of the loss of the mRNA with the plasma levels.

The transgene (which contains rat liver *cpt1* cDNA and the rabbit globin gene) is being used as a probe to clone the insertion sites transgenic genomic DNA.

Results: The islet initially appears morphologically normal then between 3-5 days after birth the amount of pancreatic insulin (both protein and mRNA) begins decreasing. The plasma glucose level remains normal until 22 days of life when

it begins increasing. At that time the plasma insulin level and insulin:glucose ratio begins falling as compared to wild-type. Interestingly, the amount of apoptosis in the islet remains similar to that seen in the wild-type until ~day 13-18 of life suggesting there is an alteration in function prior to the onset of cell death.

Conclusions: This information suggests that the gene we have altered may play a very important role not only in the health of the β -cell but also in maintaining the integrity of the whole pancreatic islet. Refinement of the timing of the β -cell changes will allow us to study the function of this gene in great detail. Additionally, we will be able to study the cell biology of the pancreatic islet and the interrelation of each specific cell type with a goal of preventing the loss of the pancreatic cells prior to the onset of clinically apparent diabetes. These studies may have applications in the prevention of Type 1 Diabetes in humans.

Evidence for the Role of a Novel bHLH Family Member, BETA4, Pancreas and Brain Development

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Group 1

Gene Therapy and
Beta Cell Growth
and Differentiation

Mentor: Ming-Jer Tsai

Purpose: The basic helix-loop-helix (bHLH) family of transcriptional regulators are characteristically involved in tissue-specific gene regulation and cellular differentiation. We are studying the role of several members of this transcription factor family in pancreas development to further delineate components of the etiology of diabetes. For this purpose, the expression pattern of a novel bHLH factor BETA4 has been assessed and BETA4 knockout animals have been generated.

Methods: BETA4 is a basic helix-loop-helix (bHLH) factor that was cloned by way of a homology based screen of a cDNA library derived from β TC cells. BETA4 expression was detected in the developing pancreas and brain by *in situ* hybridization. Thus, to determine the function of BETA4 during development, BETA4 knockout mice were generated through gene targeting technology. Furthermore, analysis of the BETA4 knockout mice has been facilitated by replacing a portion of the BETA4 coding region with the LACZ gene. The cellular expression pattern of BETA4 can be visualized by a simple b-galactosidase staining procedure. We have examined BETA4 expression in adult and embryonic tissues of wild type and BETA4 deficient animals through *in situ* hybridization and β -galactosidase staining.

Results: BETA4 expression was examined by *in situ* hybridization and was detected in non-epithelial cells associated with the pancreatic ducts exclusively during embryonic pancreas development. The timing and location of BETA4 expression in the embryonic pancreas is co-incident with differentiating pancreatic endocrine cell precursors that emerge from the duct epithelium at this stage. BETA4 does not appear to be expressed in the adult pancreas. BETA4 is also strongly expressed in the dorsal thalamus and habenula regions of the diencephalon during development. However, preliminary evidence shows BETA4 knockout animals are viable and maintain normal weights when fed a low fat high protein diet. Further experiments are underway to determine the effect of the loss of BETA4.

Conclusions: BETA4 is expressed in the developing embryo during the pancreatic endocrine cell and thus is likely involved in endocrine cell differentiation. It is also likely that BETA4 plays a role in diencephalic development. Because BETA4 knockout animals are viable, we conclude that BETA4 plays a subtle and highly specific role in development. Additional analysis of the BETA4 knockout pancreas is necessary before conclusions as to its role in pancreas development can be made. Furthermore, the inclusion of the LACZ knock-in will serve as a useful marker in the analysis of the developing pancreas.

Structural basis of dimerization, coactivator recognition and MODY3 mutations in HNF-1 α

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Purpose: Maturity-onset diabetes of the young type 3 (MODY3) is associated with mutations in hepatocyte nuclear factor 1 α (HNF-1 α). This dimeric transcriptional activator regulates genes in pancreatic islet cells. Three MODY3 mutations alter the dimerization domain of HNF-1 α , which is required for DNA binding and association of the transcriptional coactivator DCoH (dimerization cofactor of HNF-1). We characterized the functional defect of these mutations, and correlated them with the structure of the dimerization domain.

Methods: The structure of the dimerization domain of HNF-1 α (HNF-p1), alone and in complex with the coactivator DCoH, was determined by x-ray crystallography. The MODY3 mutations in HNF-1 α were characterized by gel shift, Western blot and temperature melting assays.

Results: The structures demonstrate that HNF-p1 forms a unique four-helix bundle. DCoH undergoes a dramatic tetramer to dimer conversion upon binding to HNF-1 α .

Unexpectedly, a single, bifunctional interface in DCoH mediates both homotetramerization and HNF-1 α binding. Consistent with the crystal structures, two MODY3 mutations in HNF-p1 (Leu12His and Gly20Arg) inhibit dimerization, DNA binding and DCoH recognition. A third mutation (Gly31Asp), situated on the solvent exposed surface of the dimerization domain, does not disrupt dimerization or the associated recognition of DNA and DCoH.

Conclusions: Loss-of-function mutations in HNF-1 α are dominant, demonstrating that HNF-1 α is haploinsufficient for glucose homeostasis. As predicted from the structures, the mutations Leu12His or Gly20Arg prevent the interaction of HNF-1 α with DCoH, dimerization, and subsequent DNA binding. These mutations argue strongly for the idea that glucose homeostasis in humans is sensitive to the dose of HNF-1 α . An additional function for the HNF-1 α dimerization domain is suggested by the Gly31Asp mutation, which does not affect dimerization.

Abstracts

Group 2

Insulin Action

Faculty

Kenneth Polonsky, MD, PhD

Stimulation of Skeletal Muscle 3-O-methylglucose Transport by AICAR Varies With Fiber Type

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Group 2

Insulin Action

Mentor: Neil B. Ruderman

Purpose: Exercise increases skeletal muscle glucose uptake (i.e. contraction-stimulated glucose transport) that can result in hypoglycemia in Type I diabetics. Recent studies suggest activation of the enzyme AMP activated protein kinase (AMPK) may be responsible for contraction-stimulated glucose transport. The purpose of this study was to evaluate if activation of AMPK by hypoxia or 5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR) have similar effects on the rate of 3-O-methylglucose (3MG) transport in isolated muscles composed of predominantly type IIb (i.e. extensor digitorum longus, EDL) or type I (i.e. soleus) fibers. In addition, we also examined the effects of two putative inhibitors of AMPK, iodotubercidin or adenine arabinofuranoside (Ara), on hypoxia- and AICAR-stimulated 3MG transport, as well as insulin-stimulated 3MG transport, which operates via an AMPK-independent mechanism.

Methods: Following dissection, isolated soleus and EDL muscles were allowed to recover in the presence or absence of iodotubercidin and Ara prior to specific glucose transport

stimuli. Thereafter, muscles were incubated in the presence or absence of either hypoxia, AICAR, or insulin. Ultimately, muscles were incubated in the presence of [^3H]-3MG, removed, frozen and then processed to determine the rate of 3MG transport into the muscles.

Results: Hypoxia and insulin increased the rate of 3MG transport in both muscles, whereas, AICAR stimulated 3MG transport only in the EDL. Iodotubercidin did not affect basal or insulin-stimulated 3MG transport in either muscle, however hypoxia-stimulated 3MG transport was reduced and AICAR-stimulated 3MG transport was less than basal values in both muscles. Ara had no effect on insulin-stimulated 3MG transport, reduced basal 3MG transport, modestly decreased hypoxia-stimulated 3MG transport in both muscles and totally inhibited the stimulation of 3MG by AICAR in the EDL.

Conclusions: These findings question the role of AMPK activation in modulating contraction-stimulated glucose transport in the soleus. They also suggest that hypoxia, unlike AICAR, may increase 3MG transport by AMPK-independent mechanism(s).

Refinement of IGF-1 receptor gene structure and search for potential mutations in genetic syndromes of severe insulin resistance and intrauterine growth retardation

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Purpose: Since many genetic syndromes display alterations in both insulin signaling and intrauterine growth, we raise the question whether mutations in IGF-1 receptor gene may have a pathogenic role. In this goal we refined IGF-1 receptor gene structure and established protocols for mutation detection from genomic DNA.

Methods: Sequencing of 12 clones (pBluescript II SK +/- Stratagene) covering the entire IGF-1 receptor gene was performed on an automatic sequencer ABI 373A (Perkin Elmer). This allowed to define 200-400 bp flanking intron sequences of all 21 exons. Since putative DNA interaction sites were also suspected between exons 13 and 14, this entire intron sequence was equally determined. Refinement of this gene structure allowed the screening for potential mutations in genomic DNA using PCR amplification and direct sequencing of 24 fragments of 300pb.

Results: The screening of several samples of human genomic DNA in normal population allowed the identification of 18 silent polymorphisms, 7 located in exons and 11 in introns. Examination of IGF-1 receptor gene in the Type A syndrome

was performed in 2 patients where both IGF-1 binding and tyrosine phosphorylation of the receptor were altered. These alterations were concomitant with mutations in the insulin receptor (one homozygous Tyr646Cys and another compound heterozygous Asp59Gly and Leu62Pro). Despite clear alteration in IGF-1 signaling, no mutations were found in these two patients. Extended studies over 19 other patients with various degree of insulin resistance or resistance at growth hormone treatment failed to detect missense mutations. A leprechaun patient with two new mutations in the insulin receptor (Cys524OPAL and Arg1092Trp) and characterized by intrauterine growth retardation did not display missense mutations in IGF-1 receptor gene.

Conclusion: We conclude that despite alterations in IGF-1 signaling in syndromes of severe insulin resistance, pathogenic mutations are absent from the IGF-1 receptor gene. The absence of mutations in the leprechaun patient also suggest that mutations in the IGF-1 receptor gene are rare in humans. It may be possible that deleterious mutations are associated with very severe syndromes with early intrauterine death.

Two-dimensional gel electrophoresis-based analysis of protein phosphorylation related to high glucose effect

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Insulin Action

Purpose: Hyperglycemia is regarded to be an activator of Protein kinase C (PKC) signaling pathway in vascular tissues leading to downstream protein phosphorylation and subsequent alteration of vascular cell function.

Methods: We therefore investigated influence of high glucose condition (25 mmol/L) in cultured, ortho-³²P-labeled vascular smooth muscle (VSMC) with regard to two-dimensional gel electrophoresis (2-DE)-based phosphoprotein pattern.

Results: We therefore investigated influence of high glucose condition (25 mmol/L) in cultured, ortho-³²P-labeled vascular smooth muscle (VSMC) with regard to two-dimensional gel electrophoresis (2-DE)-based phosphoprotein pattern. Over-expression of PKCbeta-wildtype, the mainly activated isoform, using adenoviral mediated gene transfer revealed that increase in PKC levels can result in altered silver stained total protein array. This indicates that changes in PKC levels can result in altered protein expression in vascular tissues. Exposure of the VSMC to high glucose condition lead to some distinct changes although total phosphoprotein pattern remained rather

unchanged. One series of phosphorylated proteins (MW: ~30kD; pI: 5.4 and 5.7), however, had been identified by comparable immunoblot and phosphoprotein analysis as heat shock protein (HSP) 27 which is involved in cellular migration by modulating actin polymerization. Both proteins showed an 1.5-2fold phosphorylation increase. Immunoprecipitation/immunoblot analysis showed two additional non-phosphorylated forms of HSP 27 (pI 6.2 and 6.4). Analysis of HSP27 in-vivo-phosphorylation in aorta from control (duration of diabetes; 4 weeks; body weight: 315 g; blood glucose: 113 mg/dl) and STZ-induced diabetic rats (4 weeks; 260 g; 422 mg/dl) compared to subsequent immunoblot analysis revealed a shift from non-phosphorylated form at pI 6.4 (23 % decrease) to progressively more acidic/phosphorylated form with pI at 5.4 (50 % increase).

Conclusions: This study demonstrates that 2-DE-based analysis of protein phosphorylation is a valuable approach to investigate signal transduction events. High glucose effect regarding cellular protein phosphorylation and activation requires further investigation.

The Interactive Effects of insulin, IGF-1 and Hyperglycemia in Murine Skin Cells—An IR-and IRS1-Null Mouse Models

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Insulin Action

Impaired wound healing of skin is one of the serious complications resulting from the diabetic state. One can hypothesize that the abnormal insulin signaling and the resulting hyperglycemia, characteristic of the diabetic state, might lead to the impaired skin wound healing. Little is known, however, on the role of insulin receptor (IR) family of proteins in normal skin physiology, in order to support such a theory.

We have investigated the relative roles of insulin, IGF-1 and hyperglycemia, all of which are abnormal in the diabetic state, in skin cells. The model used is primary keratinocytes isolated from wild type (WT) and transgenic mice lacking the expression of IR (IR-KO) or of IRS1 (IRS1-KO). These cells can proliferate and differentiate *in vitro* similarly to skin *in vivo*. These models enable us to study skin cells exhibiting insulin resistance.

We have found that while both insulin and IGF-1 induced keratinocyte proliferation, these hormones had contrasting effects on keratinocyte differentiation. Chronic insulin stimulation

facilitated the Ca²⁺-induced keratinocyte differentiation, while IGF-1 inhibited this process. Accordingly, in differentiating keratinocytes, insulin induced IR autophosphorylation was sustained while autophosphorylation of IGFR by IGF-1 was abrogated. These results were further confirmed in IR-KO keratinocytes where lack of IR expression was associated with induction of keratinocyte proliferation and abnormal spinous differentiation. Furthermore, IRS1-KO keratinocytes exhibited abnormal keratinocyte differentiation *in vitro* and abnormal skin development *in vivo*. The importance of glucose metabolism to keratinocyte growth and differentiation was shown when incubation of keratinocytes in high glucose had no effects on IR activity, while it markedly inhibited the IGFR activity.

Our results demonstrate that IR and IGFR are distinctly regulated, have different interactions with glucose and differentially affect keratinocyte differentiation suggesting an active role for these hormones in normal skin physiology as well as pathological conditions such as Diabetes Mellitus.

Freeze-drying offers an improved way to analyze early insulin signaling events from muscle biopsies

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Insulin Action

Purpose: FD offers the possibility to 1) purify muscle fibers from connective tissue, blood and vessel structures, 2) dissect and divide glycolytic and oxidative fibers and, 3) aliquote material precisely prior to the subsequent analysis. Our aim was to compare early insulin signaling events and the expression of slow and fast myosin heavy-chain (MHC) isoforms in FD vs wet homogenized tissue.

Methods: Male Wistar rats (n=6) were anesthetized and insulin (15U) or saline was injected into the portal vein. Gastrocnemius muscle was snap-frozen in liquid nitrogen at 3 minutes after insulin. Prior to the analysis the biopsy was aliquoted for wet and FD homogenization and the FD samples were freeze-dried for 12 h. Muscle fibers were purified from connective tissue, blood and vessels structures. Homogenization of wet and FD samples was performed in a homogenization buffer containing protease and phosphatase inhibitors. Expression of insulin receptor (IR), IRS-1, GSK-3, Akt/PKB, and p85 subunit of PI 3-kinase (PI3K), and tyrosine phosphorylation of IR as well as MHC expression were analyzed using SDS-PAGE and Western

blotting from straight homogenates or after immunoprecipitation with appropriate antibody. IRS-1 associated PI3K activity was analyzed *in vitro* from IRS-1 immunoprecipitates. Western blots and PI3K activity assays were visualized using autoradiography and analyzed by ImageGauge software.

Results: The autoradiography signals from FD and wet homogenized samples were identical regarding IR, IRS-1, GSK-3, Akt/PKB, and p85 subunit of PI3K. Insulin stimulated tyrosine phosphorylation signal of IR was of the same magnitude and increased similarly in FD (20-fold) and wet homogenized (20-fold) samples. Insulin stimulated activity of IRS-1 associated PI3K was comparable between FD (5-fold) and wet (4-fold) homogenized samples. MHC isoform expression profile was identical between FD and wet homogenized tissue.

Conclusions: These data indicate that freeze-drying does not affect the recovery of the expression, post-translational modifications (tyrosine phosphorylation) or enzymatic activity (PI3K) of some key insulin signaling proteins.

Insulin-stimulated GLUT4-containing Vesicle Docking and Fusion Visualized in 3T3-L1 Adipocytes

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Group 2

Insulin Action

Mentor: Jeffrey E. Pessin, Ph.D.

Purpose: The insulin-stimulated glucose transport process requires the trafficking of GLUT4 vesicles to the plasma membrane, docking of these vesicles and finally vesicle fusion. We examined the effect of reduced temperature on insulin receptor signal transduction and GLUT4 translocation.

Methods: For all studies 3T3L1 adipocytes were pre-equilibrated at 23°C or 37°C for 2 h in serum free medium and were then treated in the absence or presence of 100 nM insulin for the times indicated. The effect of reduced temperature on insulin receptor signal transduction and GLUT4 translocation was assessed by several means: (1) Plasma membrane sheets were prepared and subjected to GLUT4 immunofluorescence microscopy. (2) Whole cells were fixed, permeabilized and subjected to GLUT4 immunofluorescence confocal fluorescence microscopy. (3)

Results: At 23°C insulin-stimulated GLUT4 vesicle trafficking demonstrated an approximate 7-fold increase in the half-time for plasma membrane translocation ($t_{1/2}$ ~30 min)

compared to 37°C ($t_{1/2}$ ~4 min) without a significant change in the extent of GLUT4 translocation. Localization of GLUT4 in intact cells demonstrated that at 23°C there was a time-dependent accumulation of GLUT4 vesicles adjacent to the inner face of the cell surface membrane but which was not contiguous and/or physically incorporated into the plasma membrane. Proximal insulin signaling events including insulin receptor autophosphorylation, IRS protein tyrosine phosphorylation, stimulation of PI 3-kinase activity and *in vivo* formation of PI(3,4,5)P3 were not significantly different between cells maintained at 23°C versus 37°C. The extent, but not the rate, of insulin-stimulated Akt activity at 23°C was markedly decreased compared to that observed at 37°C. However, a dose-response study demonstrated that the extent of activity elicited with 100 nM insulin at 23°C is comparable to that elicited at 37°C with 1 nM insulin, a dose which stimulates maximal GLUT4 translocation.

Conclusions: These data are consistent with the concept that the temperature-dependent decrease in the rate of GLUT4 translocation results from a reduction in GLUT4 vesicle fusion to the plasma membrane.

Potential Role of the Trimeric GTP-binding protein (Gq/11) in the regulation of insulin-stimulated GLUT4 translocation

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Group 2

Insulin Action

Mentor: Jeffrey E. Pessin

Purpose: Previous studies have suggested that the heterotrimeric GTP binding proteins (G-proteins) may play an important role in the regulation of GLUT4 translocation. In this study, we investigated the potential role of G-proteins regulating GLUT4 translocation in adipocytes.

Methods: We co-expressed the cDNAs for enhance green fluorescent protein epitope tagged GLUT4 (GLUT4-EGFP) with the α subunits of wild type Gq (Gq/WT), Gi2 (Gi2/WT), Gs (Gs/WT) and constitutively active Gq (Gq/Q209L), Gi (Gi/Q205L) and Gs (Gs/Q227L) in 3T3L1 adipocytes. To evaluate PI3 kinase activity, we took advantage of EGFP tagged Grp1-PH domain which binds specifically to PI(3,4,5)P3. To attenuate G-protein activation, we over-expressed RGS (regulators of G-protein signal) proteins and microinjected antibodies against α subunits.

Results: Expression of Gq/Q209L induced GLUT4-EGFP translocation which was further increased following insulin stimulation. In contrast, expression of neither Gi2/WT,

Gs/WT, Gi2/Q205L, nor Gs/Q227L had any effect on GLUT4 translocation. Insulin induced GLUT4 translocation was significantly inhibited by over-expression of RGS protein and microinjection of anti-Gq/11 antibody without affect on PI3 kinase activation. However, insulin had no effect on Gq activation as determined by changes in intracellular calcium concentration or tyrosine phosphorylation whereas endothelin-1 (ET) potently activated Gq in 3T3L1 adipocytes. Despite Gq activation by ET, there was only a slight increase in GLUT4 translocation. These data indicate that Gq function may be required but is not sufficient as a signal transducer of GLUT4 translocation. Consistent with this interpretation, Gq/Q209L expression was also observed to induce the translocation of GLUT1 and the mannose-6-phosphate/IGF2 receptor to a greater extent than that of insulin. However, expression of Gq/Q209L had no effect on the rate of transferrin receptor endocytosis, suggesting that Gq/Q209L accelerates the rate of constitutive exocytosis.

Conclusions: Gq/11 probably plays an important role in general vesicle exocytosis including GLUT4-containing vesicles and is permissive for insulin-induced vesicle trafficking.

GLUT4 traffic in a L6 muscle cell line stably expressing a GFP- and myc-tagged GLUT4 chimera

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Purpose: The glucose transporter GLUT4 cycles to and from the plasma membrane in muscle and adipose cells. Insulin regulates GLUT4 traffic to increase its presence on the cell surface. In cells transiently expressing high levels of GLUT4-GFP chimeras, the traffic elements may become saturated and low transfection efficiency precludes biochemical analysis. Therefore, we generated and characterized a stable L6 myoblast line expressing GFP-GLUT4myc where GFP is at the GLUT4 N-terminus and a myc tag is inserted in the first exofacial loop. This cell line allow for the study of GLUT4 traffic in a system where low levels of GFP-GLUT4myc are uniformly expressed.

Methods: 2-deoxy glucose transport was employed to evaluate the ability of the chimera to respond to insulin stimulation in stably transfected cells as compared to wild type L6 myoblasts that do not express GLUT4. Cell surface GFP-GLUT4 myc was measured using an Elisa-like assay. GFP-GLUT4 myc localization and movement were assessed using confocal fluorescence microscopy.

Results: Expression of myc-tagged GFP-GLUT4 elevated basal glucose uptake. Insulin increased glucose uptake by approximately 2-fold. The cell surface myc signal was also increased by approximately 2-fold upon insulin stimulation with a t_{1/2} of 7-9 min. Stimulation of the cells with insulin followed by insulin removal resulted in a decrease of both glucose transport and cell surface myc signal. By confocal microscopy, the GLUT4 chimera was largely perinuclear in the basal state and insulin caused a significant increase in GFP-GLUT4myc at the cell periphery. Upon insulin stimulation followed by insulin removal, real time fluorescence showed the disappearance of the chimera from the cell surface within 30 min.

Conclusions: GFP-GLUT4myc expressed at low levels in L6 myoblasts traffic in response to insulin in a similar manner to non-chimeric GLUT4. This clonal line will allow for biochemical and morphological studies of basal and insulin-stimulated GLUT4 traffic.

Mice without hepatic cytosolic phosphoenolpyruvate carboxykinase (PEPCK) maintain normal fasting plasma glucose concentrations but have impaired lipid metabolism

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Group 2

Insulin Action

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Purpose: PEPCK is widely thought to catalyze the rate-determining step for gluconeogenesis in the liver and kidney. We generated mice with both global and conditional knockout alleles to determine the actual role of PEPCK on energy metabolism in an intact animal model system.

Methods: Conditional (pck^{lox}), functionally-impaired ($pck^{lox+neo}$), and null (pck^{del}) PEPCK alleles were generated using *cre/loxP* gene targeting strategy. Liver-specific PEPCK knockout mice ($pck^{lox+lox}+Alb-cre$) were made by intercrossing an albumin-cre transgene with pck^{lox} mice.

Results: Mice that globally lack PEPCK die within 3 days after birth, apparently from hypoglycemia, however heterozygous PEPCK null mice ($pck^{w/del}$) do not exhibit any obvious metabolic alterations. Moreover, $pck^{lox+neo/lox+neo}$ mice that have over 80% reduction in PEPCK gene expression are euglycemic after fasting although they have mildly enlarged liver and elevated hepatic and renal malate concentrations. Liver-specific PEPCK knockout mice that essentially lack all PEPCK activity

in the liver also maintain euglycemia after fasting. However, these mice manifest major changes in lipid metabolism including hepatomegaly and hepatic steatosis, increased fasting plasma FFA and triglyceride concentrations and decreased β -hydroxybutyrate concentration. In fasted liver-specific PEPCK knockout mice mRNA abundance for a variety of enzymes involving hepatic mitochondrial and perxisomal FFA oxidation as well as for enzymes in the TCA cycle, malate-aspartate shuttle, and substrate anaplerosis are elevated compared to controls. After a 26-h fast, these mice have similar glucose turnover rate but diminished hepatic gluconeogenesis compared to controls as determined in an isotopic study in which mice were infused with [$3-^3H$]glucose and [$U-^{14}C$] lactate.

Conclusions: Mice that either have diminished global expression of PEPCK or lack PEPCK only in the liver maintain euglycemia after fasting. While these results indicate that the control strength of PEPCK on blood glucose concentrations is low unexpectedly, they also demonstrate that this enzyme is essential for normal hepatic lipid metabolism.

Direct and Indirect Effects of Insulin on Net Hepatic Glucose Uptake (NHGU)

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Purpose: The aim of this study was to determine how much of insulin's effect on hepatic glucose uptake arises from direct hepatic action versus indirect (extrahepatic) action.

Methods: Experiments were carried out on 42-h fasted dogs. Each experiment consisted of an equilibration (-120 to -30 min), a basal (-30 to 0 min), and an experimental period (0 to 240 min). During the experimental period, somatostatin was infused to inhibit the endocrine pancreas and glucagon was replaced intraportally at a basal rate in each group. Glucose was infused into the portal vein at a constant rate (4mg/kg/min) and into a peripheral vein (at variable rates) to keep the hepatic glucose load 2-fold basal. In the first group (**BI**, n=5), insulin was infused intraportally (0.3mU/kg/min) to keep insulin basal. In the second group (**PoI**, n=5), insulin was infused intraportally at 4-fold basal (1.2mU/kg/min). In the third group (**PeI**, n=6),

insulin was infused into a peripheral vein (0.6mU/kg/min) to create a selective increase in the arterial insulin level as occurred in PoI

Results: Arterial and portal insulin levels ($\mu\text{U/ml}$) were 6 ± 1 and 23 ± 3 in BI, 18 ± 2 and 91 ± 16 in PoI, and 18 ± 2 and 15 ± 1 in PeI, respectively. In BI, arterial nonesterified fatty acid and glycerol levels were reduced 61% and 41% from basal to the last 120 min. On the other hand, their reductions were 84% and 61% in PoI, and 87% and 57% in PeI, respectively. Arterial glucagon levels were reduced by ~15% in all three groups. During the last 120 min NHGU (mg/kg/min) averaged 1.8 ± 0.4 , 4.2 ± 0.7 , and 3.2 ± 0.7 in BI, PoI, and PeI, respectively. NHGU was significantly greater in PoI ($P<0.005$) and PeI ($P<0.05$) than in BI.

Conclusions: About half of the increment in NHGU in PoI could be explained by the rise in arterial insulin.

Serine789 is the phosphorylation site in IRS-1 for enhanced serine kinase activity in insulin resistant rodents

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Insulin Action

Mentor: Xiao Jian Sun

Purpose: Serine phosphorylation of IRS-proteins has been implicated in attenuation of insulin signaling and is hypothesized to be a mechanism for insulin resistance. The purpose for this investigation is intended to identify the serine phosphorylation site in IRS-1 for enhanced serine kinase activity in insulin resistant rodents.

Methods: After IRS-1 was phosphorylated in vitro by enhanced serine kinase, the tryptic phosph-peptides were purified through HPLC. Manual amino acid radio-sequencing was performed to determine the serine phosphorylation site in IRS-1.

Results: There was an enhanced serine kinase activity in insulin resistant rodents JCR:LA-cp rats and Zucker rats.

2) IRS-1 was phosphorylated at serine789 by the enhanced serine kinase. 3) Converting serine789 to glycine completely abolished ability of IRS-1 to be phosphorylated by the enhanced serine kinase. 4) Serine789 is in the sequence motif that is completely different from consensus sequences for mitogen-activated protein kinase, glycogen synthase kinase-3, protein kinase C, cAMP-dependent protein kinase, phosphatidylinositol 3-kinase and mTor.

Conclusions: The unique sequence motif for the enhanced serine kinase indicated that the enhanced serine kinase is most likely to be a new serine kinase. The fact that the serine kinase activity increased in both JCR:LA-cp and Zucker insulin resistant animal models suggests that it may play an important role in the development of insulin-resistance by phosphorylating IRS-proteins.

Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression

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Group 2

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Purpose: Identify the molecular mechanisms underlying the regulation of mRNA translation in response to insulin, at the level of PHAS-I and eIF4E. Therefore we have determined:

1) the regulation of phosphorylation of the five (S/T)P sites of PHAS-I by insulin, amino acids and rapamycin; 2) the role of each site in the dissociation of the PHAS-I/eIF4E complex; 3) the role of each site in insulin-stimulated cap-dependent translation.

Methods: We have generated a panel of mutants having Ser/Thr to Ala mutations in the five (S/T)P sites (Thr36, Thr45, Ser64, Thr69 and Ser82). The mutants were transiently transfected in HEK293 cells. The regulation of phosphorylation of the sites was evaluated by labeling cells with ^{32}P i. The association between eIF4E and the PHAS-I mutants was determined by purifying eIF4E with $m^7\text{GTP}$ -Sepharose. The cap-dependent translation was measured by co-transfecting with PHAS-I mutants a bicistronic vector encoding two reporter elements that reflect cap/eIF4E-dependent and cap/eIF4E-independent translation rates, respectively.

Results: There are three different levels of regulation of the five (S/T)P sites of PHAS-I. At one extreme in nonregulated phosphorylation, exemplified by constitutive phosphorylation of Ser82. At an intermediate level, amino acids and insulin stimulate the phosphorylation of Thr36, Thr45 and Thr69 via mTOR-dependent processes that function independently of other sites in PHAS-I. At the third level, the extent of phosphorylation of one site modulates the phosphorylation of another. This control is represented by Ser64 phosphorylation, which depends on the phosphorylation of all three TP sites. The five sites have different influences on the affinity of PHAS-I for eIF4E and on cap-dependent translation, with the maximal effect for Thr36 and Thr45, followed by Thr69, and finally Ser82 and Ser64.

Conclusions: Each of the (S/T)P sites of PHAS-I is able to influence the dissociation of PHAS-I/eIF4E complex and insulin-stimulated mRNA translation, and there is a good correlation between these two events.

Basis of ARI binding using thermodynamics and X-ray Crystallography

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Insulin Action

Purpose: To understand the basis for aldose reductase inhibitor binding. To design more specific aldose reductase inhibitors that will alleviate diabetic complications without toxic side effects.

Methods: ARIs are for the most part competitive in the 'backward reaction' (alcohol \rightarrow aldehyde). The competitive inhibition constants of a series of phenylacetic acid derivatives have been determined for a temperature range of 4C to 40C. The three dimensional structure of representative inhibitors in this series bound to both mutant and wild type human aldose reductases have been determined using X-ray crystallography.

Results: Using the relatively simple pharmacophor, phenylacetic acid, we have found that substituents that are β to the carboxylic acid are detrimental to binding. Substituents at the 4 position on the ring are not important for binding. The majority of substituents (F, CH_3 , Cl, Br) at the 2 or 6 position on the ring improve binding by about ten-fold per substituent. For these substituents the improvement in binding is due to increases in ΔS . Further, the crystal structures show that there

is little difference in binding by these inhibitors compared to the parent compound. Based on this analysis, these substituents are increasing ΔS by decreasing the entropy of the free compound. By comparison, the 2-hydroxy substituent is bound to aldose reductase about 100-fold tighter than the parent compound, and the increase is due to changes in ΔH . Interestingly, the binding effects for 2-hydroxy phenylacetic acid are not seen in the C298A/W219Y double mutant.

Conclusions: This analysis has provide two new routes for designing specific aldose reductase inhibitors. First, derivatives of phenylacetic acid at the 4 position may be made to explore the "specificity" site without compromising the inherent binding affinity of phenylacetic acid. Second, a hydroxyl group at the 2-position of the ring may provide for the increased specificity that is needed to differentiate aldose reductase from the other members of the aldo-keto reductase super family. Further investigations are underway.

Ceramide Blocks Adipogenesis and Inhibits the Transcriptional Activity of CCAAT Enhancer Binding Protein (C/EBP)

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Purpose: Adipogenesis of 3T3-L1 preadipocytes is an established model for investigating molecular mechanisms regulating terminal cell differentiation (TCD). Ceramide is a bioactive sphingolipid metabolite which is a potent regulator of TCD. We examined the effect of ceramide on adipogenesis of 3T3-L1 preadipocytes to identify novel molecular targets by which ceramide regulates TCD.

Methods: Preadipocytes were induced to undergo adipogenesis in the presence and absence of exogenous short chain ceramide analogs. The effect of ceramide on the adipocyte phenotype was determined by staining for triacylglycerol accumulation. Levels of adipogenic transcription factors were determined by western blot analysis. The effect of ceramide on the transcriptional activity of C/EBP β was determined using a luciferase reporter construct.

Results: Elevation of endogenous ceramide levels blocked adipogenesis of 3T3-L1 preadipocytes and was associated with decreased expression of the requisite adipogenic transcription factors CCAAT enhancer binding protein α (C/EBP α) and

peroxisome proliferator activated receptor γ (PPAR γ). The expression of C/EBP α and PPAR γ is dependent upon an increase in the expression and transcriptional activity of LAP, a 34 kDa form of C/EBP β . C2-ceramide had no effect on the expression of LAP but increased the expression of a 20 kDa form of C/EBP β (LIP) which may act as an inhibitor of transactivation. Importantly, C2-ceramide significantly inhibited C/EBP β -mediated transcription from a reporter construct in both 3T3-L1 cells and HEK 293 cells. However, LIP formation may not be necessary for inhibition of C/EBP β -mediated transcription since ceramide increased LIP formation in 3T3-L1 cells but not in HEK 293 cells. Inhibition of C/EBP β transactivation may be linked to a ceramide-induced decrease in the phosphorylation state of C/EBP β potentially through increased protein phosphatase activity.

Conclusions: These results suggest that ceramide blocks adipogenesis by inhibiting C/EBP β -mediated transcription. The identification of the molecular mechanism of this block may provide novel targets for the development of novel anti-adipogenic small molecule drugs.

Abstracts

Group 3

Immunology—I

Faculty

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Neonatal TNF α promotes diabetes in NOD mice by CD154-independent antigen presentation to CD8 $^+$ T cells

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Group 3

Immunology—I

Purpose: To establish the relationship between localized inflammation and the breakdown in peripheral tolerance in NOD mice.

Methods: Transgenic NOD mice that express TNF α exclusively in their islets (TNF α -NOD mice) were generated were subsequently crossed to the NOD-SCID, NOD- $\beta 2\mu^{-/-}$, NOD-CIITA $^{-/-}$ and NOD-CD154 $^{-/-}$ backgrounds. For diabetes monitoring, TNF α -NOD mice and non-transgenic littermates were tested weekly for glycosuria. For histological studies, pancreata were excised at various time-points, and 5-7 μ m frozen sections were stained with the appropriate antibodies. For antigen presentation assays, the pancreas was extracted, and the islets hand-picked. Islet-infiltrating antigen presenting cells (APCs) were sorted into dendritic and B cell populations. The respective APCs were cultured with NOD CD4 $^+$ T cells and the ability to present islet antigen determined *in vitro*. Promotion of autoaggression of islet-specific CD4 $^+$ and CD8 $^+$ T cells was determined by adoptive transfer of CD4 $^+$ TCR-transgenic T cells or cloned insulin-specific CD8 $^+$ T cells into TNF α -SCID mice.

Results: Islet-specific expression of TNF α in neonatal NOD mice accelerated diabetes onset in comparison to non-transgenic littermates. In neonatal transgenic mice, disease was preceded by apoptosis of some β -cells, upregulation of MHC class I molecules on residual islet cells, and influx and activation of both APCs bearing MHC-islet peptide complexes and T cells. Infiltrating dendritic cells (DCs), but not B cells, from neonatal islets activated islet-specific T cells *in vitro*. Further, neonatal expression of TNF α could promote autoaggression of both effector CD4 $^+$ and CD8 $^+$ T cells. Whereas CD8 $^+$ T cells are critical for diabetes progression, CD4 $^+$ T cells play a lesser role. TNF α -mediated diabetes development was not dependent on CD154-CD40 signals or activated CD4 $^+$ T cells.

Conclusions: It appears that TNF α can promote cross-presentation of islet antigen to CD8 $^+$ T cells using a unique CD154-CD40-independent pathway. These data provide new insights into the mechanisms by which inflammatory stimuli can bypass CD154-CD40 immune regulatory signals and cause activation of autoreactive T cells.

Reduced incidence of diabetes and impaired TH1 responses in GM-CSF-deficient NOD mice

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Immunology—I

Purpose: To understand the role of granulocyte-macrophage colony stimulating factor (GM-CSF) in autoimmune diabetes and whether inhibition of GM-CSF may be a useful therapeutic strategy. GM-CSF is a potent growth factor for dendritic cells (DCs) *in vitro* but its role in immune responses *in vivo* remains incompletely understood.

Methods: GM-CSF-deficient mice backcrossed for 8 generations on the NOD mouse background were produced. Standard methods of analysing insulinitis (H&E and immunoperoxidase staining) and diabetes incidence were used. T cell responses to recombinant human glutamic acid decarboxylase (GAD) 65 (gift of T Dyrberg) were measured after immunization in CFA. Autoantibodies were measured by routine radio-precipitation assays.

Results: Insulinitis was delayed and reduced and diabetes was rarely seen in female GM-CSF $^{-/-}$ NODs (1/20 at 250d) compared with GM-CSF $^{+/+}$ mice (18/20). Transfer of splenic T cells from diabetic NOD mice to neonatal GM-CSF $^{+/+}$ or $^{-/-}$ NOD mice showed that there was a delay in diabetes in

GM-CSF $^{-/-}$ recipients but no delay when activated CD4 $^+$ T cells from BDC2.5 T cell receptor transgenic NOD mice were transferred. Interferon- γ production in response both to the beta cell autoantigen GAD65 and to a general immune stimulus (P. Acnes/lipopolysaccharide) was markedly reduced in knockout mice. Anti-GAD antibody titres were more modestly decreased. Surprisingly serum IL-12 p75 levels after P Acnes/LPS were elevated, perhaps due to reduced IL-12 receptor expression.

Conclusions: GM-CSF $^{-/-}$ NOD mice were almost completely protected from diabetes and insulinitis was significantly reduced. T cell immune responses, including Th1 cytokine responses associated with the pathogenesis of diabetes, were inhibited in these mice. CD4 T cell activation may be less affected than CD8. Decreased T cell activation was possibly due to a previously unrecognised requirement for GM-CSF for IL-12 responsiveness and IFN γ production. GM-CSF warrants further investigation as a therapeutic target in diabetes and other autoimmune diseases.

Purpose: Our group has recently characterized a unique population of T lymphocytes that coexpress two functionally competent antigen receptors (dual TCR⁺ T cells) and are potentially autoreactive. This study examines the role of dual TCR⁺ cells in the immunopathogenesis of autoimmune diabetes.

Methods: Dual TCR⁺ cells were determined flow cytometrically as T cells coexpressing two species of TCR α ⁺ and/or TCR β ⁺. Islet reactive dual TCR⁺ T clones were established by limiting dilution from spleen and lymph node of NOD mice.

Results: Dual TCR⁺ T cells are abundant in the lymphoid tissues of NOD mice and appear to expand throughout the preclinical period. There is a relative enrichment of dual TCR⁺ cells in the pancreatic lymph nodes, suggesting the

involvement of this cell type in a local immune response. To formally evaluate the autoreactive content of the dual TCR⁺ repertoire, we have succeeded in establishing long term islet reactive dual TCR⁺ T cell lines. A panel of islet reactive dual TCR⁺ T cell clones has been derived from these lines by limiting dilution and are presently under evaluation for their ability to transfer diabetes and to be regulated through modulation of TCR coexpression.

Conclusions: NOD mice exhibit an abundance of dual TCR⁺ T cells which are amenable to establishment in culture as long term lines and clones. Such clones express the stable surface expression of two distinct TCR β ⁺ species of antigen receptor and are reactive to β -granule membrane fraction. The existence of cells with two antigen receptor species imposes unique regulatory problems for the maintenance of self tolerance.

Type 1 diabetes-susceptible and resistant MHC class II molecules modulate insulinitis in HLA transgenic mice

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Group 3

Immunology—I

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Purpose: To evaluate the role of predisposing and resistant MHC class II molecules in the initiation of insulinitis in A β o/DR3/DQ8 transgenic mice.

Methods: Pancreata from A β o/DR3/DQ8, A β o/DR3/DQ6 [control], A β o/DR2/DQ8 [control], A β o/DR3, A β o/ DQ8, A β o/DR2 [control], A β o/DQ6 and A β o [controls] mice were evaluated histopathologically for insulinitis. The animals that showed insulinitis were monitored till about 30 weeks of age for the development of symptoms of clinical diabetes. Recombinant human glutamic acid decarboxylase [GAD 65] protein or a GAD peptide [mouse sequence 250-270] was used to immunize A β o/DR3/DQ8 mice to determine whether it could modulate insulinitis in these mice.

Results: HLA DR3 [DRB1*0301] and DQ8 [DQB1*0302] are associated with susceptibility to type 1 diabetes while HLA DR2 [DRB1*1502] and DQ6 [DQB1*0601] are protective and neutral respectively. Pancreata from the 3 groups of double transgenic mice as well as the single transgenic controls -

A β o/DR3, A β o/DQ8, A β o/DR2, A β o/DQ6 and A β o were evaluated for insulinitis. A β o/DR3 and A β o/DQ8 single transgenic mice showed mild insulinitis [7-8% of the total islets were affected] while A β o/DR2 and A β o/DQ6 mice did not show any insulinitis. **Naive** A β o/DR3/DQ8 mice had lymphocytic infiltration in about 17% of the islets, however, in the A β o/DR3/DQ6 and A β o/DR2/DQ8 there was no insulinitis, suggesting that the presence of a neutral [DQ6] or resistant [DR2] HLA allele in the presence of either DR3 [DR3/DQ6] or DQ8 [DR2/DQ8] can prevent islet inflammation in these transgenic mice. The A β o/DR3/DQ8 animals also showed spontaneous T cell reactivity to human GAD 65 [which is 96% homologous to mouse GAD]. Immunization with the recombinant human GAD 65 diminished the intra-islet inflammation in A β o/DR3/DQ8 when compared to naive animals but the peptide -m250-270 immunization did not alter the pattern of insulinitis in these mice.

Conclusions: It appears, therefore, that while diabetes-susceptible HLA molecules are sufficient for the insulinitis in the transgenic mouse model system, the onset of diabetes probably requires other genetic and environmental factors.

Progression of islet inflammation to autoimmune diabetes driven by affinity maturation of a CD8⁺ T cell population

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Immunology—I

Mentor: Pere Santamaria

For unknown reasons, type 1 diabetes develops after a long, protracted period of mononuclear cell inflammation of pancreatic islets. Here we show that progression of benign pancreatic islet inflammation to overt diabetes in nonobese diabetic (NOD) mice is driven by the "affinity maturation" of a prevalent, pancreatic beta cell-specific CD8⁺ T-cell population that recognizes two related peptides (NRP and NRP-A7) in the context of H-2Kd major histocompatibility complex class I molecules. As pre-diabetic NOD mice grow older, their pancreatic islet-associated CD8⁺ T-cells contain increasing numbers of NRP-A7-reactive CD8⁺ T-cells, and the T cell receptors (TCRs) expressed by these T-cells bind NRP-A7/H-2Kd tetramers with increased specificity, increased affinity and longer half-life. This affinity

maturation of the NRP-A7-reactive CD8⁺ T-cell population occurs during the time when penetrance of diabetes through the NOD colony follows an exponential distribution. Repeated treatment of pre-diabetic NOD mice with soluble NRP-A7 peptide does not prevent the development of insulinitis or the accumulation of low affinity NRP-A7-reactive CD8⁺ T-cells in islets, but blunts the affinity maturation of the NRP-A7-reactive CD8⁺ T-cell population by deleting clonotypes bearing high affinity TCRs. This results in a marked reduction in the local production of beta cell-cytotoxic CD8⁺ T cells (CTL) and halts the progression of insulinitis to diabetes. Affinity maturation of pathogenic autoreactive T-cell populations may therefore represent the long-sought-after key event in the progression of benign islet inflammation to overt diabetes.

T cells deficient in the down-regulatory molecule CTLA-4 can bypass Stat-6 to differentiate into TH2 cells

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Immunology—I

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Purpose: CTLA-4 is a down-regulatory molecule expressed by activated T cells. Mice deficient in CTLA-4 develop a lethal lymphoproliferative disease due to hyperproliferation of activated CD4⁺ Th2 cells. To study the role of CTLA-4 and Th2 cells in the development of diabetes, we generated NOD-SCID mice deficient in CTLA-4. Even though the onset of the disease was delayed as compared to CTLA-4^{-/-} mice, NOD-SCID-CTLA-4^{-/-} mice similarly died from a Th2 cell-mediated autoimmune disease targeting multiple organs, but strikingly did not develop diabetes. This unexpected result led us to examine if CTLA-4 deficiency was associated with abnormal Th2 cell differentiation.

Methods: Stat-6 is a transcription factor that plays a critical role in the differentiation of Th2 cells. Proliferation and cytokine production by splenic and lymph nodes T cells from Stat-6^{-/-} CTLA-4^{-/-} and control mice were studied.

Results: Stat-6^{-/-} CTLA-4^{-/-} mice died at 2 to 3 weeks of age from a lymphoproliferative disease resembling the pathology observed in CTLA-4^{-/-} mice. T cells from Stat-6^{-/-} CTLA-4^{-/-} mice displayed an activated phenotype and were autoproductive for a short period of time *in vitro*. Surprisingly, Stat-6^{-/-} CTLA-4^{-/-} T cells were able to produce the Th2 cytokines IL-4, IL-5 and IL-10 after a primary stimulation *in vitro*, whereas Stat-6^{-/-} CTLA-4^{+/-} T cells produced only the Th1 cytokines IL-2 and IFN- γ after primary or secondary stimulation.

Conclusions: T cells deficient in the down-regulatory molecule CTLA-4 can bypass Stat-6 to differentiate into Th2 cells. This result has important implications for the understanding of the signaling pathways and transcription factors implicated during early Th2 differentiation. Moreover, this finding may be particularly relevant to diabetes with the recent finding that diabetes can be prevented by autoreactive regulatory T cells through an IL-4 and Stat-6-dependent mechanism.

Lack of neonatal tolerance to proinsulin elicits the pathogenesis of IDDM in NOD mice

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Immunology—I

Mentor: Terry L. Delovitch

Purpose: To investigate whether a lack of neonatal tolerance to islet autoantigen (s) other than GAD65 and insulin exists in NOD mice that may predispose to the development of Type I diabetes.

Methods: We determined whether peripheral T cells in female NOD mice respond to proinsulin and other islet autoantigens. We analyzed T cell reactivity to proinsulin and other islet autoantigens at different ages, and examined the effects of various immunization schedules on the progression to onset of IDDM.

Results: T cell reactivity to three islet autoantigens, proinsulin, GAD67 and ICA69 was detectable in neonatal NOD mice.

Peripheral T cell responses to proinsulin arose in the spleen and pancreatic lymph nodes of three week-old NOD mice despite the expression of proinsulin in the thymus. Neither oral administration nor parenteral immunization of six week-old NOD female mice with proinsulin prevented the onset of diabetes. However, neonatal vaccination with proinsulin started at 18 days of age delayed the onset and reduced the incidence of diabetes.

Conclusions: NOD mice lack neonatal tolerance to several islet autoantigens. The ability to protect against Type I diabetes by modulation of T cell reactivity to proinsulin suggests that neonatal T cell reactivity to proinsulin may predispose to the pathogenesis of disease (Supported by MRC of Canada and JDFI)

T Cells from Autoimmune Mouse Strains are Hyporesponsive to TCR-mediated activation

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Group 3

Immunology—I

Mentor: Donald Bellgrau, PhD

Purpose: It has been suggested that autoimmunity has evolved as a consequence of the evolution of a “superior” immune system. We wished to address this hypothesis by comparing the reactivities of T cells from autoimmune-prone mice, including diabetes-prone NOD mice and lupus-prone (NZBxNZW)F1 mice, to non autoimmune-prone strains.

Methods: We purified T cells from lymph nodes of various mice. The T cells were incubated with increasing doses of plate-bound anti-T cell receptor (TCR) +/-anti-CD28 antibodies. After two days in culture, the T cells were incubated with fluorescence-tagged antibodies specific for activation markers CD25, CD29, and CD154. The presence of activation markers was analyzed using flow cytometry. The data were calculated as percentage of T cells positive for CD25, CD69, or CD154 proteins.

Results: The percentage of cells expressing the CD25, CD69 and CD154 activation markers increased in a dose-dependent

manner to TCR-mediated stimulation. T cells from diabetes-prone NOD mice consistently showed a *hyporesponsive* activation phenotype compared to Balb/c T cells. The hyporesponsive phenotype of the NOD was not age-dependent and was reproduced in in vivo activation experiments. The hyporeactivity is not MHC-dependent as T cells from MHC congenic NOD.B mice respond equivalently to NOD T cells. The phenotype appears to be polygenic as Balb/cxNOD F2 mice show a range of intermediate phenotypes. Analysis of several strains of mice reveal that other autoimmune strains share the hyporeactivity according to the following hierarchy: Balb/c>C57Bl/6, 129, C3H, B10.D2>A/J, NZB, NZW, (NZBxNZW)F1, NOD, and SJL.

Conclusions: We conclude that a polygenic effect results in a hyporeactive reactivity of T cells from diabetes-prone NOD mice, as well as other mice predisposed to other forms of autoimmunity. We hypothesize that this hyporeactive phenotype may contribute to autoimmunity by increasing the threshold for negative selection in the thymus resulting in a repertoire of higher-affinity autoreactive T cells.

Synthesis of Third Form of Glutamic Acid Decarboxylase in Human Islets

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Group 3

Immunology—I

Mentor: Åke Lernmark

Purpose: Two forms of glutamic acid decarboxylase (GAD) have been identified in human tissues: a 65 kDa form (GAD65) and a 67 kDa form (GAD67). Because GAD has unclear function in non-neural tissues and is a key autoantigen in type 1 diabetes, it is important to understand its pattern of expression. Unlike GAD65, GAD67 is not produced in human pancreatic islets. Autoreactivity to GAD67 is frequently detected in the serum of patients with type 1 diabetes, including some patients without reactivity to GAD65; the origin of this autoreactivity is unclear.

Methods: We used Northern and Western blot analysis to test for the expression of other forms of GAD in pancreatic islets and other tissues. A novel GAD transcript was detected and cloned by rapid amplification of cDNA ends. It was expressed by in vitro transcription and translation and the resulting protein tested for GAD activity and—using serum samples from a group of 115 newly diagnosed subjects with type 1 diabetes—in a standard serum assay for humoral autoimmunity.

Results: Our results show that a novel splice variant of GAD67 is produced in human islets, testis, adrenal cortex and perhaps other endocrine tissues but not in brain. This transcript directs the synthesis of a protein without GAD enzymatic activity: GAD25. A unique peptide sequence at the carboxyl terminus of GAD25 is highly conserved between mice, rats and humans. Preliminary data indicate there is a low prevalence of autoimmunity to this protein at the clinical onset of type 1 diabetes mellitus. Reactivity to GAD25 may underlie some but not most instances of GAD67 reactivity.

Conclusions: We conclude that humans produce a third form of GAD in non-neural tissues and that human islets, although they do not synthesize full-length GAD67, express this shortened variant. Autoreactivity to GAD25 may be associated with some cases of type 1 diabetes, though anti-GAD25 autoantibodies do not underlie autoreactivity to GAD67, which is more prevalent. The role of anti-GAD25 autoreactivity early in the course of insulinitis and in autoimmune polyglandular disease remains to be determined.

Comparison of a T Cell Clone and of T Cells from a T Cell Receptor (TCR) Transgenic (Tg) Mouse: TCR Tg T Cells Specific for Self-Antigen are Atypical

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Group 3

Immunology—I

Mentor: Kathryn Haskins

Purpose: The purpose of this study was to investigate whether autoreactive T cells arising in the presumably more physiological environment of a TCR Tg mouse differed from T cell clones cultured *in vitro*.

Methods: We compared the *in vivo* activity and *in vitro* characteristics of a CD4⁺, V-beta-4⁺, islet-specific, diabetogenic T cell clone, BDC-2.5, with that of T cells from a TCR Tg mouse expressing the TCR genes of the BDC-2.5 T cell clone (2.5 TCR Tg/NOD-*scid*). The 2.5 TCR Tg/NOD-*scid* mice have a monoclonal T cell repertoire consisting exclusively of CD4⁺ T cells that express the BDC-2.5 TCR.

Results: As we previously published, the BDC2.5 T cell clone requires CD8⁺ T cells from a diabetic NOD mouse in order to transfer diabetes to adult NOD-*scid* mice. In contrast, we have found that T cells from 2.5 TCR Tg/NOD-*scid* mice are able to efficiently transfer diabetes to adult NOD-*scid* mice without help from CD8⁺ T cells. Phenotypic analysis of the

in vitro propagated BDC-2.5 T cell clone demonstrates that, like diabetogenic CD4⁺ T cells from NOD mice, the BDC-2.5 T cell clone expresses LPAM-1 and low levels of CD45RB but does not express CD62L. Despite their pathogenicity, CD4⁺ T cells from 2.5 TCR Tg/NOD-*scid* mice more closely resemble NOD cells with a protective potential; that is, they express high levels of CD62L and CD45RB but do not express LPAM-1. In addition, although perforin expression and perforin-dependent CTL activity are normally associated with CD8⁺ not CD4⁺ T cells, our data demonstrates that CD4⁺ T cells from 2.5 TCR Tg/NOD-*scid* mice express perforin but that the CD4⁺ BDC-2.5 T cell clone does not.

Conclusions: Overall, our data suggest that functionally and phenotypically the *in vitro* cultured BDC-2.5 T cell clone is more representative of pathogenic CD4⁺ T cells from the NOD mouse than are the 2.5 TCR Tg/NOD-*scid* cells. In addition, our results suggest that caution should be exercised when using autoreactive T cells from TCR Tg mice, particularly TCR Tg mice with monoclonal T cell repertoires, to draw inferences about spontaneous autoimmune disease.

Study on the correlation between dysfunction of the NKT cells/CD1 pathway and pathogenesis of insulin-dependent diabetes mellitus (IDDM) in the non-obese diabetic mice (NOD)

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Group 3

Immunology—I

Mentor: Nora Sarvetnik

Purpose: There is increasing evidence that lack of function of the NKT cell subset is associated with autoimmunity. Aim of this study was to characterize the defect of the NKT cells in the NOD mice and establish a clear link between NKT cell function and immunoregulation of autoimmune diabetes.

Methods: The CD1 molecule was transgenically expressed in the pancreatic islets of NOD mice under the control of the human insulin promoter. Diabetes incidence in ins-CD1 NOD and their negative littermates was determined by weekly measurements of blood glucose values. NKT cells of NOD mice were isolated from spleens by FACS single cell sorting of CD3⁺CD122⁺Ly49A⁺ cells. Activation and cytokines secretion upon TCR as well as IL-12 stimulation were analyzed by mean of ³H-thymidine incorporation and ELISA assay and compared to that C57BL/6 mice.

Results: The expression of CD1 in the pancreatic islets recruited a large number of NKT cells and protected NOD mice from autoimmune diabetes (p<0.05). In contrast with previous studies, we found that NKT cells of NOD mice were not defective in IL-4 secretion, but rather, in their ability to proliferate and secrete IFN- γ in response to TCR and IL-12 activation. Interestingly, the defective activation also affected the clearance of NKT cells upon *in vivo* anti-CD3 stimulation in NOD mice.

Conclusions: NKT cells could be critical in the balancing immune responses between protective immunity and avoidance of autoimmunity. In the NOD mice the NKT cell dysfunction involved lack of activation and clearance of inflammatory NKT cells. We hypothesize that during inflammation defective NKT cells of NOD mice could sustain pathogenic Th1 autoimmune responses.

The role of the HPA axis in the inhibition of IDDM following immune stimulation

Group 3

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Immunology—I

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Purpose: The purpose of this study is to determine if inhibition of IDDM in the BB rat, following immune challenge, is dependent on the activation of the hypothalamic-pituitary-adrenal axis, and if so, whether IL-1 β and/or TNF- α are key mediators of this effect.

Methods: Aim 1 will confirm proinflammatory cytokine synthesis and HPA activation following immune challenge with CFA. This will be done by assaying for IL-1 β and TNF- α and adrenocorticotrophic hormone and corticosteroid. Aim 2 will determine the specific role of the corticosteroid response by adrenalectomy or metyprone treatment (a glucocorticoid synthesis inhibitor) of BB rats prior to immune challenge and the following of these animals for the development of IDDM. Aim 3 will determine if IL-1 β and/or TNF- α are key mediators of this inhibition by treating animals with IL-1 receptor antagonist and/or TNF binding protein just prior to immune challenge and following for the development of IDDM.

Conclusions: We have previously demonstrated that non-islet-antigen specific therapies can be safely used to modulate the natural history of type I diabetes. An understanding of the mechanism(s) mediating this inhibition of IDDM is now required. The nervous and endocrine systems have been shown to be major factors in the regulation of immunity via the HPA axis. During periods of inflammatory challenge, the HPA axis is activated through the release of proinflammatory cytokines. We can now consider a mechanistic basis for non-islet-antigen specific therapies to inhibit the development of IDDM. Immune challenge of diabetes-prone animals might result in the release of proinflammatory cytokines. These cytokines would signal the CNS, which in turn would activate the HPA axis with the ultimate production of glucocorticoids, thereby altering the development of disease. It is the interaction between the neuroendocrine and the immune systems that may maintain tolerance or induce reactivity to the insulin producing islet beta-cells.

NK-T cell and T cell defects associated with immune mediated (Type 1) diabetes in humans

Group 3

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Immunology—I

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Mentor: Noel Maclaren

Purpose: Numerous *in vitro* studies in humans have reported immunological disorders associated with immune mediated (Type 1) diabetes (IMD). These include elevated numbers of activated T cells, subnormal production of IL-2 and a suggested polarization of T cell responses from a protective Th2 to a Th1 bias. These could be secondary to abnormal T cell cytokine production. We therefore studied intracellular T cell cytokine production after their *in vitro* stimulation by phorbol myristate and Calcium Ionomycin. Since an important immunoregulatory role has been attributed to CD1d-restricted natural killer T (NK-T) cells, we next studied NK-T cell numbers in various groups of patients. We also studied the invariant CD1d gene for mutations as may be associated with IMD.

Methods: Groups of newly diagnosed, and long standing diabetics, as well as their ICA⁺ relatives were studied and compared to normal controls for their intracellular staining for Th1 and Th2 cytokines by flow cytometry. Similarly, NK-T cells

were characterized using antibodies to CD3 and to their receptors as restricted by their Va24 and Vb11 gene repertoire usage. CD1d gene was sequenced using ABI 377 automatic sequencer.

Results: The patients exhibited significantly reduced percentages of both Th1- (IFN- g^+) as well as Th2-biased (IL-4⁺) cells. Similarly, NK-T cell numbers were reduced in the patient groups compared to the normal controls, with a severity that was related to the impediment in the T cell cytokine secretion. On sequencing CD1d gene and its promoter region we found no mutations associated with IMD.

Conclusions: These data suggest the presence of a broad, non-polarized, T cell signaling defect resulting in defective cytokine production. NK-T cells were also reduced in the patients. These two defects may have separate causations or result from a common defect. Either way, we speculate that defective regulatory functions result, and thus predispose to autoimmunity.

Characterization of CD8⁺ T lymphocytes which persist after peripheral tolerance to a self antigen expressed in the pancreas

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Group 3

Immunology—I

Mentor: Linda A. Sherman

Purpose: Self antigen-specific CD8⁺ T lymphocytes (CTL) are part of a normal T cell repertoire in the peripheral immune system. In most cases, these lymphocytes are not auto-aggressive. However, this is not the case in autoimmune diseases such as diabetes. We wanted to determine how self-specific CTL remain tolerant. This in turn could provide insight as to how these lymphocytes can be activated to become auto-aggressive.

Methods: These experiments utilized a transgenic mouse system where mice express a well-characterized viral protein, influenza hemagglutinin (HA), on the beta-islet cells of the pancreas (B10.D2 InsHA mice). These mice do not succumb to diabetes following immunization with influenza virus or vaccinia viruses recombinant for HA. CTL were isolated from influenza-immunized InsHA mice, propagated *in vitro*, and cloned by limiting dilution. For comparison, clones were also isolated from conventional, HA-negative B10.D2 mice. CTL clones, as well as CTL populations, were compared

phenotypically and functionally to determine what, if any, differences could be detected between a diabetogenic (B10.D2) and a nondiabetogenic (InsHA) T cell repertoire.

Results: InsHA HA-specific CTL were observed to be of lower functional avidity than B10.D2 HA-specific CTL. This was not due to functional deficiencies, such as lysis through the Fas/FasL pathway, or impaired signaling through the TCR. Moreover, all CTL expressed comparable levels of CD8, TCR, and a host of other cell surface-expressed proteins involved in adhesion and activation. However, InsHA CTL consistently bound peptide-coated MHC tetramer less efficiently than B10.D2 CTL.

Conclusions: HA-specific InsHA CTL within a tolerant peripheral T cell repertoire possess a TCR that is of lower affinity than conventional B10.D2 CTL that recognize HA as a foreign antigen. We have also generated a TCR transgenic mouse line, using genomic DNA sequences from a selected InsHA CTL clone (Clone 1). Findings from studies using this mouse line will also be discussed.

Suppression of autoimmune diabetes by expression of the anti-apoptotic gene CrmA in NOD pancreatic beta cells

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Immunology—I

Mentor: Robert S. Sherwin

Purpose: Type 1 diabetes is caused by the autoimmune destruction of insulin-producing pancreatic beta cells. Although multiple mechanisms may be involved, it is commonly thought that the activation of apoptosis is a critical pathway by which beta cells die. Strategies aimed at interfering with beta cells apoptosis could therefore be of potential therapeutic value.

Methods: To this end, we generated NOD transgenic mice with targeted expression of the anti-apoptotic gene Cytokine Response Modifier A (CrmA), a potent inhibitor of caspases 1 and 8, to pancreatic beta cells. To create these novel transgenic (tg) mice, we used the rat insulin promoter and the reverse tetracycline transactivator to express CrmA in a time controlled manner. Four lines of transgenic mice were generated whose expression of CrmA (detected by RT-PCR) could be achieved only after feeding with Doxycycline food (2g/kg). Two lines of CrmA tg mice and their negative littermates were further studied for the diabetes protective effect of CrmA using 3 different models of diabetes. For those studies, both groups were fed with Doxycycline food since birth.

Results: In both CrmA tg mice, following transfer of diabetic spleen cells, we observed a significant delay in the onset of diabetes. In one line, four weeks after adoptive transfer, 100 % of the control mice (n=8) developed diabetes whereas only 44.4 % of CrmA tg mice did (n=9). Similarly, we observed a significant delay in the onset of diabetes following transfer of potent diabetogenic insulin-specific CD8 T cells (F.S. Wong, *Nat. Med.* 1999, 5:1026). Three weeks after transfer, 100 % of the control mice developed diabetes compared with 60 % of CrmA tg mice. In addition, beta cell expression of CrmA suppressed the development of diabetes in female NOD mice. In the CrmA tg mice, the onset of diabetes was delayed and its incidence reduced. At 6 month of age, 77.7 % of control mice (n=9) had developed diabetes, compared with 41.6 % of CrmA tg mice (n=12).

Conclusions: We conclude that the expression of the anti-apoptotic gene CrmA in pancreatic beta cells reduces (a) the susceptibility of the beta cells to destruction by diabetogenic T cells and (b) the onset of diabetes in NOD mice. Thus CrmA gene may have therapeutic potential as a candidate for islet directed gene therapy in diabetes.

Role of CD4 T cells in the induction of diabetes

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Immunology—I

Mentor: Richard A. Flavell

Purpose: Determine if CD4 T cells are required to induce apoptosis in beta cells.

Methods: NOD mice deficient in CIITA (Class II Trans-activator) and therefore, deficient in peripheral CD4 T cells were crossed to transgenic mice expressing Fas Ligand on beta cells (RIP-FasL), and monitored for diabetes on a weekly basis.

NOD/SCID mice were adoptively transferred with either CD8 and/or CD4 T cells from diabetic NOD females and immunohistochemistry of pancreas was performed.

Results: NOD/CIITA deficient mice do not develop diabetes but some degree of pancreatic infiltration, very rarely insulinitis. NOD/RIP-FasL mice exhibit accelerated diabetes. However NOD/RIP-FasL transgenic mice deficient in CIITA do not develop diabetes while the CIITA sufficient littermates do. On the other hand, adoptive transfer of CD4 T cells from diabetic females in to NOD/SCID recipients, is able to cause insulinitis (not diabetes), while CD8 T cells on their own are not. We have also observed by immunohistochemistry that CD4 infiltration in the pancreas is associated with Fas-positive cells.

Conclusions: CD4 T cells seem to be required to induce Fas on beta cells although further experiments need to be done to confirm this hypothesis.

Expression of a Soluble Lymphotoxin- β Receptor-fusion Protein Inhibits Diabetes Development in Non-obese Diabetic Mice

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Group 3

Immunology—I

Mentor: Hugh O. McDevitt

Purpose: The role of lymphotoxin- β in diabetes development is unknown. To explore this issue, we have crossed mice deficient in Lt $\alpha\beta$, but not TNF or Lt α , onto the NOD background to examine insulinitis and the incidence of diabetes.

Methods: BALB/c mice expressing a soluble murine LT β R-humanFc fusion protein (driven by the cytomegalovirus promoter) were crossed onto the NOD background for nine generations. This fusion protein specifically neutralizes Lt $\alpha\beta$, but not TNF or Lt α activity. Both transgenic and nontransgenic littermates were followed for at least one year. Immunohistology and Hematoxylin and eosin stains were performed on sectioned tissue to examine the organization of the spleen and lymph nodes, as well as infiltrates into the salivary gland and pancreas.

Results: Two lines of NOD mice which express either high or intermediate levels of the Lt β R-Fc fusion protein were followed for insulinitis, sialitis, and diabetes development. As reported previously in BALB/c mice, NOD mice expressing high concentrations of the soluble Lt β R-Fc fusion protein also

do not develop splenic primary follicles, follicular dendritic cell networks, MOMA-1 or MAdCAM-1 expression. However, in aged NOD mice expressing high levels of the fusion protein, FDC did develop.

Importantly, the concentration of circulating fusion protein was directly related to a reduction diabetes incidence (but not insulinitis or sialitis). Only 4% (1/26) of mice expressing a high concentration of the fusion protein developed type 1 diabetes by 24 weeks of age, compared to 77% (24/31) of the transgenic-negative littermates. A second mouse line expressing a lower level of the fusion protein had a 56% diabetes incidence rate at 24 weeks of age, while 78% of nontransgenic littermates were diabetic. However, in all animals expressing the high level of the fusion protein examined, lymphocytic pancreatic infiltration was found by 10 weeks of age.

Conclusions: This study demonstrates that LT, like TNF, is also an important mediator in autoimmunity. However, the mechanisms of action of these two related cytokines appear to be distinct. Whereas TNF neutralization blocks both insulinitis and IDDM, LT β neutralization inhibits diabetes development, but allows for pancreatic lymphocytic infiltration.

Abstracts

Group 4

Genetics and Beta Cell Biology

19 Abstracts

Faculty

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*Genetics and
Beta Cell Biology*

Mentor: John A. Todd

Fine mapping of genes involved in the predisposition to multifactorial diseases is one of the great challenges in human genetics. We have investigated the accuracy of currently available fine mapping methods using the example of an established type 1 diabetes locus mapped to the HLA region, designated *IDDM1*. In a two-stage approach, first linkage was tested in 385 affected sib-pair families from the UK over a 14 Mb region on chromosome 6p21 with a map of 13 equally spaced microsatellite markers. The whole region showed strong linkage. Analysis of allelic association of these markers revealed disease association only for marker D3A. In the second stage another 12 markers located around D3A were analysed. All of these markers were highly associated with the disease. Strongest association ($p=7.5 \times 10^{-34}$) was found with the marker closest to the HLA class II genes *HLA-DQB1* and *-DRB1* known to be the primary determinants of *IDDM1*.

This marker (*D6S2444*) is located 85 kb centromeric of *DQB1*. Given the highly significant linkage disequilibrium within the HLA region, extending in our data set over a long distance of 5 Mb, we were surprised about the accuracy these genes were mapped with. Despite extraordinary linkage disequilibrium in the region it was reassuring that the marker located closest to the aetiological locus is the one showing strongest association with the disease. We have analysed the influence of recombination and marker informativity on the mapping accuracy. We demonstrate that the disease association observed with other markers can almost be entirely explained by linkage disequilibrium with the class II genes *DQB1* and *DRB1* with the exception of marker *D6S2223*, located 5.9 Mb telomeric of *DRB1*, which showed minor independent association. Our results indicate that the effects of other putative susceptibility genes in the HLA region are likely to be relatively minor compared to those of DQ and DR.

CD4 promoter polymorphisms in Danish Type 1 diabetes families

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Mentor: Thomas Mandrup-Poulsen

Purpose: The A4 allele of a (CTTTT)-repeat in the *CD4* promoter has been shown to link to Type 1 diabetes (IDDM) in Danish patients. Hence, the aims were to screen the *CD4* promoter for further polymorphisms and to analyze for linkage between novel polymorphisms and IDDM in Danish patients.

Methods: SSCP screening (386→1523 bp; GenBank acc. # M86525) and sequencing (224→658 bp) of the *CD4* promoter was performed in 20 IDDM and 10 healthy subjects. The identified SSCP polymorphic sites were sequenced and PCR based assays for novel polymorphisms were established. Linkage was investigated by TDT analysis in 249 Danish IDDM families. Transmission to 418 affected and 249 non-affected offspring was evaluated.

Results: Three novel polymorphisms were identified: 1) a 474T C, 2) a 1003G C and 3) a 1343C G polymorphism. The G474-allele was in complete linkage disequilibrium with the IDDM non-associated A8-allele of the *CD4* (CTTTT)-

repeat and not further investigated. Random transmission to affected offspring was found for both the 1003G C and the 1343C G alleles, but a non-significant trend to a distortion in the transmission was observed for both polymorphisms: 80 (55%) G- and 66 (45%) C-alleles ($p_{\text{TDT}}=0.25$) and 160 (54%) C- and 134 (46%) G-alleles ($p_{\text{TDT}}=0.13$) were transmitted, respectively. A 1003G C-1343C G haplotype was established. Transmission of the identified haplotypic alleles G-C, C-G and G-G were 55% (164/300; $p_{\text{TDT}}=0.11$), 43% (65/151; $p_{\text{TDT}}=0.09$) and 48% (113/233; $p_{\text{TDT}}=0.6$), respectively.

Conclusions: Three novel polymorphisms in the *CD4* promoter were found. None of the novel polymorphisms or the 1003G C-1343C G haplotype were significantly linked to IDDM in Danish patients. Thus, it is less likely that the identified polymorphisms confer susceptibility to IDDM. Consequently, the *CD4* (CTTTT)-repeat variations may be the *CD4* promoter variation conferring susceptibility to IDDM. Hence, reporter-assay studies investigating the putative functional impact of the *CD4* (CTTTT)-repeat variations are in progress.

Genetic and Functional Analysis of IDDM12 on Chromosome 2q33

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Group 4

Genetics and
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Mentor: Jin-Xiong She

Purpose: To identify additional genes within the *IDDM12* interval, and to identify polymorphisms/mutations within *CTLA4* which could confer susceptibility to type 1 diabetes.

Methods: A human PAC library was screened with primers specific for *CTLA4*. A 100 kb clone containing *D2S72*, *CTLA4*, and *D2S105* (PAC 321F19) was identified. PAC 321F19 DNA was sonicated and subcloned into a pBluescript SK- vector. Using vector specific primers, 750 clones with an average insert of 750 bp were sequenced. The sequences were compared to dbEST sequences to identify additional genes within the *IDDM12* interval. The *CTLA4* gene was sequenced in pooled DNA from U.S. Caucasians, Mexican-Americans and Asians (15 individuals per pool). Sequencing of pooled samples allowed rapid detection of common polymorphisms in the populations. *CTLA4* was also sequenced in 15 Mexican-American individuals to identify less common polymorphisms. The polymorphic loci were typed in a multiethnic collection of 528 families.

Results: Sequences from PAC 321F19 were compared to GenBank dbEST by BLAST search. No matches were identified between PAC 321F19 and any published EST sequences. Sequencing of *CTLA4* with specific primers revealed one SNP located within intron 1, which had a high frequency in all populations. This marker showed association with type 1 diabetes, similar to the association detected for the exon 1 and 3' UTR polymorphisms. Linkage disequilibrium (LD) analysis of the region showed strong LD between markers within *CTLA4*. The LD was weaker between *CTLA4* and the two flanking loci, *D2S72* and *D2S105*. There was no LD between *CTLA4* and other markers in the region.

Conclusions: Sequencing of the *IDDM12* region has revealed no additional genes within the interval. *CTLA4* remains the strongest candidate for *IDDM12*, although the mutation which confers susceptibility remains to be identified.

Variability in the *IKBKB* gene does not play a major role in the etiology of early-onset, autosomal dominant type 2 diabetes

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Mentor: Alessandro Doria

Purpose: Tumor necrosis factor α (TNF- α) inhibits insulin action through an increased serine phosphorylation of insulin receptor substrate 1. In this study, we investigated whether genetic variability in *IKBKB*—a serine-threonine kinase activated by TNF- α —plays a role in the etiology of early-onset, autosomal dominant type 2 diabetes.

Methods: The *IKBKB* gene was finely mapped on chromosome 8p11 by radiation hybrid (RH) screening. Linkage between two markers flanking the *IKBKB* gene and diabetes was evaluated in 32 multigenerational families with early-onset, autosomal dominant type 2 diabetes including 233 diabetic (mean age at Dx = 37 ± 18) and 152 non-diabetic subjects. Families with the best evidence of linkage were screened for sequence differences in the *IKBKB* coding region by PCR-dideoxyfingerprinting and sequencing.

Results: The *IKBKB* gene was mapped to a 3 cM interval between markers *D8S532* and *D8S538*. The overall lod scores for linkage between these markers and diabetes were negative (-47.7 for *D8S532* and -40.7 for *D8S538*) suggesting that variability in *IKBKB* was not a major determinant of diabetes in these families. Positive lod scores, however, were observed in selected pedigrees, indicating that a role in selected pedigrees could not be excluded. After isolating and sequencing two genomic BAC clones (137-M-4 and 384-C-8), the *IKBKB* gene was found to include 22 exons ranging in size from 50 to 194 bp. After all exons were screened for sequence differences in the 18 families with highest lod scores, we identified silent polymorphisms in exon 11 (1226 G A, L361L) and in intron 12 (+14 T A). However, no mutations segregating with diabetes could be found in these families.

Conclusions: Variability in the coding sequence of the *IKBKB* gene does not play a major role in the development of early-onset, autosomal dominant type 2 diabetes. We are currently screening the promoter region for sequence differences linked with diabetes in these families.

Linkage disequilibrium mapping of the IDDM-8 interval on chromosome 6q27 in different ethnic populations

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Mentor: Jin-Xiong She

Purpose: Insulin Dependent Diabetes Mellitus (IDDM) is a multifactorial disease resulted from a combination of genetic and environmental factors. We have previously reported the first evidence of confirmed linkage for IDDM-8 on chromosome 6q27. In this study, we attempted to narrow the interval to a small region suitable for positional cloning and candidate gene analyses.

Methods: Initially, two hundred and fifty one American families (Caucasian origin) with two affected sibs in each family were screened with a total number of eight markers. Association between disease gene and marker allele was evaluated by transmission disequilibrium test (TDT).

Results: A significant association was found with one of microsatellite marker named 38K at the telomeric region of chromosome 6q27. Then, we extended our screening to another 96 American families, 41 French families, 46 Italian families and 92 British families. Our data showed a strong association in Americans as well as in Italian and French families but not in British families. This finding encourage us to screen more families and then we included our simplex families including 84 Mexican-American, 28 French families, 44 Italian families, 33 Korean families and 31 Chinese families.

Conclusions: The association was also found in Mexican-American, Italian and French families. The presence of strong association in different population is a manifestation of major contribution of a gene in the pathogenesis of disease.

***In vivo* Evidence for the Contribution of HLA-DQ Molecules to the Development of Diabetes**

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*Genetics and
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Purpose: Although DQA1*0301/DQB1*0302 is the HLA-class II gene most commonly associated with human type 1 diabetes, direct *in vivo* experimental evidence for its diabetogenic role is lacking. We, therefore, decided to study its diabetogenic role in an *in vivo* model.

Methods: To generate C57BL/6 transgenic mice that bear DQ8 or DQ6 molecule and do not express mouse MHC-class II molecules (DQ8+/mII- or DQ6+/mII-) and double transgenic mice expressing DQ8 or DQ6 together with expressing co-stimulatory molecule B7.1 on beta cells (RIP-B7.1).

Results: Single transgenic mice, DQ8+/mII- or DQ6+/mII- or RIP-B7.1, do not develop spontaneous diabetes. However, 81% of the DQ8+/mII-/RIP-B7.1+ mice developed spontaneous diabetes. The diabetes was accompanied by severe insulinitis composed of both T cells (CD4+ and CD8+) and B cells. T cells from the diabetic mice secreted large amounts of IFN γ , but not IL-4, in response to DQ8+ islets and the puta-

tive islet autoantigens, insulin and GAD. Diabetes could also be adoptively transferred to irradiated non-diabetic DQ8+/mII-/RIP-B7.1+ mice. In striking contrast, none of the transgenic mice in which the diabetes protective allele (DQA1*0103/DQB1*0601, in short DQ6), was substituted for mouse MHC-class II molecules but remained for the expression of B7.1 on pancreatic beta cells (DQ6+/mII-/RIP-B7.1+) developed diabetes. Only 7% of DQ-/mII-/RIP-B7.1+ mice developed diabetes at an older age and none of the DQ-/mII+/RIP-B7.1+ mice or DQ8+/mII+/RIP-B7.1+ mice developed diabetes.

Conclusions: Substitution of HLA-DQA1*0301/DQB1*0302, but not HLA-DQA1*0103/DQB1*0601, for murine MHC class II provokes autoimmune diabetes in non-diabetes prone RIP-B7.1 C57BL/6 mice. Our data also provide direct *in vivo* evidence for the diabetogenic effect of this human MHC class II molecule and a unique "humanized" animal model of spontaneous diabetes.

Dissection of the Genetic Basis for Cytokine-mediated Immunoprotection and Acceleration of Insulin-dependent Diabetes Mellitus

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Mentor: Nora Sarvetnick

Purpose: To study 1) the genes involved in the protection from type-1 diabetes in the IL-4 transgenic NOD mouse and 2) the genes responsible for accelerated onset and progression of IDDM in the IL-10 transgenic NOD mouse.

Methods: Pancreatic poly(A)⁺ RNA was prepared from NOD mice, IL-4 NOD mice and IL-10 NOD mice. These poly (A)⁺ RNAs were reverse transcribed into cDNAs and used as “*tester cDNAs*” (where specific transcripts are to be found) and “*driver cDNAs*” (reference cDNAs) for polymerase chain reaction (PCR)-based subtractive hybridizations. cDNA sequences differentially expressed in the IL-4 NOD mouse and in IL-10 NOD (as compared to the NOD mouse) were further enriched by suppression PCR and the subtracted cDNA pools used for preparation of cDNA subtracted libraries. 92 clones from these libraries were selected for DNA sequencing.

Results: Nucleotide sequence comparison with the National Center for Biotechnology Information (NCBI) database identified novel cDNA sequences in mice protected from autoimmune diabetes and in mice with accelerated onset of disease. Other (known) genes, not yet linked to type-1 diabetes pathogenesis, were also found associated with protection or progression of type-1 diabetes. Characterization and study of the role of these genes in IDDM are in progress.

Conclusions: New genes have been found associated with cytokine-mediated immunoprotection or acceleration of type-1 diabetes in the NOD mouse. Characterization and study of these genes can give new clues on the mechanisms regulating the evolution of IDDM.

An ER mediated mechanism for conditional amplification of Ca²⁺ signaling by co-incidence detection

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Purpose: Some Ca²⁺ dependent processes including exocytosis require high intracellular free Ca²⁺ concentrations. We hypothesized that b-cells have intracellular mechanisms for amplifying Ca²⁺ signaling. We intended to identify such processes and elucidate the molecular mechanisms involved such processes.

Methods: Intracellular free Ca²⁺ concentration was measured by single-cell fluorometry in b-cells or INS-1 cells loaded either with fura-2 or fluo-3. In some experiments, strontium, instead of Ca²⁺ was present in the extra-cellular medium, and incoming Sr²⁺ was used to trigger release of Ca²⁺ from intracellular stores. Pharmacological agents like thapsigargin, forskolin, xestospongine C, ryanodine and 8-bromo cADPR were used to identify the mechanisms involved in the amplification process.

Results: The integral of [Ca²⁺]_i change over a 300 s period of depolarization by KCl was significantly higher in control cells as compared to the cells whose intracellular Ca²⁺ pools were depleted with thapsigargin suggesting that depolarization-induced Ca²⁺ signaling is amplified by Ca²⁺ release from intracellular stores. In fluo-3 loaded cells, and in the presence of extracellular Sr²⁺ entry of Sr²⁺ into the cytoplasm gave an ini-

tial small increase in fluorescence due to low fluorescence of Sr²⁺-bound fluo-3. However, this was followed by a large spike-like increase in fluorescence caused by Ca²⁺ released from the intracellular stores. This protocol thus allowed us to dissect the trigger Ca²⁺ (Sr²⁺) from the released Ca²⁺. When cells were depolarized by KCl in the presence of glucose and forskolin, large Ca²⁺ spikes were observed which were abolished by thapsigargin or omission of extracellular Ca²⁺ but were resistant to verapamil. These large Ca²⁺ spikes typically required elevation of camp level either by forskolin or GLP-1. sGLP-1 also resulted in large Ca²⁺ spikes resistant to verapamil. The spikes were enhanced by 8-Bromo cADPR a partial agonist of ryanodine receptor. Carbachol and cholecystokinin-induced Ca²⁺ signaling were also amplified by a mechanism involving Ca²⁺-induced Ca²⁺ release as the magnitude of signaling was reduced by high concentration of ryanodine in a use dependent manner.

Conclusions: The ER associated Ca²⁺ apparatus amplifies Ca²⁺ signals initiated by depolarization or inositol 1,4,5,-triphosphate. The amplified Ca²⁺ signals take the form of large periodic spikes, which is conditional in that it requires detection of co-incidence of camp-dependent signaling and the initial Ca²⁺ trigger. The amplified Ca²⁺ signal may play a role in triggering exocytosis or bringing about membrane repolarisation.

Functional and Molecular Characterization of the Pancreatic Neuroendocrine Calcium Channel

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Purpose: Develop a regulated gene delivery system that allows the isolation as well as the functional and molecular characterization of β -cell L-type voltage-dependent calcium channels (VDCCs) in both mammalian cell lines and the intact pancreatic islet

Methods: Generation of recombinant, replication-deficient adenoviruses (Ad) that overexpress specific sense and anti-sense mRNAs to the alpha 1 subunit ($\alpha 1$) of the neuroendocrine class (D) VDCC. Adenoviral-mediated transfer and expression of specific $\alpha 1$ D sense and anti-sense mRNA into the insulinoma HIT T15 cell line to determine the effect of their overexpression on insulin secretion. Cloning the long C-terminal isoform of the $\alpha 1$ D subunit from HIT T15 cells total mRNA by reverse-transcription (RT) polymerase chain reaction (PCR). Construction of fusion proteins between the amino terminus of the $\alpha 1$ D and the yellow variant of

enhanced green fluorescent protein (YEFP- $\alpha 1$ D). Similarly, generation of functional fusion proteins at the C-terminal end of each β subunit and the cyan variant of EGFP (β -ECFPs) Electrophysiological and fluorescence resonance energy transfer (FRET) analyses in a heterologous expression system using YEFP- $\alpha 1$ D in combination with each of the individually labeled β -ECFPs.

Results: Ad-mediated overexpression of $\alpha 1$ D anti-sense mRNA, but not sense, results in approximately 69% inhibition of insulin secretion in HIT T15 cells. All cloned fluorescent-labeled subunits are functional. YEFP- $\alpha 1$ D subunit preferentially associates with $\beta 1$ -ECFP and $\beta 2$ -ECFP.

Conclusions: $\alpha 1$ D appears to be the predominant functional L-type channel in insulin secretion in association with either the $\beta 1$ or $\beta 2$ subunit.

Pyruvate and Lactate Handling in Pancreatic β -cells

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Mentor: Claes B. Wollheim

Purpose: The significance of low lactate dehydrogenase (LDH) and monocarboxylate transporter (MCT) activities in the β -cell has been explored by overexpressing MCT-1 and LDH-A in rat insulinoma INS-1 cells and primary rat islets.

Methods: Overexpression of LDH-A in INS-1 cells was achieved using a tetracycline-inducible expression system. LDH-A cDNA and MCT-1 cDNA were introduced into rat isolated islets by recombinant adenoviral vectors.

Results: Adenovirus-mediated overexpression of MCT-1 increased lactate transport activity 3.7-fold in INS-1 cells. Although overexpression of LDH-A and/or MCT-1 did not affect glucose-stimulated insulin secretion, LDH-A overexpression resulted in stimulation of insulin secretion even at

a low lactate concentration (2 mM) with a concomitant increase in its oxidation in INS-1 cells regardless of MCT-1 co-overexpression. Adenovirus-mediated overexpression of MCT-1 caused an increase in pyruvate (10 mM) oxidation and conferred pyruvate-stimulated insulin release to isolated rat islets. Although lactate (10 mM) did not stimulate insulin secretion from control and MCT-1-overexpressing islets, co-overexpression of LDH-A and MCT-1 evoked lactate-stimulated insulin secretion with a concomitant increase in lactate oxidation in rat islets.

Conclusions: These results suggest that low expression of MCT and LDH is requisite to the specificity of glucose in insulin secretion, protecting the organism from undesired hypoglycemic actions of pyruvate and lactate during exercise and other catabolic states.

3-D Structure of the Golgi Complex in the Pancreatic β Cell Line, HIT-T15

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Mentor: J. Richard McIntosh

Purpose: We are analyzing the organelles involved in insulin synthesis, processing and secretion in 3-D at ~6nm resolution, using ultrastructural tools that provide for improved preservation and imaging of organelle morphogenesis.

Methods: High pressure freezing/freeze-substitution fixation and cellular tomography were employed.

Results: The Golgi complex is typically comprised of 7-9 elements. The first element sometimes possesses a structure intermediate to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and cis-most cisterna observed in the NRK cell. There are multiple trans cisternae that are structurally and functionally distinct. A specialized form of ER adheres closely to each of these trans elements, and is directly continuous with ER that intimately apposes mitochondria.

Dense core granules are often connected to Golgi elements and to each other by membranous tubules, and are found attached to cis, medial and trans Golgi elements.

Conclusions: Our findings reveal an unsuspected complexity of tubulo-vesicular events in the Golgi complex which may account for the disparities between current morphological and biochemical data. These data provide structural evidence that new Golgi elements are generated when ERGIC clusters dock and fuse, using the existing cis-most cisterna as a template. Moreover, our finding that the volumes and surface areas of trans Golgi elements decrease in the cis- to trans- direction strongly suggests that the Golgi complex is consumed at its trans aspect. Relating these structural data to the biochemical mechanisms for protein sorting and secretory granule biogenesis will lead to a more comprehensive understanding of the biology of regulated secretion and exocytosis in the pancreatic β cell.

Molecular and Pharmacological Manipulation of 5'-AMP-activated Kinase Activity in Engineered INS-1 Cells Increases Fatty Acid Oxidation but is Without Effect on Insulin Secretion

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Genetics and
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Mentor: Christopher B. Newgard

Purpose: To improve the performance of insulin-secreting cell lines.

Methods: Parental INS-1 cells were transfected with pUCMV8/INS/IRES/Neo containing the human insulin cDNA. Colonies were selected and characterized with respect to insulin secretion at 3 and 15 mM. One clone, 832/13, was further characterized, using HPLC and secretion studies. Then, 832/13 cells were transduced by a recombinant adenovirus expressing the catalytic subunit of 5'-AMP-activated Kinase (α 1-AMPK). Glucose-stimulated insulin secretion (GSIS), fatty acid oxidation (FAox) and activities of AMPK and acetyl-CoA Carboxylase (ACC) were examined.

Results: Of all clones, 16% exhibited a 5-13-fold increase in GSIS. Differences in responsiveness could not be attributed to insulin content. The clone 832/13 maintained its responsiveness for ~11 months in culture. HPLC confirmed the presence

of human insulin and efficient proinsulin processing at high and low passages. GSIS in 832/13 cells was potentiated by IBMX, fatty acids, and GLP-1, while carbachol was without effect. Tolbutamide potentiated GSIS at 3 and 15 mM glucose, while GSIS was abolished by diazoxide. In the presence of 35 mM K⁺ and diazoxide, GSIS was increased, thus demonstrating both robust KATP-dependent and -independent glucose sensing in the engineered cells. Overexpression of α 1-AMPK increased FAox from 79 \pm 18 to 287 \pm 49 pmol/mg/h (P<0.05) at 3 mM glucose and from 22 \pm 11 to 153 \pm 34 pmol/mg/h (P<0.05) at 15 mM glucose, while GSIS (~20-fold) was unaffected. AICAR, increased AMPK activity both at 3 mM and 15 mM glucose. In parallel, ACC activity was inhibited. AICAR increased FAox similarly to AMPK overexpression; again GSIS remained unchanged.

Conclusions: We have created a stable insulin-secreting cell line with improved performance, using a transfection/selection strategy.

Impaired Glucose Tolerance and Abnormal Pancreatic Islet Structure in Type 1 Sulfonylurea Receptor-Deficient Mice

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Mentor: Mark A. Magnuson

Purpose: To directly assess the role of ATP-sensitive potassium (K_{ATP}) channel in glucose-stimulated insulin secretion we made and characterized type 1 sulfonylurea receptor (Sur1) knock-out (KO) mice.

Methods: A gene targeting vector containing two *loxP* sites flanking the proximal promoter and exon 1 region of Sur1 was electroporated into ES cells. A correctly targeted cell line was used to generate mutant mice. This was followed by microinjection of a *cre* expression plasmid vector in order to create a Sur1 null allele for this study. Hyperglycemic clamp and oral glucose tolerance tests were performed to assess glucose-stimulated insulin secretion in 12–16 week old fasted Sur1 KO mice. Pancreata from Sur1 KO mice at various ages were analyzed by immunohistochemistry.

Results: Sur1 KO mice lack all K_{ATP} channel activity as assessed by patch clamp electrophysiology, but exhibit normal growth and fertility. Both hyperglycemic clamp and oral glucose tolerance tests demonstrated impaired glucose tolerance in Sur1 KO mice. Basal plasma insulin levels in Sur1 KO mice were not different from control mice. During a 120 min hyperglycemic clamp test period (300 mg/dl), plasma insulin levels were increased 1.6-fold in Sur1 KO mice compared to 5.9-fold for wild type controls. Immunohistochemical analysis revealed that both glucagon- and pancreatic polypeptide-positive cells were scattered throughout pancreatic islet in adult Sur1 KO mice. These changes in islet structure became more pronounced with age.

Conclusions: These studies indicate that glucose-stimulated insulin secretion occurs largely through a K_{ATP} -dependent pathway, but that a second, K_{ATP} -independent pathway also exists.

Sequences in the Untranslated Regions of the Rat II Proinsulin mRNA Regulate its Translation

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Genetics and
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Mentor: Christopher J. Rhodes

Purpose: The translation of approximately 100 proteins of pancreatic β -cells is stimulated in response to glucose, expression of proinsulin being the most strongly stimulated. We are interested in identifying sequences within the rat II proinsulin mRNA that subject it to this specific regulation.

Methods: Histidine tagged rat II proinsulin genes were engineered so as to be flanked by the rat II proinsulin 5' and 3' untranslated regions (UTRs) or in which the 5' and/or 3' UTRs were replaced with neutral sequences. These gene constructs were introduced into isolated rat islets by recombinant adenoviruses. The translational response to glucose in infected islets was measured by the incorporation of [³⁵S]methionine into the histidine tagged proinsulin.

Results: Characterization of the adenovirally expressed genes revealed the expected 5' terminus and polyadenylation sites.

Incubation at either 2.8mM or 16.7mM glucose of isolated rat islets infected with the adenoviruses revealed a strong stimulation by glucose of the translation of the mRNAs carrying both the rat II proinsulin 5' and 3' UTRs, and the rat II proinsulin 5' with a neutral 3' UTR. The translation of mRNAs lacking the rat II proinsulin 5' UTR appeared to respond only modestly, if at all to glucose. The mRNAs carrying the rat II proinsulin 3' UTR (either in the presence or absence of the rat II proinsulin 5'UTR) appeared to be expressed at a lower level than those in which the rat II proinsulin 3' UTR was replaced with neutral sequences.

Conclusions: These data show that the 5' UTR is crucial for the glucose regulated translation of the rat II proinsulin mRNA. They also indicate that the 3' UTR may be involved in negatively regulating translation of the mRNA in the absence of glucose. Therefore, both 5' and 3' UTRs may be necessary for normal regulation of the rat II proinsulin mRNA.

Retrovirus Mediated Overexpression of Ca²⁺-Independent Phospholipase A₂ in INS-1 Cells Increases Insulin Secretion Response to Glucose

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Purpose: Stimulation of islets with glucose leads to insulin secretion and hydrolysis of arachidonate from β -cell membrane phospholipids. The nonesterified arachidonate appears to participate in glucose-stimulated insulin secretion by amplifying the crucial Ca²⁺ signal. Phospholipase A₂ (PLA₂) enzymes catalyze the hydrolysis of fatty acids esterified in the *sn*-2 position of membrane phospholipids. Purpose of this study is to examine the function of cloned iPLA₂ in glucose-stimulated insulin secretion.

Methods: A retrovirus system was used to overexpress iPLA₂ in INS-1 cells. Infected INS-1 cells were selected to establish iPLA₂-overexpressing cell lines.

Results: Several iPLA₂-overexpressing cell lines were established. The iPLA₂ activity expressed in these cell lines was 2-20 fold higher than that of control INS-1 cells and is stimulated 3 to 4 fold by 1 mM ATP. Late passage INS-1 cells respond poorly to glucose alone and secrete little insulin in

response to glucose alone but their secretion rate increases 3-fold when stimulated with the combination of glucose (22 mM), carbachol (500 μ M), and IBMX (100 μ M). In contrast, iPLA₂-overexpressing INS-1 cells exhibit a tripling of the insulin secretion rate when stimulated with glucose (17 mM) alone and adding carbachol, and/or IBMX does not further increase insulin secretion. This suggests that insulin secretion by iPLA₂-overexpressing cell line has reached maximum level in response to glucose alone. Further investigation revealed that the overall insulin secretion in low glucose concentration (3 - 5 mM) by iPLA₂-overexpressing INS-1 cells is about 25-50% higher than that by control INS-1 cells, indicating that glucose sensitivity in iPLA₂-overexpressing cells has increased compared to that of the INS-1 cells.

Conclusions: These results provide further evidence for the hypothesis that iPLA₂ could be a target of a signal, e.g. ATP generated by glucose metabolism and may be a component of the fuel-sensing apparatus of the β -cell.

Inducible expression of human Growth Hormone in the pancreatic beta cells of transgenic mice

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Mentor: Sophia Y. Tsai

Purpose: Insulin-dependent (type I) diabetes mellitus results from the functional alteration of the endocrine pancreas. To study the molecular mechanisms that occur during the development of this disease, it is necessary to generate a number of *in vivo* experimental models. We established in our study an animal model of transgenic mice to specifically regulate the expression of a target transgene in the endocrine pancreas, using a regulatory system recently developed in our laboratory.

Methods: This system consists of a chimeric regulator, GLVP, that is composed of a mutated human progesterone receptor-ligand binding domain (PR-LBDD) fused to the yeast GAL4 DNA binding domain and the herpes simplex virus transcriptional activation domain VP16. In this study, GLVP was targeted for expression in the endocrine pancreas by a rat insulin II gene promoter (INS-GLVP). In the presence of RU486, this chimeric regulator bound to a target gene containing the 17-mer GAL4 upstream activation sequences. The binding induced the transactivation of the target gene, human growth hormone (hGH).

Results: Expression of hGH in the pancreas of transgenic mice was induced in a RU486-dose dependent manner. Both regulator and the target gene expressions were specific to the pancreas and not to others tissues. Immunohistochemistry analysis and immunofluorescence microscopy further demonstrated that only the beta cells in the endocrine pancreas expressed human growth hormone. Human Growth Hormone was detected in the blood of bigenic animals only after RU486 was administrated.

Conclusions: We established a new animal model in which the expression of the transgene occurred specifically in the pancreatic beta cells in an inducible and regulable manner. This *in vivo* system of regulatable gene expression in the endocrine pancreas represents an important research tool and may contribute to a better understanding of the function of the factors involved in the development of type I diabetes.

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Mentor: Catherine J. Pallen

Purpose: IAR is a receptor-like protein tyrosine phosphatase (PTP) cloned from a human pancreas cDNA library. Unlike other plasma membrane localized receptor-like PTPs, IAR is found on the membrane of peptide hormone-containing secretory granules of neuroendocrine cells. The 'intracellular' region of IAR is in the cytoplasm, and the 'extracellular' region of IAR is in the granule lumen. In this study, pancreatic β -cell lines were used to study the regulation of IAR activity by insulin secretagogues.

Methods: Pancreatic β -cells (bTC-3, HIT-T15, or RIN) were treated with insulin secretagogues (high KCl or glucose, TPA) or control (low KCl or glucose, DMSO) compounds. At various times, insulin secretion into the medium was measured by RIA, and the cells were harvested and extracts assayed for PTP activity. Secretagogue treatment followed by cell fractionation enabled localization of secretagogue-responsive PTP activity to a particular subcellular compartment. Secretagogue-regulated PTP activity was also analyzed in whole cell extracts, cell fractions, and IAR immunoprecipitates prepared from β -cells transiently expressing wild-type and catalytically inactive forms of IAR.

Results: A peak of elevated PTP activity was detected in whole cell lysates at 15-20 min treatment with the insulin secretagogues high KCl, high glucose, or TPA, which did not appear upon treatment with control compounds. The activated PTP was localized to the cytoskeleton fraction. Secretagogue-stimulated PTP activity was not detected in cells that do not undergo regulated secretion, such as COS-1 or NIH 3T3 cells. By transient transfection of β -cells with wild-type and mutant IAR, we found that anti-IAR immunoprecipitates from secretagogue-treated cells possessed elevated PTP activity with identical properties to the endogenous secretagogue-activated PTP.

Conclusions: Insulin secretagogues effect the transient activation of an endogenous, cytoskeleton-associated PTP in pancreatic β -cells, with maximum PTP activation occurring 15-20 min after treatment. The receptor-like PTP IAR associates with the cytoskeleton, and we have identified IAR as the secretagogue-activated PTP (or as a component of it). The kinetics of IAR activation suggest that it is not required for insulin secretion *per se*, but may play a role in secretion-linked events in the β -cell.

Regulation of Inositol 1,4,5-Triphosphate Receptor Isoform Expression in Rat Pancreatic Beta Cells

Group 4

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Mentor:

Purpose: The regulation of inositol 1,4,5-triphosphate receptor (IP3R) mRNA and protein expression was investigated in rat pancreatic islets.

Methods: RNA was extracted from pancreatic islets and RT-PCR was performed to quantitate mRNA levels for IP3Rs and beta-actin. Protein was analyzed by Western blot.

Results: In rat islets and RINm5f cells, the expression of IP3R mRNA-III > IP3RI=IP3R-II. Culture of islets with glucose (G, 20mM) for 30 min increased IP3R-III mRNA expression above control and actinomycin D inhibited the increase. Culturing islets for one day or 7 days with G (11 mM) reduced the expression of IP3R-III mRNA but increased the expression of IP3R-II mRNA compared to control (5.5 mM G). IP3R-I mRNA levels remained unchanged. Islet IP3R-III protein levels increased after 2 h islet culture at 20 mM G; however, after 7 days the levels decreased below control. IP3R-II levels increased after 7-day culture at 11 mM

G, whereas IP3R-I protein levels remained unchanged.

When forskolin, 8-bromo-cAMP, or glucagon-like peptide were added to islets after 4 days of culture in 1 mM G, IP3R-II and IP3R-III mRNA levels returned to levels of control islets within 2-3 hours. The level of IP3R-I mRNA was unaffected by forskolin. H-89, an inhibitor of protein kinase A, inhibited the action of forskolin on the changes in IP3R-II and IP3R-III mRNA. Ca-2+ ionophore mimicked the effects of forskolin in IP3R mRNA expression, whereas blockade of voltage-dependent Ca-2+ channels or chelation of intracellular Ca-2+ inhibited the action of forskolin.

Conclusions: Glucose metabolism plays a role in the regulation of IP3R genes and the regulated expression of IP3R genes are mediated in part by cAMP/PKA and intracellular Ca-2+ availability.

Abstracts

Group 5

Metabolism and Complications

Faculty

Kenneth Polonsky, MD, PhD

A Randomized, Prospective, Controlled Study of Continuous Subcutaneous Insulin Infusion Versus Insulin Injection Therapy in Very Young Children with Type 1 Diabetes

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Group 5

Metabolism and Complications

Purpose: The management of diabetes in young children presents unique challenges. Despite vigilant management, wide swings in blood glucose levels are common. In addition, fear of the effects of hypoglycemia on neurocognitive development creates extreme levels of parental anxiety. Interest in insulin pumps is increasing dramatically, with more children being started on continuous subcutaneous insulin infusion (CSII). CSII therapy affords patients greater flexibility, while allowing better glycemic control. We have begun a randomized, prospective pilot trial of CSII versus insulin injections in diabetic children under the age of five.

Methods: We are selecting approximately twenty children from motivated families in our clinic population. Patients are randomly assigned either to begin CSII or to continue insulin injection therapy. After education and training, patients are followed for two six consecutive month blocks. At the six-month time point patients who are on pumps will discontinue and resume insulin injections, and the injection group will

undergo pump training and begin pump therapy. In addition to intensive phone follow-up, clinic visits occur at study initiation, then at three, six, nine, and twelve months.

Results: We plan to compare the two groups to each other and also to compare each patient's six-month profile from the CSII period to his/her profile while on injections. We will do this evaluation using multiple means, including overall glycemic control, variance in individual blood sugar measurements, safety indices, stress level of parents, and neuropsychological profiles of children. To date we have enrolled eight patients.

Conclusions: We hypothesize that CSII, used in very young children with motivated and compliant parents, will result in a decreased frequency of hypoglycemia and hyperglycemia and an alleviation of parental anxiety as compared to traditional therapy with multiple insulin injections.

The JEVIN2000 trial — A population-based survey on the quality of diabetes diabetic care in Germany: 1989/1990 up to 1999/2000

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Group 5

Metabolism and Complications

Purpose: In 1989/90 the JEVIN2000 trial was started as a system of continuous quality management in the treatment of patients with diabetes mellitus, to prove the implementation of the St. Vincent Declaration and to evaluate the effect of new therapeutic strategies.

Methods: The JEVIN2000 trial (Jena's St. Vincent Trial) was designed as a prospective, population-based survey of all insulin treated diabetic patients aged 16 to 60 years and living in the city of Jena, Thuringia (about 100,000 inhabitants). In 1989/90 190 patients (83%) of the target population were examined. In 1994/95, after a period of 5 years, 244 patients (90%) of the target population were re-examined. The results were compared with the baseline examination of 1989/90.

Results and Conclusions: In 1989/90 HbA1c (HPLC, Diamat, mean normal 5%) of patients with type 1 diabetes mellitus under specialised care was similar to 1994/95: 7.9 ± 1.35 (n=47) versus 7.6 ± 1.55 (n=131, p=0.18). The mean HbA1c of patients under non-specialised care was higher:

8.55 ± 1.9 (n=80, p=0.0087). In the total group of patients with type 2 diabetes there were no changes: 8.75 ± 2.0 (n=117) versus 8.9 ± 2.0 (n=59, p=0.67). The incidence of acute (severe hypoglycaemia, ketoacidosis) and the prevalence of long-term complications (nephropathy, retinopathy, neuropathy) were also comparable (Schiel et al., *Diabetologia* 1997; 40: 1350-1357). Based on these results and in particular regarding the ongoing changes in health care systems in other Eastern European countries, specialised care and modifications of the private health care systems are mandatory to prevent inconvenient and costly treatment of diabetes complications in the future.

In 1999/2000, ten years following the baseline examination, the target population of insulin treated diabetic patients aged 16 to 60 increased to 333. Now, in patients with type 2 diabetes insulin therapy was started at an earlier point of time. Up to the present, the results 149 (45%) patients (type 1: n=61, age 42.0 ± 10.6 [range, 16-58] years, type 2: n=77, age 52.6 ± 6.7 [22-60] years) were analysed.

Is the Glucose Transporter GLUT 2 Important in Brain Glucose Sensing?

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Purpose: Brain hypoglycemia sensing is important in the generation of counterregulatory responses and may share common mechanisms with pancreatic beta cell glucose-sensing. We examined the effects of brain intracerebroventricular (ICV) administration of streptozotocin (STZ), a specific toxin which targets GLUT 2 containing cells, on counterregulatory responses to subsequent hypoglycemia.

Methods: Healthy Sprague Dawley male rats (n=9) underwent vascular catheter and ICV guide cannula insertion on day 1. On days 2, 3 and 4, ICV injections of either 5 mg/kg STZ or vehicle (ECF) alone were given. On day 7, catecholamine and glucagon responses to a hyperinsulinemic (10 mU/kg/min) hypoglycemic (50 mg/dl) challenge were made.

Results: Despite a more profound hypoglycemic challenge (50 + 1 vs 54 + 1 mg/dl, STZ vs ECF), epinephrine and glucagon responses were markedly lower in STZ injected animals compared with ECF injected animals (peak glucagon 87 + 14 vs 257 + 70 mg/dl, p = 0.015; peak epinephrine 1874 + 368 vs 3940 + 826 pg/ml, p = 0.032; STZ vs ECF). Nor-epinephrine responses to hypoglycemia were not altered by STZ (peak 661 + 64 vs 684 + 84 pg/ml, STZ vs ECF, p = NS).

Conclusions: Counterregulatory responses to hypoglycemia may be triggered by sensitive brain areas that utilise the glucose transporter GLUT 2. Brain glucose-sensing may share mechanisms with those used by pancreatic beta-cells, and these brain areas may be more important in initiating glucagon responses to hypoglycemia than local pancreatic hypoglycemia-sensing.

Prolonged, Moderate Exercise Blunts Counterregulatory Responses to Subsequent Hypoglycemia

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Metabolism and Complications

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Purpose: We tested the hypothesis that prior exercise may blunt counterregulatory responses to subsequent hypoglycemia.

Methods: 29 healthy subjects (14f/15m, age 28±3 yrs, BMI 22±1 kg/m², HBA1c 5.0±0.5%) underwent 2-day experiments. Day 1 implied either two 90-min cycle ergometer exercise bouts (morning and afternoon) at 50% VO₂max (PriorEXE, n=16, 8m/8f), or two similar periods of rest (PriorREST, n=13, 7m/6f). Day 1 plasma glucose was maintained at 94±2 mg/dl in all subjects. Day 2 consisted of a 2 h morning hypoglycemic clamp in all 29 subjects. Endogenous glucose production (EGP) was measured using [3-³H]glucose. Muscle sympathetic nerve activity (MSNA) was measured with microneurography.

Results: On Day 2, insulin (87±6 mU/ml) and plasma glucose (55±2 mg/dl) were equivalent in PriorEXE and PriorREST. Results from the last 30 min of day 2 hypoglycemic period are:

	PriorREST	PriorEXE
Norepinephrine (pg/ml)	341±25	273±19*
Epinephrine (pg/ml)	754±85	481±61*
Glucagon (ng/ml)	163±23	95±15*
Growth Hormone (ng/ml)	39±4	14±2*
Panc. Polypeptide (pg/ml)	1161±137	665±74*
EGP (mg/kg/min)	1.6±0.2	0.6±0.3*
MSNA (Δ burst/min)	+10±4	+1±1*
Glucose inf.rate (mg/kg/min)	0.2±0.1	1.2±0.3*

Day 2 cortisol, glycerol and FFA levels were similar in both groups of subjects.

**p*<0.01, AUC, PriorEXE vs PriorREST

Conclusions: Prolonged, moderate exercise (90 min at 50% VO₂max) significantly blunted metabolic (EGP) and neuroendocrine (glucagon, catecholamines, growth hormone, pancreatic polypeptide) responses to subsequent hypoglycemia.

Hydrocortisone Regulates Endothelial Cell Occludin Content, Phosphorylation State and Distribution Coincident with Decreased Water and Solute Flux.

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Group 5

Metabolism and Complications

Purpose: Increased vascular permeability characterizes diabetic retinopathy and results, at least in part, from increased growth factors like VEGF. We and others have shown a decrease in tight junction proteins at the cell border after receptor tyrosine kinase activation. This study determined whether hydrocortisone could increase endothelial barrier properties coincident with changes in occludin phosphorylation state, content, and distribution.

Methods: BREC were grown to confluence and stepped to hydrocortisone containing media or 10% FBS for the indicated time. Occludin distribution was determined through immuno-cytochemistry and confocal microscopy. Occludin protein content and phosphorylation state were determined by western blot analysis and RNA content through real time PCR. Permeability was measured by recording accumulation of a fluorescent-labeled solute across cell monolayers or by real time monitoring of fluorescence and water accumulation across cell monolayers.

Results: Hydrocortisone decreased BREC permeability to 70 kDa RITC-dextran 5 fold and to FITC-albumin 1.6 fold. Real time measures of dextran flux revealed identical difference in diffusive flux and a 13-fold difference after application of 10 cm of water pressure. Furthermore, hydrocortisone reduced water flux by 11-fold. Immunoblot analysis of occludin revealed a 2-fold increase in content after two days of hydrocortisone treatment, a change that was reflected in RNA content as well. In addition, hydrocortisone reduced occludin phosphorylation in a dose dependent manner by 4 h. Finally, treatment of BREC with hydrocortisone caused a dramatic increase in occludin and ZO-1 staining at the cell border.

Conclusions: The opposite effects of hydrocortisone and VEGF on permeability coincide with opposing changes in tight junction phosphorylation state, content, and morphology implying a role for tight junction regulation of paracellular permeability in retinal endothelial cells.

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Regulation of vascular endothelial growth gene expression in retinal cells by insulin

Group 5

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Metabolism and
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Purpose: It has been shown by clinical trials that intensive insulin therapy causes a transient worsening of retinopathy. Since vascular endothelial growth factor (VEGF) is known to play an important role in diabetic retinopathy, we hypothesized that insulin indirectly worsens diabetic retinopathy by increasing VEGF gene expression.

Methods: The effect of insulin therapy on VEGF mRNA expression in diabetic rats was evaluated using *in situ* hybridization analysis. Human retinal pigment epithelial (RPE) cells were exposed to insulin, and VEGF mRNA levels were quantified with ribonuclease protection assays (RPA). The bioactivity of the VEGF protein in the conditioned-media of insulin-treated cells was examined using endothelial cell proliferation analysis. The capacity of insulin to stimulate the VEGF promoter linked to a luciferase reporter gene was characterized in transient transfection assays.

Results: Insulin increased VEGF mRNA levels in the ganglion, inner nuclear, and RPE cells layers. *In vitro*, insulin up-regulated VEGF mRNA levels in human RPE cells and enhanced VEGF promoter activity without affecting transcript stability. Insulin treatment also increased VEGF protein levels in conditioned RPE cell media in a dose-dependent manner. The insulin conditioned RPE cell media stimulated capillary endothelial cell proliferation the effect that was completely blocked by anti-VEGF neutralizing antibody.

Conclusions: Insulin increases VEGF mRNA and secreted protein levels in RPE cells through enhanced transcription of the VEGF gene. Intensive insulin therapy may cause a transient worsening of retinopathy in diabetic patients through increased retinal VEGF gene expression.

Regulation of Tonicity-responsive Enhancer Binding Protein (TonEBP)

Group 5

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Metabolism and
Complications

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Mentor: H. Moo Kwon

Purpose: The purpose of this project is to test the hypothesis that, in tissues such as lens where sorbitol production is high, hyperglycemia reduces the activity of TonEBP leading to decreased expression HSP70.

Methods: Using MDCK cells, changes in TonEBP and its mRNA abundance in various conditions were measured by immunoblot and Northern blot analysis, respectively. Nuclear distribution of TonEBP was examined using immunocytochemistry. A large number of reagents were screened to identify agents that inhibit or augment the TonEBP activity by examining their effect on expression of downstream genes such as SMIT and BGT1. Finally, stable cell lines expressing a dominant negative form of TonEBP were generated and used to investigate the role of TonEBP in gene expression.

Results: 1. Activity of TonEBP correlates with its amount in the nucleus. 2. Exposure of cells to hypertonic medium results in increased activity of TonEBP due to a combination of increased amount (induction) and increased proportion of TonEBP in the nucleus (nuclear distribution). 3. Induction is achieved by an increase in mRNA abundance and increased

synthesis of TonEBP without changes in turnover rate.

3. Inhibition of proteasome activity prevents the nuclear redistribution of TonEBP in response to hypertonicity leading to a decrease in expression of downstream genes.

4. TonEBP is active in isotonic conditions; it is up regulated in response to hypertonicity and it is down regulated in response to hypotonicity. 5. In isotonic conditions, inhibition of TonEBP by over expression of the dominant negative

TonEBP leads to down regulation of down stream genes.

6. Excess accumulation of compatible osmolytes such as betaine and inositol leads to down regulation of TonEBP.

Conclusions: TonEBP responds to changes osmolarity (tonicity) in a bi-directional manner, i.e., both up and down directions. TonEBP appears to respond to changes in the cellular ionic strength. As such, TonEBP is responsible for expression of HSP70 under basal conditions and plays an important house keeping role. In lens epithelial cells, cellular accumulation of sorbitol (a compatible osmolyte) during hyperglycemia leads to down regulation of SMIT. It is very likely that down regulation of TonEBP is responsible for the decreased expression of SMIT and that HSP70 expression is also decreased contributing to the toxicity of hyperglycemia.

Aberrant glomerular expression of Type 1 collagen experimental diabetes: amelioration with renoprotective therapy

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Group 5

Metabolism and Complications

Purpose: The accumulation of increased extracellular matrix in the glomerulus is a central pathogenetic mechanism in diabetic nephropathy ultimately leading to glomerular capillary obliteration and renal failure. The composition of the sclerotic matrix and its cell specific sources within the glomerulus are, however, poorly understood. The present study sought to examine whether the fibrillar, scar-associated type I collagen, not normally present in the glomerulus, was aberrantly expressed in the diabetic context. Furthermore, the cell-specific sources of collagen I expression and the effects of renoprotective therapy were also examined.

Methods: Rats were randomised to control (n=12) and diabetic (STZ) groups and sacrificed at 6 months. Diabetic rats were further randomised to receive the ACE inhibitor, perindopril (8 mg/l in drinking water, n=12); the inhibitor of advanced glycation, aminoguanidine (1 g/l in drinking water, n=12); or no treatment (n=12). Gene expression of type I collagen was localised and quantitated by *in situ* hybridization using a riboprobe coding for $\alpha 1$ (I) collagen. Type I collagen protein content was assessed immunohistochemically using a polyclonal antibody.

Results: Type I collagen mRNA and protein were not detected in the glomerulus of control rats and was present only in renal arterial vessels. In contrast, glomeruli from diabetic rats demonstrated abundant type I collagen gene transcription and protein content. This *de novo* expression was localised exclusively to glomerular podocytes. Renoprotective therapy with both perindopril and aminoguanidine significantly reduced type I collagen mRNA and immunostaining in diabetic rats. Collagen I mRNA: control: not detected, diabetes: 4.9 ± 0.7 AU (arbitrary units); diabetes + perindopril: $2.2 \pm 1.4^*$ AU; diabetes + aminoguanidine $1.4 \pm 0.4^\dagger$ AU. Collagen I immunostaining: control: not detected, diabetes 2.8 (0-3); diabetes + perindopril 1 (0-2) † , diabetes + aminoguanidine 0.8 (0-2) † ; * p < 0.05 and † p < 0.01 versus diabetic.

Conclusions: These findings indicate that; (1) the composition of glomerular extracellular matrix is altered in experimental diabetic nephropathy, (2) the glomerular podocyte is the source of *de novo* type I collagen synthesis in diabetes, (3) treatment with aminoguanidine and ACE inhibition reduced this aberrant type I collagen expression.

Renal Hypertrophy, Low Birth Weight and Diabetic Nephropathy

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Group 5

Metabolism and Complications

Purpose: Using an animal model, we sought to test the hypothesis that low birth weight (LBW) individuals who develop diabetes will, due to reduced total glomerular filtration surface area, be more likely to develop diabetic nephropathy that will progress more rapidly than in normal birth weight individuals.

Methods: Female Wistar rats that received either 6% protein diet isocaloric to standard rat chow (LPD n = 18) or standard chow (NPD n = 18) in utero were weighed at birth and cross-fostered onto dams receiving NPD until weaning. Rats were divided into three groups: control (Cont n = 6), diabetes no insulin (Diab n = 6) and diabetes plus insulin (D+I n = 6). Diabetes was induced at 12 weeks with streptozotocin 50 mg.kg⁻¹ i.v. All rats were weighed weekly and urine albumin excretion and systolic blood pressure (SBP) measured at weeks 7,10 and 13. Renal size (renal weight: body weight) was measured at week 13 after perfusion fixation. Mean glomerular number was determined by the fractionator technique.

Results: All data are expressed as mean + SD. Overall, LPD rats were of LBW ($5.2 + 0.6$ g vs $6.3 + 0.8$ g (NPD), p < 0.0001) and had higher SBP prior to diabetes ($134 + 8$ mmHG vs $125 + 6$ mmHG, p < 0.001). LPD rats had smaller kidneys than NPD rats: (Cont $0.77 + 0.02$ vs $0.90 + 0.11$, p = 0.041). LPD rats had a greater proportional increase in renal size in response to diabetes (D diab:cont $50 + 12\%$ vs $20 + 4\%$, p = 0.003) LPD rats also had fewer glomeruli ($18,641 + 2,537$ vs $26,017 + 2,455$, p < 0.0001).

Conclusions: Rats exposed to LPD in utero have smaller kidneys with reduced mean glomerular number. LPD rats also exhibit a greater proportional increase in renal size in response to diabetes. Thus, intra-uterine environment may influence the early stages of diabetic nephropathy. It could be postulated that the observed reduction in glomerular number induced by LPD in utero may in turn, lead to a more rapid deterioration in renal function.

Long-term Follow-up of Normoalbuminuric Longstanding Type 1 Diabetic Patients: Progression is Associated with Worse Baseline Glomerular Lesions, Lower Glomerular Filtration Rate and Hypertension

Group 5

Metabolism and
Complications

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Purpose: To evaluate the risk factors for the development of diabetic nephropathy (DN) in longstanding normoalbuminuric (NA) type 1 diabetic patients.

Methods: This prospective study will ultimately include 86 NA type 1 diabetic patients that had a kidney biopsy performed for research purposes 5–20 [12.9±4.8 (mean±SD)] yrs ago. To date, 32 patients [baseline age 36±10 yrs; baseline diabetes duration 21±11 yrs] have been contacted. They do not differ in any baseline characteristic from the 54 patients not yet contacted. Glomerular filtration rate (GFR) was estimated by multiple in hospital creatinine clearances (CCr); albumin excretion rate (AER) by immuno assay; mesangial fractional volume [Vv(Mes/glom)] and glomerular basement membrane (GBM) width by electron microscopic morphometric analysis.

Results: After 5–17 (mean 7.3) yrs of follow-up, 5 (16%) of these 32 patients were classified as progressors (P): one developed persistent microalbuminuria (MA; AER 20–200µg/min) and 4 had overt DN (AER >200µg/min), 3 of whom had died. There were no differences between P and non-progressors (NP) for baseline age, diabetes duration, gender, AER, or HbA1c. Although not different in baseline blood pressure (BP), more P (3 of 5) than NP (2 of 27) were classified as hypertensive (p<0.02). Baseline CCr was lower in P than NP (82±6 in P vs. 106±20 ml/min/1.73m² in NP; p=0.01). Baseline Vv (Mes/glom) was higher in P (0.37±0.06) than in NP (0.28±0.07; p=0.01). Similarly GBM width was greater in P (587±159 nm) than in NP (454±103 nm; p=0.02).

Conclusions: Longstanding NA type 1 diabetic patients who progress to MA or overt DN have worse baseline glomerular lesions, lower baseline GFR and are more frequently hypertensive than patients who remain NA. Measurements of kidney biopsy specimens, GFR, and BP may be better predictors of DN risk than AER in long-term NA D patients.

TGF-beta Receptors in Diabetic Nephropathy

Group 5

Metabolism and
Complications

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Purpose: Diabetic nephropathy affects 40% of individuals with type I diabetes. The hallmark of diabetic nephropathy is excess deposition of extracellular matrix in the kidney. The soluble ligand TGF-β has been shown to be a major inducer of increased ECM deposition. However, regulation of TGF-β receptors remains poorly understood in this setting.

Methods: We used primary mesangial cells isolated from Sprague-Dawley rats to investigate TGF-β receptor expression patterns and their regulation by high glucose. Cell extracts were analyzed for TGF-β receptor expression by Northern and Western Blotting, as well as by radioligand-binding. Subcellular expression patterns of TβRs were investigated by fluorescence microscopy.

Results: Primary rat mesangial cells express all three TGF-β receptors (TβRI, TβRII, and TβRIII) as shown by RNA and protein analyses. Ligand binding was prominent to TβRI and TβRIII, but comparably weak to TβRII. This corresponded to strong expression of TβRI and TβRIII protein in the cytosol with absence of nuclear staining. In contrast, TβRII exhibited strong nuclear and cytoplasmic staining patterns. Interestingly, high glucose specifically upregulated TβRI RNA and protein expression. This led to enhanced ligand binding and biological responses to TGF-β.

Conclusions: TβRs present as novel molecules involved in the mesangial cell's response to high glucose. Specific upregulation of TβRI leads to enhanced biological responses to TGF-β, and therefore may drive a fibrotic response by itself. Intervention of this pathway is an attractive target for future therapy of diabetic nephropathy.

Gene Transfer Approaches to Limiting Vascular Dysfunction in Experimental Diabetes Mellitus: Endothelial Nitric Oxide Synthase vs. Superoxide Dismutase

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Metabolism and Complications

Purpose: Reduced vascular nitric oxide (NO) bioavailability is characteristic of diabetes mellitus and may be one of the earliest features of atherosclerosis. The etiology of this defect is unclear but may involve reduced generation of NO or increased scavenging of NO via interaction with superoxide anion. Potential molecular strategies to enhance NO bioavailability in the blood vessel wall include gene transfer of endothelial nitric oxide synthase (eNOS) or the copper zinc or manganese isoforms superoxide dismutase (CuZnSOD, MnSOD). The aim of the current study is to compare the effect of these strategies on endothelial dysfunction in the alloxan-induced diabetic rabbit.

Methods: Rabbits were rendered diabetic by the injection of alloxan. Ten weeks later the aorta was harvested and adenoviral vectors were used to transfer the genes for eNOS, CuZnSOD or MnSOD to vessel segments *ex vivo*. Vessels were placed in

organ culture and 24 hours later transgene expression was sought and vascular function was analyzed in organ chambers.

Results: Abnormal endothelium dependent vasorelaxation was observed in the diabetic aorta. Expression of recombinant eNOS, CuZnSOD and MnSOD was confirmed by Western Blot analysis, immunohistochemistry and SOD activity assay. While eNOS gene transfer altered vascular reactivity, endothelial dysfunction was not reversed. In contrast, gene transfer of both CuZnSOD and MnSOD reversed endothelial dysfunction in this diabetic model.

Conclusions: SOD and not eNOS gene transfer reverses diabetic endothelial dysfunction and thus strategies aimed at overexpression of SOD in the diabetic vasculature may be a useful gene therapy approach to diabetic vascular dysfunction.

Monocyte-macrophage Triglyceride-rich Lipoprotein Receptor Expression and Activity in Diabetes

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Metabolism and Complications

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Purpose: Individuals with IDDM and NIDDM have increased risk for atherosclerosis and cardiovascular disease (CVD). Most diabetics have elevated triglyceride (TG) and TG-rich lipoproteins (TGRLP), which are risk factors for CVD. We identified a novel TGRLP receptor (apoB48-R) in reticuloendothelial cells (REC) that binds apoB48, the apoB species of dietary TGRLP. Uptake by this R causes macrophage foam cell formation and EC dysfunction *in vitro*. Goals are to determine if structural/functional alterations of TGRLP increase uptake via this R and/or if changes in the apoB48-R pathway occur in diabetic subjects, increasing their CVD risk.

Methods: Standard molecular biology techniques were used to clone the apoB48-R. TGRLP subfractions were tested for rapid (≤ 3 h) lipid accumulation via the apoB48-R in R-transfected cells and for binding by ligand blotting.

Results: Confounders of apoB48-R-specific TGRLP uptake in REC include uptake by LDL-R family members and inter-

actions with apoE and lipoprotein lipase. We expressed the apoB48-R in R-negative cell lines (CHOK1 and IdIA7 CHO, lacking the LDL-R) conferring ligand specificities and kinetics equivalent to those observed for native macrophage apoB48-R. The transfected apoB48-R binds and internalizes chylomicrons (apoB48 as the only apoB) and VLDL from hypertriglyceridemic (HTG) subjects, but not VLDL and LDL from normal subjects. Using this model of apoB48-R activity, the relative apoB48-R-specific uptake of TGRLP in diabetic and non-diabetic subjects can be measured. Thus we anticipate that TGRLP from HTG diabetic subjects will, as previously determined in macrophages, cause apoB48-R-mediated lipid accumulation in R-transfected CHOs.

Conclusions: The novel apoB48-R is a nutritional receptor for chylomicrons in REC, providing an efficient pathway for lipid-soluble vitamins, TG, and cholesterol. In diabetics and nondiabetics with HTG, this receptor may contribute to foam cell formation, EC perturbation, and atherosclerosis. In diabetic subjects with elevated TG levels the apoB48-R pathway may contribute to increased risk for macro- and micro-vascular disease.

Structure-Function Relationship of Advanced Glycation Endproducts

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Metabolism and
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Purpose: To clarify the mechanism by which advanced glycation endproducts (AGE-proteins) induce oxidative stress in vascular cells exposed to albumin modified by reducing sugars and oxoaldehydes

Methods: AGE proteins were prepared by incubating 50mg/ml bovine serum albumin (BSA) with 20mM methyl glyoxal (MGO) for 1 week in 0.1M-phosphate buffer under sterile conditions. RAW 264.7 cells (a monocyte/macrophage-like cell line) was obtained from ATCC and cultured according to manufacturers recommendations. The assay uses the principle that the non-fluorescent dye- dichlorofluorescein diacetate (DCFH-DA) becomes fluorescent (DCF) upon intracellular oxidation. Quiescent cells are preloaded with DCFH-DA for 45 minutes. Following this, the cells are washed and treated with 10mM test proteins- BSA or MGO-BSA. The resulting fluorescence is used as a marker of intracellular oxidation induced by the test samples.

Results: Cells treated with MGO-BSA show a significant increase in DCF fluorescence (162%) as compared to cells treated with BSA. DCF-fluorescence in cells treated with

BSA is 30% less than cells treated with medium (which does not contain any protein), whereas it is 36% higher in cells treated with MGO-BSA. The oxidative activity of MGO-BSA is also found in a minimally modified preparation of BSA viz., MM-BSA (prepared by incubating 0.5mM MGO with BSA for 6 hours). This property of BSA is unique to its primary sequence and not generalizable to albumins from all species. Other proteins such as RNaseA, chicken egg ovalbumin and a-crystallins were also unlike BSA. The oxidation induced by MGO-BSA is suppressed in metal-free medium. Enzymatic hydrolysis results in complete loss of activity of BSA.

Conclusions: BSA tends to protect cells against oxidation by serving as an anti-oxidant. When modified by MGO, BSA not only loses its protective anti-oxidant property, but also becomes pro-oxidative. The fact that MM-MGO has similar properties suggests that such modifications may be of physiological relevance in the diabetic state. Another important conclusion is that intact BSA is required for this protective activity. Conformational changes in the modified proteins may be responsible for their loss in protective activity. These results provide new insight into the structure-function relationship of AGE-proteins and their relevance to diabetic complications.

Apo E-enriched triglyceride-rich lipoproteins from subjects with Type 2 diabetes do not demonstrate increased binding to biglycan, a vascular proteoglycan

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Purpose: Retention of atherogenic lipoproteins by arterial wall proteoglycans is thought to be an important step in the development of atherosclerotic lesions. Proteoglycans can bind to apolipoprotein (apo) B- and E- containing lipoproteins via ionic interactions, leading to their retention in the artery wall. Triglyceride (TG)-rich lipoproteins contain both apo B and apo E and may be important sources of artery wall cholesterol, especially in diabetic subjects in whom the levels of TG-rich lipoproteins are increased. In this study we tested the hypothesis that TG-rich lipoproteins from individuals with type 2 diabetes are enriched in apo E compared to nondiabetic controls, and that this apo E enrichment would result in increased retention by proteoglycans as a mechanism to explain, in part, the increased atherosclerosis in diabetes.

Methods: TG-rich lipoproteins ($d < 1.019$ g/ml) were isolated from fasting type 2 diabetic subjects (n=7) and age-matched controls (n=7) using density gradient ultracentrifugation. The

TG-rich lipoproteins were incubated with purified 35S-SO₄ labeled biglycan, a small dermatan sulfate proteoglycan, and lipoprotein binding was assessed using an electrophoretic gel-shift assay.

Results: TG-rich lipoproteins from type 2 diabetic subjects were significantly enriched in the relative proportion of apo E to apo B levels compared to age-matched controls (1.97 ± 0.20 vs 1.36 ± 0.11 molar ratio, $p=0.02$). However, when an equal number of TG-rich lipoprotein particles (based on apo B content) was incubated with purified biglycan, the TG-rich lipoproteins from the diabetic subjects demonstrated lower binding affinity than from controls (Kd of 8.1×10^{-8} M vs 4.5×10^{-8} M apo B for diabetic and control subjects respectively, $p=0.03$)

Conclusions: Thus, although the TG-rich lipoproteins from subjects with diabetes are enriched in apo E compared to age-matched controls, this appears to result in decreased proteoglycan binding and, therefore, may not explain the increased atherosclerosis seen in type 2 diabetes.

Effect of protein kinase C β 2 overexpression in the heart

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Metabolism and Complications

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Purpose: Protein kinase C (PKC) activation in the myocardium is associated with a variety of cardiac abnormalities including hypertrophy, fibrosis, cardiomyocyte lysis and failure. Cardiac specific activation of the PKC β 2 isoform is reported to produce specific cellular and functional changes leading to cardiomyopathy of diabetic or non-diabetic origin (Wakasaki et al. 1997, Proc. Natl. Acad. Sci., 94, 9320-9325). The purpose of this study is to further characterize the alterations produced with increasing age in myocardium from transgenic mice overexpressing the PKC β 2 isoform.

Methods: A transgenic mouse line overexpressing the PKC β 2 isoform in the heart was utilized. The transgene consisted of rat myosin heavy chain promoter ligated to mouse PKC β 2. Southern blot analysis of DNA extracted from tail samples was used to confirm gene transfer. Male and female heterozygous mice bearing the PKC β 2 transgene and non-transgenic control littermates were used. Ventricular tissue was obtained from mice at 4-16 weeks of age for experimental studies.

Results: Histological assessment of ventricular sections obtained from 10-week old transgenic mice revealed areas of fibrosis and calcification, in addition irregularities in the outer surface of the heart were noted. Connective tissue growth factor (CTGF) has been identified at sites of extracellular matrix accumulation and fibrosis in vascular tissue. CTGF functions as a downstream mediator of transforming growth factor (TGF) β , the expression of which has been reported previously to be increased in these transgenic mice. Assessment of CTGF mRNA identified increased expression in hearts obtained from 4, 8 and 10 week old mice. Preliminary immunoblot experiments have also indicated increases in mitogen-activated protein kinase (MAPK) levels at these durations.

Conclusions: PKC β 2 may be involved in the regulation of CTGF expression either directly or via TGF β , and in the induction of the MAPK pathway. Alterations in these pathways induced by overexpression of PKC β 2 could contribute to the development of fibrosis as observed in cardiomyopathy of diabetic or non-diabetic origin.

High Glucose Leads to Decreased Glucose 6-Phosphate Dehydrogenase (G6PD) Activity and Cell Survival in Bovine Aortic Endothelial Cells (BAEC). Whereas High Glucose Leads to Increased G6PD Activity and Cell Growth in Renal Mesangial Cells (RMC)

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Group 5

Metabolism and Complications

Mentor: Robert C. Stanton

Abstract: Hyperglycemia is thought to be the primary cause of diabetic complications. However, the effects of high glucose vary depending on cell type. Specifically, high glucose leads to increased cell death in BAEC. In contrast, high glucose causes increased cell hypertrophy/growth in renal mesangial cells. Research from our lab has shown that appropriate G6PD activity is required for normal cell growth (JBC 273: 10609, 1998) and prevention of cell death (AJP 276: C1121, 1999). Considering the important role of G6PD in cell survival, we hypothesized that in endothelial cells high glucose would cause inhibition of G6PD activity leading to cell death, whereas in mesangial cells high glucose would cause increased G6PD activity leading to cell growth. BAEC and RMC were exposed to normal (5.5 mM) and high glucose (up to 25 mM) with/without G6PD inhibitors. G6PD activity, reactive oxygen

species (ROS), and NADPH were measured. Cell death and apoptosis were determined by Trypan Blue, DAPI and DNA fragmentation. In BAEC, 1) High glucose concentrations as low as 10 mM caused up to a 70% decrease in G6PD activity; 2) Decreased G6PD activity was evident within 1 hour of exposure to increased glucose; 3) Increased glucose as low as 10 mM increased cell death within 3 hours of exposure to high glucose; 4) Inhibition of G6PD led to a 2-fold increase in high glucose-induced cell death; 5) High glucose increased apoptosis which was also significantly enhanced by G6PD inhibition; 6) High glucose increased ROS and decreased NADPH. In RMC, 1) High glucose increased G6PD activity; 2) High glucose led to an increase in cell number; 3) Inhibition of G6PD abrogated the effects of high glucose RMC. Conclusion: Differential effects of high glucose on various cell types is mediated at least in part by high glucose-induced changes in G6PD activity.

Role of c-jun in Dorsal root ganglia apoptosis

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Group 5

*Metabolism and
Complications*

Mentor: Bruce D. Carter

Purpose: To assess the role of c-jun in apoptosis of dorsal root ganglia (DRG) neurons following nerve growth factor (NGF) withdrawal. This study will provide a mechanistic understanding of neuronal degeneration which occurs in diseases such as diabetes, where reduced peripheral NGF levels have been observed.

Methods: DRGs were isolated from E12.5 c-jun +/+, +/- or -/- mouse embryos. The isolated DRGs were grown in supplemented F-14 medium with varying concentrations of NGF and plated at approximately 7000 cells per well of a 24-well plate. Phase bright neurons bearing neurites twice the length of the soma were counted. E12.5 embryos of all 3 genotypes were also fixed, frozen and will be sectioned through the DRGs. Every 5th section will be TUNEL stained, to detect apoptotic cells, and the percent of dying DRG neurons quantitated. Since increased levels of phospho-jun and phospho-ATF2 have been implicated in apoptosis, we will immunostain cultured neurons that have been deprived of NGF with P-jun and P-ATF2 antibodies.

Results: We found that the NGF-dependence curve was similar for neurons from all genotypes. We have also determined the time course of cell death for c-jun +/+, +/- and -/- neurons upon NGF withdrawal. There was no apparent difference in the kinetics of programmed cell death. Currently, we are determining if the in vitro findings correlate to the in vivo situation by TUNEL staining sections from wild type and c-jun -/- DRGs. Further, we are examining the levels of phospho-jun and phospho-ATF2, both substrates of c-jun kinase (JNK), following NGF withdrawal. JNK is a stress-activated kinase which has been shown to be activated in various paradigms of cell death.

Conclusions: The above results imply that c-jun is not necessary for either survival or apoptosis, induced by trophic factor withdrawal, of DRG neurons. These findings suggest that additional factors are involved in sensory neuron degeneration. One candidate under investigation is another substrate for JNK, the transcription factor ATF2.

Polymorphisms in endothelin system genes and the development of nephropathy in insulin dependent diabetes mellitus (IDDM)

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*Metabolism and
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Mentor: Andrzej S. Krolewski

Purpose: The endothelin system, consisting of three genes, is implicated in the development and progression of diabetic nephropathy. The genes considered are: endothelin (ET-1), endothelin converting enzyme 1 (ECE-1), and endothelin receptor (ER). The aim of this study is to examine whether DNA polymorphisms in these genes contribute to genetic susceptibility to diabetic nephropathy.

Methods: Direct sequencing of genomic DNA from 8 cases and 8 controls was used to screen the regulatory and structural parts of these genes for DNA polymorphisms. The identified polymorphisms are being genotyped in 191 individuals with advanced diabetic nephropathy (cases) and 188 individuals with normoalbuminuria despite diabetes duration ≥ 15 years (controls). So far, the two first genes have been examined.

Results: Two polymorphisms in the ET-1 gene were identified: one in the promoter region (-1370t/g) and the other a

lysine to asparagine substitution at amino acid codon 198 (K198N). The distributions of the alleles of these two polymorphisms did not differ significantly between cases and controls ($p=0.10$ and $p=0.15$, respectively). Three polymorphisms in the ECE-1 gene were identified: two in the promoter region (-b25g/t and -b338g/t) and one silent polymorphism in exon 9 (ex9 T45T). The distributions of alleles of the -25g/t and ex9 T45T polymorphism did not differ significantly between cases and controls. For the -b338g/t polymorphism, the frequency of the t allele was somewhat higher in cases than controls ($p=0.10$), and carriers of the t allele had a significantly higher risk of diabetic nephropathy than non-carriers ($p=0.04$).

Conclusions: The -b338g/t polymorphism in ECE-1 gene is associated with the development of diabetic nephropathy; however, the function of this polymorphism is unknown. To confirm our finding, we need to conduct a family based study and test the association using the transmission disequilibrium test.

Oxidative stress and HNE conjugation of GLUT3 are increased in the hippocampus of diabetic rats subjected to stress

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Group 5

Metabolism and Complications

Mentor: Bruce S. McEwen

Purpose: Recent studies demonstrate that cellular, molecular and morphological changes induced by stress in rats are accelerated when there is a pre-existing strain upon their already compromised adaptive responses to internal or external stimuli, such as may occur with uncontrolled diabetes mellitus. Compensatory mechanisms are activated in response to increases in allostatic load in the hippocampus of diabetic rats subjected to stress. Nonetheless, the deleterious actions of diabetes and stress increase hippocampal neuronal vulnerability, possibly resulting from increases in oxidative stress. Since increases in oxidative stress are associated with both diabetes and stress, the present study examined whether oxidative stress is increased in the hippocampus of streptozotocin (STZ) diabetic rats, rats subjected to restraint stress, and STZ diabetic rats subjected to stress.

Methods: Radioimmunochemistry (RIC) was performed using polyclonal antisera that recognize proteins conjugated by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE). RIC was performed in vehicle treated, non-stressed controls, STZ diabetic rats (70 mg/kg, iv), vehicle treated rats subjected to 7 days of restraint stress, and STZ diabetic rats subjected to 7 days of stress. In addition, immunoprecipitation and immunoblot analysis was performed on hippocampal synaptosomal membranes isolated from diabetic rats subjected to stress and control rats.

Results: Radioimmunochemistry revealed that HNE protein conjugation is increased in all subregions of the hippocampus of streptozotocin (STZ) diabetic rats, rats subjected to restraint stress and STZ diabetic rats subjected to stress. Such increases were not significant in the cortex. Because increases in oxidative stress may contribute to stress- and diabetes-mediated decreases in hippocampal neuronal glucose utilization, we examined the stress/diabetes mediated HNE protein conjugation of the neuron specific glucose transporter, GLUT3. GLUT3 immunoprecipitated from hippocampal membranes of diabetic rats subjected to stress exhibited significant increases in HNE immunolabeling compared to control rats, suggesting that HNE protein conjugation of GLUT3 contributes to decreases in neuronal glucose utilization observed during diabetes and exposure to stress.

Conclusions: Collectively, these results demonstrate that the hippocampus is vulnerable to increases in oxidative stress produced by diabetes and stress. In addition, increases in HNE protein conjugation of GLUT3 provide a potential mechanism for stress and diabetes mediated decreases in hippocampal neuronal glucose utilization.

Acceleration of Atherosclerosis, Vascular Inflammation and Hypercoagulability by Hyperhomocysteinemia in a Murine Model

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Immunology—II

Mentor:

Introduction: Hyperhomocysteinemia (HHC) is a known cardiovascular risk factor and found in a high prevalence in people with diabetic nephropathy. However, the pathogenesis of homocystein (HC) induced vasculopathy is not fully defined.

Methods: Male Apo E null mice, 4 weeks of age, were maintained on a normal rodent chow (= diet A), or on a methionine rich and vitamin depleted diet to induce HHC (= diet B) or on a methionine rich and vitamin enriched diet (= diet C) and sacrificed 8 weeks later. Mean lesion area and molecular markers of vascular dysfunction were measured.

Results: Apo E null mice receiving diet B demonstrated a 19-fold increase in plasma HC which results in increased oxidative stress measured as lipid hydroperoxide (28 vs 22 nmol/ml plasma, $p < 0.05$). Similarly, NF kappa B, the receptor of advanced glycation endproducts (RAGE), Carbomethyllysine (CML) and

EN-RAGE, a newly identified proinflammatory RAGE ligand, tissue factor and metalloproteinase-9 were upregulated. Quantification of atherosclerotic lesions using serial sections of the aortic arch stained with oil red O revealed a 2-fold increase in mean lesion area which could be significantly reduced by vitamin enriched diet C.

Conclusions: (1) This murine model supports a role for HHC in the induction/perturbation of increased atherosclerosis and vascular dysfunction. (2) HC-mediated expression of RAGE and its ligand, EN-RAGE and CML might contribute to the increased development of macrovascular disease in diabetes. (3) It is suggested that vitamin enrichment (folic acid, B6, B12) may suppress HC induced vascular dysfunction.

Abstracts

Group 6

Immunology—II

22 Abstracts

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Autoimmune diabetes, in several experimental systems, is opposed by active control mechanisms that either delay or prevent the onset of full-blown disease. One of these systems is the BDC2.5/NOD T cell receptor transgenic mouse where most T cells express a diabetogenic TCR: only a fraction of these mice develops diabetes, and not before 3 months of age, in spite of having huge numbers of diabetogenic T cells from birth and massive insulinitis from 3 weeks of age. By crossing these transgenic mice with immunodeficient strains, we found

that the control of disease requires T cells bearing non-diabetogenic TCRs (i.e., endogenously encoded TCRs, by opposition to the transgene-encoded TCR). More dramatic was the result from crosses with RAG-null mice: diabetes appeared in all mice by 30 days of age. Experiments involving reconstitution of these BDC2.5-transgenic RAG-null mice with various cell populations confirmed the existence of protective T splenocytes in young NOD mice: protection was specifically transferred by the CD4⁺ subpopulation of ab T cells. These cells were quite potent as a few hundred thousand were able to prevent diabetes in this system.

Targeted immune regulation for treatment of type 1 diabetes

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Purpose: Phenotypic and functional analysis of regulatory cells capable of suppressing diabetogenic T cell responses and preventing type 1 diabetes.

Methods: Transgenic mice expressing a protein of lymphocytic choriomeningitis virus (LCMV) under control of the rat insulin promoter in the pancreatic β -cells have a normal phenotype but develop diabetes following LCMV infection due to the activity of virus-specific T cells destroying the viral transgene expressing β -cells. Regulatory cells were generated by a) oral insulin immunization, b) i.m. DNA vaccination or c) CD40 signaling blockade (anti-CD40L treatment).

Results: Both oral (insulin) and DNA (insulin B-chain) immunization lead to generation of insulin B-chain-specific CD4⁺ regulatory T cells. Isolated regulatory cells produce IL-4, IL-10 and IFN γ . After adoptive transfer into prediabetic recipients, CD4⁺ regulatory T cells proliferate selectively in the pancreas draining lymph node resulting in localized suppression of diabetogenic T cell activity to the antigenically

unrelated viral transgene and complete protection from diabetes. IL-4 is essential for diabetes prevention since regulatory T cells cannot be induced in the absence of IL-4 or the signal transducer and activator of transcription 6 (Stat6, IL-4 signaling pathway). Both factors are critical for modulating antigen presenting cells (APCs) which act as a “bridge” between T cells of differential specificity. Furthermore, blockade of CD40 signaling during APC activation induces CD11c⁺ “regulatory” APC that are also capable of diabetes prevention.

Conclusions: Targeted immune regulation can be achieved by regulatory T cells with a specificity distinct from diabetogenic T cells. Localized modulation of APC activity appears to be a critical event as further demonstrated by therapeutically effective “regulatory” APCs. Thus, effective and specific forms of immune regulation can be designed in the absence of a clearly defined disease initiating autoantigen. These findings have implications for devising immune-therapeutic strategies aimed at preventing type 1 diabetes in individuals at risk.

Interleukin-4 suppresses virus-induced diabetes by limiting the development of antigen-specific effector CTLs

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Group 6

Immunology—II

Mentor: Nora Sarvetnick

Purpose: The mechanisms underlying suppression of immune responses by interleukin-4 remain unclear. In this report we investigate the ability of IL-4 to suppress CD8⁺ T cell mediated responses in the LCMV virus-induced diabetes model.

Methods: RIP-NP transgenic mice express the nucleoprotein (NP) of the lymphocytic choriomeningitis virus (LCMV) under the control of the rat insulin promoter (RIP) in their pancreatic β cells. Breeding NOD mice, which express interleukin-4 in their pancreatic islets, with RIP-NP (H2d) mice, generated double-transgenic IL-4+NP⁺ mice. Mice were infected with LCMV and blood glucose values measured twice weekly. One week following infection, spleens and pancreatic lymph nodes were examined for the evidence of cell death, activation, the percent CD8⁺ T cells that recognized NP118-126 by flow cytometry and killer activity against NP 118-126 peptide pulsed targets.

Results: Transgenic IL-4+NP⁺ mice were protected from diabetes following infection with LCMV. Pancreatic islet expression of IL-4 prevented diabetes by inhibiting the development of antigen specific effector cytotoxic T lymphocytes (CTLs). The effect of IL-4 on CD8⁺ T cell function was stage specific since the numbers of antigen specific CD8⁺ T cell precursors in the periphery were unaffected. IL-4 inhibited the CTL response in the pancreas directed against the immunodominant NP peptide 118-126. Transfer of NP peptide 118-126 pulsed dendritic cells reversed the suppressive effect of IL-4, resulting in diabetes in all recipient mice. This effect was not observed following transfer of IL-4 treated dendritic cells.

Conclusions: Islet expression of IL-4 can act via antigen presenting cells to inhibit the development of an effective CTL response in the pancreas.

Autoimmune insulinitis and diabetes in the absence of antigen-specific contact between T cells and islet-b cells

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Group 6

Immunology—II

Mentor: Harald von Boehmer

Purpose: To determine whether antigen-specific cell to cell contact between T cells and beta cells is necessary to induce insulinitis and diabetes

Methods: We have used an adoptive transfer model of diabetes in which monospecific T cells expressing a class II-restricted TCR specific for a peptide from the hemagglutinin (HA) are transferred into immunodeficient mice expressing the HA under the insulin promoter. We constructed bone marrow chimeras in which the host antigen presenting cells (APCs), but not the beta cells, would be able to present the HA to the transferred CD4⁺ T cells. As positive controls, we used recipients where both the beta cells and APCs could present the HA epitope and as negative controls we used recipients where neither beta cells nor APCs could present the HA epitope.

Results: We found evidence for the activation of HA-specific T cells in the lymph nodes draining the pancreas of mice expressing HA in the pancreas, suggesting transfer of the tissue specific antigen to the APCs draining the tissue. We then proceeded to determine whether a) this “cross-presentation” of pancreatic antigen required previous recognition and destruction of beta cells by T cells and b) whether beta cell death could proceed in the absence of antigen-specific contact between T cells and islet beta cells. By using the bone marrow chimeras described in methods, we show that both insulinitis as well as beta cell destruction can proceed in the absence of islet beta cell surface antigen recognition by T cells.

Conclusions: Our results demonstrate in a definitive manner that class II restricted monospecific T cells can cause diabetes without need for antigen-specific contact between the former and the latter. Thus, beta cell death can be induced by distinct effector mechanisms such as secretion of soluble factors by activated T cells and APCs present in the islets. Such mechanisms are currently under investigation.

Intrinsic defects in the T cell lineage result in NKT cell deficiency, leading to autoimmune diabetes in the NOD mouse

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Immunology—II

Purpose: Type I diabetes is a T cell-mediated autoimmune disease. However, it is unclear whether T cells express defective genes contributing to the pathogenesis of autoimmune diabetes. In addition, a major regulatory T cell population, NKT cell population is deficient in NOD mice. This study is initiated to determine 1. whether intrinsic defects of T cell lineage in NOD mice play a critical role in the development of autoimmune diabetes; 2. whether the NKT cell deficiency of NOD mice is due to intrinsic or developmental defects.

Methods: In order to determine whether the intrinsic defects of T cell lineage contribute to the pathogenesis of the disease and NKT cell deficiency, we reconstituted the T cell compartment of NOD.*scid* or BALB.*scid* mice with T cells derived from NOD, NOR or AKR thymic precursor cells. We then examined the development of the T cell and NKT cell populations and autoimmune diabetes in these recipient mice.

Results: We found that major T cell populations developed well in all the reconstituted recipient mice. However, NKT cells developed well only from AKR thymic precursor cells but not from other precursor cells in both NOD.*scid* and Balb.*scid* recipient strains. Insulinitis and diabetes developed only in the NOD.*scid* recipients of NOD or NOR precursor cells. In contrast, NOD.*scid* recipient mice of AKR precursor cells were free of insulinitis and diabetes. When thymic precursor cells of beta 2-microglobulin gene-deficient AKR mice, which have a deficient NKT population, were introduced into NOD.*scid* recipients, both CD4⁺ and CD8⁺ T cell populations developed and the recipient mice developed insulinitis and diabetes.

Conclusions: We conclude that NKT cells originated from a T cell-committed thymic precursor population, and the deficiency in the NKT cell population in NOD mice results from intrinsic defects within the T cell lineage and plays an important role in the development of autoimmune diabetes in the presence of both the NOD thymus and antigen presenting cells.

Autoreactive CD4⁺ T Cell Responses in Children with Type 1 Diabetes Mellitus and Healthy Controls

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Group 6

Immunology—II

Mentor: Darrell M. Wilson

Purpose: 1) To verify that the immunodominant epitopes of glutamic acid decarboxylase (GAD) 65 and preproinsulin (PPI) previously determined in HLA-transgenic mice are also immunogenic in humans.

Methods: We have used a number of HLA-DR4 and DQ8 transgenic mice with diabetes-associated HLA alleles to determine the immunogenic T cell epitopes of recombinant human GAD65 and PPI. Patients with recent-onset (within one month of diagnosis) type 1 diabetes mellitus as well as their parents and siblings have been recruited from Lucile Salter Packard Children's Hospital at Stanford. All patients and controls have been HLA-typed and T cell responses to the immunodominant epitopes of GAD65 and PPI have been studied using a T cell proliferation assay.

Results: The immunodominant epitopes of GAD65 and PPI previously determined in HLA-DR4 and DQ8 transgenic

mice were also found to stimulate T cells from humans with type 1 diabetes mellitus. Notably, the GAD65 peptides: 76-95, 116-130, 206-220, 271-285, and 551-570 were reactive in a high percentage of patients as well as controls. However, T cell proliferative responses to the PPI peptide, 73-90, which were found in more than 25% of DR4-positive patients, were not detected in any of the DR4-positive or negative control individuals suggesting that this PPI epitope may be of significance in the disease.

Conclusions: T cell proliferative responses to GAD65 and PPI can be seen in both patients and healthy controls, and therefore, are not disease-specific. The finding that HLA DR4-positive patients respond to PPI peptide 73-90 which is on the C-peptide of the molecule (cleaved during formation of mature insulin) suggests that it is the pro-hormone which is the target of the autoimmune CD4⁺ T cell response in type 1 diabetes. Such information may be important for more precise targeting of preventive immunotherapy in individuals with disease-susceptible HLA alleles.

Role of T Cell Resistance to Activation-Induced Cell Death (AICD) in Susceptibility to Type I Diabetes

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Immunology—II

Mentor: Terry L. Delovitch

Purpose: To determine whether T cell antigen receptor (TCR)-induced and Fas-mediated signalling pathways are defective and elicit the breakdown of T cell tolerance to self antigens and onset of autoimmune type 1 diabetes (IDDM) in nonobese diabetic (NOD) mice.

Methods: The percent AICD in splenic T cells from 7-8 wk-old diabetes-susceptible NOD and diabetes-resistant (NOR, C57BL/6, BALB/c) mice was determined after stimulation for 72 hr with anti-CD3⁺ anti-CD28 mAb. The percentage of apoptotic cells was quantitated by FACS analysis of propidium iodide (PI)/Annexin V-FITC stained cells.

Results: Both CD4⁺ and CD8⁺ NOD T cells were found to be resistant to AICD compared to that observed in C57BL/6, BALB/c and NOR T cells. We also found that while there is a reduction of FLIP, a potent inhibitor of the Fas signalling pathway, in T cells from C57BL/6 mice after activation, the expression of FLIP persists in NOD T cells. Moreover, caspase-3 cleavage, as determined by disappearance of the proenzyme form of caspase-3 (Mr=32 kD) and appearance of the processed/cleaved caspase-3 subunit, p17, was significantly reduced in NOD CD8⁺ T cells.

Conclusions: NOD T cell resistance to AICD may be related to a deficiency in IL-2 production by NOD T cells after TCR stimulation, which sets a higher threshold for progression to S phase of the cell cycle and resistance to apoptosis in NOD T cells.

Role of B7 costimulation in the pathophysiology of autoimmune diabetes

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Immunology—II

Mentor: Jeffrey A. Bluestone

Purpose: The goal of this work is to develop a better understanding of the role of T cell costimulation in autoimmune diabetes with a particular emphasis on the B7/CD28/CTLA-4 pathway.

Methods: We have developed different strains of the spontaneously autoimmune NOD mouse in which the expression of the CD28 and B7 molecules were disrupted to study the role of T cell co-stimulation in this spontaneous autoimmune setting.

Results: Diabetes was exacerbated in both B7-1/B7-2-deficient and CD28-deficient NOD mice as compared to control mice. The immunoregulatory CD4⁺CD25⁺ T cells which control diabetes in prediabetic NOD mice were absent from CD28KO, B7-1/B7-2KO and CTLA4Ig-treated animals. The transfer of this regulatory T cell subset from

control NOD animals into CD28-deficient animals delayed, and in some instances, prevented diabetes. In contrast to B7-1/B7-2KO mice, the B7-2-deficient NOD mice did not develop disease. Moreover, there was minimal islet infiltration in spite of a near normal GAD antigen-specific response by splenic T cells. Diabetes was not induced after cyclophosphamide treatment or injection of diabetogenic T cells into irradiated B7-2 KO NOD mice.

Conclusions: Our studies show that the absence of CD28/B7 costimulation results in exacerbation of autoimmunity which is best explained by a deficit of CD4⁺CD25⁺ immunoregulatory T cells. In addition, the CD4⁺CD25⁺ T cells depend on CD28 co-stimulation for their survival, thus, pointing to an antigen-dependency of this novel T cell subset found to control multiple spontaneous autoimmune diseases. Furthermore, the data suggest that the absence of disease in B7-2 KO mice is due to a deficit of migration of pathogenic T cells in the pancreas.

Antigen Dependent Extravasation and Pancreatic Homing of Diabetogenic CD8⁺ T Cell Clone in NOD Mouse

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Immunology—II

Purpose: K^d-restricted CD8⁺ T cell clone the TGNFC8 previously described by Wong et al., (Nat., Med., 1999, 9, 1026-31) that recognize peptide derived from insulin β chain, (aa. 15-23) cause rapid (5-8 days) diabetes upon transfer into NOD mice. The purpose of the work was to define mechanisms of such rapid diabetes development.

Results: Experiments tracing fluorescently labeled TGNFC8 cells injected i/v into NOD mice showed its islet specific homing including their initial attachment to the walls of pancreatic blood vessels followed by extravasation and migration towards the center of the islet. Specific homing appears to depend on MHC class I-peptide complexes expression by pancreatic endothelium, since tracing of CD8⁺ cells in β 2m deficient animals did not show any significant islet infiltration within the same time period. TGNFC8 induced b cell death occurs by Fas-mediated mechanism, in contrast endothelial damage by TGNFC8 is most likely Fas-independent, as unaltered migration of labeled effectors in NOD mice bearing *lpr* mutation was observed.

Additionally, certain K^d expressing strains were found to be sensitive to the TGNFC8 diabetogenic challenge (DBA/2J and BALB/cJ), while another H2^d strain - B10.D2 was resistant. Moreover, resistance to diabetogenic CD8⁺ cells was present in (B10.D2XNOD) F1 mice, indicating that it is controlled by a dominant gene(s). Further genetic analysis of this phenomenon is discussed. The mechanism of resistance is not dependent on a lack of antigen expression, since isolated β cells from both sensitive and resistant strains were efficiently killed *in vitro* by CD8⁺ T cells. The fluorescent tracing experiments suggested that resistance is based on the failure of TGNFC8 cells to home into pancreatic islets.

Conclusions: Recognition of peptides derived from protein produced by b cells and most probably presented by endothelial cells of pancreatic microcapillary bed affects cytotoxic CD8⁺ cells homing to the pancreas and plays a significant role in progression of diabetes.

Investigation of the properties of an islet homing CD8⁺ T cell clone R1, which causes insulinitis

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Immunology—II

Mentor: Birgitte K. Michelsen

Purpose: I have previously isolated a cytotoxic GAD₆₅ specific CD8⁺ Vb8.2⁺ T cell clone R1 which produces large quantities of IFN γ . This clone home into the pancreatic islets within two days and accelerate the onset of insulinitis in NOD mice. We wish to investigate why the R1 clone traffics to the pancreas, how it initiates the insulinitic process, and to identify the earliest events taking place in the diabetogenic process.

Methods: We are using FACS analysis, ELISA and multiplex RT-PCR to study which molecules the R1 clone expresses. To further study which molecules the R1 clone induces in the pancreas when transferred to NOD mice we use RT-PCR and immuno-histochemistry. The GAD₆₅ specific non insulinitis-inducing sister clone, R7 (CD8⁺, Vb5⁺) will be used as a control. This clone is cytotoxic and produces large quantities of IFN γ just as R1, but is not reactive *in vivo*.

Results: When transferred to neonatal NOD mice R1 accelerates the onset of insulinitis as 5 week old R1 transferred NOD mice show considerable larger insulinitic infiltrates than do nontransferred control littermates. As stated earlier R1 produces large quantities of IFN γ but it does not produce IL4, IL10 or TNF α . Similar results have been obtained for R7. Preliminary results obtained through FACS analysis and multiplex RT-PCR have shown that R1 expresses IL2r, LFA-1, IL8, PSGL-1, SDF-1 α , CCR1, CCR2 and RANTES.

Conclusions: Since TNF α is a Th1 cytokine it was possible that R1 would express TNF α , however this was not the case. The expression of IL2r, LFA-1 and PSGL-1 corresponds well with what is normally expected for activated/memory T cells. Since IDDM is a Th1 mediated disease, the expression of IL-8 and SDF-1 α and RANTES is interesting, as these chemokines can push the immune response towards a Th1 response.

Differential Splicing of the IA-2 Gene in Pancreas and Lymphoid Organs as a Predisposing Factor to Autoimmune Responses against the IA-2 molecule in Type 1 Diabetes

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Immunology—II

Purpose: IA-2 is a 979 aa tyrosine phosphatase-like protein expressed in the secretory granules of pancreatic beta-cells, and an autoantigen in Type 1 diabetes. We have recently reported the pancreatic expression of an alternative spliced form of the IA-2 mRNA with a in frame deletion of exon 13, which codes for 73 aa (557-629) containing the trans-membrane domain. We investigated IA-2 mRNA splicing in lymphoid organs since these express IA-2 and other self-molecules (insulin, etc) with the likely purpose of inducing and maintaining self-tolerance.

Methods: IA-2 mRNA splicing was studied in human thymus, spleen, and pancreas (whole organ or purified islets) from 26 non-diabetic cases (11 fetal and 15 post-natal cases, age range: 21 gestational weeks-52 years, 11 females, 15 males) by RT-PCR.

Results: All pancreatic islet cell (n= 4) and whole pancreas samples (n= 5) expressed full-length IA-2 mRNA and 5/9 also expressed the -exon 13 variant. Nine of 15 thymi and 11/15 spleens expressed IA-2 message. Among these, all thymus (9/9)

and spleen (11/11) samples exclusively expressed the -exon 13 variant. We also detected another alternatively spliced IA-2 mRNA lacking 129 bp of exon 14. This results in the in frame deletion of 43 aa (653-695) in the intracellular domain. The -exon 14 variant was detected exclusively in 4/9 pancreatic samples and very weakly in 1/15 spleens.

Conclusions: We speculate that immunological tolerance to linear or conformational epitopes expressed by the full-length protein or the -exon 14 variant may not be achieved if these are never expressed in lymphoid organs. In contrast, their expression in the pancreas could generate cryptic epitopes unknown to the immune system. The exposure of such cryptic epitopes to the immune system may result in autoimmunity. Indeed, antibodies and T-cell responses against IA-2 epitopes associated with the juxta-membrane and trans-membrane domains (encoded by exons 13 and 14) have been reported and correlate with the development of diabetes. We suggest that differential tissue splicing of the IA-2 mRNA may act as a predisposing factor to the autoimmune responses to IA-2 associated with Type 1 diabetes.

Beta cells play a TNF- α dependent role in their own demise

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Group 6

Immunology—II

Mentor: Nora Sarvetnick

Purpose: To understand how cytokines influence insulinitis, it is critical to determine their effects on the assorted cell types comprising the lesion: the effector T cells, antigen-presenting cells, vascular endothelium, and target islet tissue. We found that mice transplanted with islets deficient in Fas, IFN- γ receptor, or inducible nitric oxide synthase had normal diabetes development. However, the specific lack of TNF- α receptor 1 (p55) afforded islets a profound protection from disease by altering the ability of islet-reactive, CD4⁺ T cells, to establish insulinitis and subsequently destroy islet cells. Protection from disease, however, was conditional, as normal diabetes ensued if the T cells had access to wild type islets even if they were located in discrete physical locations.

In this study we aim to determine the role of TNF- α receptor on the islets in the activation and reactivation of diabetogenic T cells.

Methods: *Mice:* BDC2.5 NOD, TNF- α receptor 1 (p55) deficient mice, C57Bl/6 and NOD.scid mice have all been previously described. Animals were housed and bred in a SPF facility at TSRI. *Reagents:* Anti-TNFSF-10 antibodies and TNFRSF-14-Fc were obtained from Drs. Hideo Yagita (Juntendo University School of Medicine, Tokyo) and Li Ping Chen (Mayo Clinic, Rochester MN) respectively.

Results: Using anti-TNFSF-10 antibodies and TNFRSF-14-Fc we demonstrate the upregulation of these TNF family members when BDC2.5 T cells are activated *in vitro*. Furthermore we demonstrate that the upregulation is dependent on the presence of the TNF- α receptor 1 (p55) on the islets. We are currently determining the role of these molecules in diabetes.

Conclusions: TNFSF-10 has previously shown to engage its receptor and induce apoptosis in immune cells. This indicates the critical role of TNF- α receptor 1 (p55) on the islets in beta cell destruction.

Chemokine expression by islet cells: a mechanism regulating inflammation in autoimmune diabetes

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Immunology—II

Mentor: David Lo and Matthias von Herrath

Purpose: This study explores the possibility that inflammatory chemokine responses of resident islet cells can play a role in the preferential recruitment of mononuclear leukocytes observed in diabetes.

Methods: The expression of C, CC, CXC, and CX3C chemokines was studied in four resident islet cell types (macrophages, fibroblasts, endothelial cells, and beta cells) after stimulation with various inflammatory mediators (endotoxin, TNF α , IL-1 β , IL-6, IFN γ , IFN α , IL-4, and IL-13). Expression patterns were determined using RNase protection assays and confirmed with reverse transcriptase polymerase chain reaction (RT-PCR). Additional analyses were performed using intact normal islet tissue stimulated *ex vivo* with endotoxin or IFN γ . Islet tissue from pre-diabetic NOD mice were analyzed similarly but without prior stimulation.

Results: Both CC and CXC chemokines are induced by a variety of stimuli in bone marrow derived macrophages, tissue

fibroblasts, and microvascular endothelium. Expression patterns vary between these cells and are dependant on the inflammatory stimuli, however all upregulate IP-10 and Mig in response to IFN γ . Interestingly, beta cell chemokine responses are extremely limited; only IP-10 is expressed in response to IFN γ , IL-1 β , or TNF α , and Mig is upregulated only by IFN γ . Examination of intact normal islet tissue following *ex vivo* stimulation with endotoxin demonstrates a pattern of chemokine upregulation consistent with fibroblast and endothelial responses, suggesting that these cells contribute most of this response. More importantly, previously unstimulated islet tissue from pre-diabetic NOD mice express IP-10 and Mig: a pattern consistent with IFN γ -stimulated normal islet tissue.

Conclusions: Our data suggest that IFN γ -induced upregulation of IP-10 and Mig occurs early in disease. Thus, islet cell regulation of chemokines suggests that they are not innocent bystanders of a destructive immune inflammatory response, but rather active participants able to selectively encourage a Th1 response.

Role of the class Ib HLA molecule HLA-G in the regulation of allogeneic immune responses to human pancreatic islets

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Immunology—II

High levels of a non-polymorphic class Ib HLA molecule, HLA-G, are selectively expressed by the fetal trophoblasts at the maternal-fetal interface and at sites of immune privilege such as the eye and the testis. Increasing evidence supports an immune-regulatory function of this HLA molecule at these sites. Thus, engagement of HLA-G by specialized killer inhibitory receptors (KIRs) expressed on NK cells and subsets of CD8⁺ T cells results in the inhibition of NK- and T cells cytolytic activity.

Recent studies demonstrate that maintenance of immune tolerance to autologous pancreatic islets as well as the establishment of tolerance to allogeneic islet transplants may involve immune privilege. These observations prompt us to investigate whether expression of HLA-G may play a role in this phenomenon in human islets.

Our work provides novel evidence that HLA-G is expressed in both human fetal and adult pancreatic islets. To investigate whether HLA-G presented by islet cells may function as an

immuno-modulatory molecule in allogeneic responses to this tissue, we performed mixed islet/lymphocyte reactions in the presence or absence of an anti-HLA-G blocking antibody. These experiments demonstrated that blockade of HLA-G on adult islets significantly enhances allogeneic proliferative immune responses to target islet cells and leads to the activation of a distinct CD3⁺CD8⁺ T cell population. These results provide direct evidence for a role of HLA-G in the down-regulation of allogeneic proliferative responses to pancreatic islets. Specifically recognition of HLA-G appears to restrain the activation of a CD8⁺ T cell subset. By enhancing HLA-G expression on islet cells, growth stimuli and/or mechanisms of cytokine-mediated immune deviation may contribute to the regulation of pancreatic islet antigenicity during transplantation. We propose that the non-polymorphic HLA-G molecule is an important mediator of immune regulatory mechanisms preventing the activation of auto- and/or allo-reactive T cells for the creation of immune privilege in certain autologous tissues, and the immunological acceptance of yet allogeneic fetal tissues during pregnancy.

Risk of pig endogenous retrovirus infection in xenotransplantation

Group 6

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Purpose: Xenotransplantation using pig organs or tissues may alleviate the human donor organ shortage. However one concern is the potential transmission of pig pathogens to humans, especially pig endogenous retroviruses (PERVs), which infect some human cell lines *in vitro*. In this paper, the cross-species *in vivo* transmission of PERV by xenotransplantation was studied using a severe combined immunodeficient (SCID) mouse model.

Methods: Twenty-one SCID mice were transplanted with fetal pig pancreatic cells for a period from three to 41 weeks before being sacrificed. Liver, spleen and brain samples were taken from these mice. DNA and RNA were extracted and examined for PERV using nested PCR and RT-PCR. The pig mitochondrial cytochrome oxidase II subunit gene (COII) was also amplified to monitor the presence of pig cell microchimerism in xenotransplanted tissues.

Results: Examination of 39 DNA samples from tissues of 21 xenografted mice identified two mice (M4 liver and M19 spleen) that were PCR positive for PERV and were negative for COII. A total of 59% of mouse tissues were positive for both PERV and COII, 16% were negative for both, and 20% were positive for COII only. Sequence analysis revealed that some of the PERV sequences in the mice varied significantly (around 15% variation) when compared to the PERV sequences in the pig donor cells. Negative RT-PCR result was obtained from three mouse samples including M4 liver and M19 spleen.

Conclusions: The PERV positive, COII negative results from the two mice indicated the presence of PERV transmission from pig cells to mouse tissue. The sequence variation detected in PERVs from mouse tissues suggested that different PERVs were transmitted from the pig cells to mouse tissues during xenotransplantation. However, the PERVs were not transcribing RNA at high levels in the mice, as indicated by negative RT-PCR results for PERV.

Expression of Gal α (1,3)Gal on porcine islet cells is age-dependent

Group 6

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Purpose: To examine the expression of Gal α (1,3)Gal (α Gal) on non-matured neonatal porcine islets (NPI), *in vitro* matured NPI, NPI grafts in SCID mice, and adult porcine islets.

Methods: Porcine islets were either dissociated into single cells or transplanted into non-diabetic SCID mice. Cells and tissues were then stained with Gal-specific IB4 lectin to detect the expression of α Gal, or antibodies to cytokeratin 7 (CK7) to detect the epithelial ductal islet precursor cells or insulin to detect the presence of β cells.

Results: Expression of α Gal on non-matured NPI cells is significantly higher ($19.7 \pm 3.3\%$, $n=14$, $p<0.01$) than *in vitro* matured NPI cells ($11.0 \pm 2.8\%$, $n=7$) and adult porcine islet cells ($5.1 \pm 1.3\%$, $n=6$). We also observed a significant decrease in CK7 expression between non-matured NPI ($28.4 \pm 4.2\%$, $n=14$, $p<0.01$), *in vitro* matured NPI ($10.5 \pm 1.8\%$, $n=9$), and adult porcine islet cells ($1.3 \pm 0.5\%$, $n=6$). In contrast, the proportion of insulin-positive cells increased significantly

($p<0.01$) with age, as non-matured NPI were composed of $33.7 \pm 3.8\%$ β cells versus $42.1 \pm 3.1\%$ and $82.8 \pm 1.2\%$ in *in vitro* matured NPI and adult porcine islets, respectively. α Gal expression on NPI grafts was less prominent in those grafts harvested at later times post-transplant. Similar findings were observed when these grafts were stained for CK7. To further investigate the association of α Gal and CK7 expression on non-matured NPI cells, we compared the proportion of CK7-positive cells in Gal-positive and -negative FACS sorted cells. Significantly more cells in the Gal-positive population were positive for CK7 ($24.4 \pm 10.4\%$, $n=7$, $p<0.01$) than Gal-negative cells ($1.8 \pm 0.7\%$, $n=9$). In contrast, higher percentage of Gal-negative cells are positive for insulin ($32.4 \pm 3.8\%$, $n=9$, $p<0.01$) compared to Gal-positive cells ($11.7 \pm 2.1\%$).

Conclusions: These data suggest that α Gal and CK7 expression is associated with less mature islet cells and disappear as these cells mature into insulin-producing cells, which may explain in part why in some studies α Gal on porcine endocrine cells can not be detected.

Macroencapsulation of neonatal porcine pancreatic cell clusters for transplantation

Group 6

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Purpose: The aim of this project is to study neonatal porcine pancreatic cell clusters (NPCCs) as potentially suitable tissue for transplantation. We are focused on macrocapsules made as flat discs using alginate hydrogel covered with a semipermeable membrane, their immune protection, and the optimal packing density of NPCCs.

Methods: NPCCs were isolated from 1-3 d. old Yorkshire pigs and cultured in serum-free modified Ham's F10. NPCCs embedded in calcium-alginate slabs (~3000 islet equivalents (IE)/ml of alginate) were cultured for 10 d. in medium with 10 % FCS. DNA and insulin content was measured. Discs (10 mm diameter) containing 2000, 4000 and 8000 IE in 100 µl of alginate were prepared. As the controls, 2000 IE suspended in the culture medium were used. MTT viability tests were performed after 1 wk culture. Empty discs produced from purified Pronova (high G), Monsanto (high M) and Kelco (intermediate G) alginates, covered with poly-l-lysine or pro-

tamine-heparin membrane, were transplanted for 2 wk into B6AF1 mice or Lewis rats intraperitoneally and subcutaneously.

Results: Culture of NPCCs in medium with serum resulted with 6 fold increase in their insulin/DNA ratio. Viability MTT tests showed that discs containing 2000 IE had the same optical density as controls, double for 4000 IE and only 2.5 fold higher for 8000 IE, which is about 60% of theoretical expected value. Generally, the cellular response to discs transplanted intraperitoneally was less severe than to transplanted subcutaneously. Preliminary results suggested that Kelco alginate is the most suitable for disc preparation.

Conclusions: Neonatal porcine pancreatic tissue matures in vitro providing insulin-producing cells for transplantation. A flat macrocapsule approach may have important advantages over the microencapsulation technique, because the possibility of graft removal in case of failure, which is an important issue in transplantation of encapsulated tissue.

Ongoing T cell activation in chronic rejection: Role of CD28-B7

Group 6

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Purpose: The pathophysiology on chronic renal allograft rejection remains poorly understood. We have already shown that blockade of the CD28-B7 T cell costimulatory pathway is capable of preventing the development of chronic rejection. Here we examine the ongoing role of T cell costimulatory activation in the progression of chronic rejection

Methods: The F344 to LEW renal transplant model of chronic rejection was used. The CD28-B7 pathway was blocked using CTLA4Ig (0.5mg ip) at either 8 or 16 weeks post-transplantation. Renal allograft function was assessed by following animals for survival and urinary protein excretion. Kidneys were harvested from representative animals at 16 and 24 weeks and along with non treated and isograft controls. Morphology, immunopathology and rt-PCR analysis of harvested kidneys was compared.

Results: Animals treated 8 weeks post transplantation did not develop progressive proteinuria when compared with controls (14.3±4.1 vs 41±12.0 mg/24 hrs p<0.05). Nor did these animals develop significant morphological changes when compared with controls at 24 weeks. There was also decreased cellular infiltration with T cells and macrophages in the treated group, with very low levels of Th1 cytokines (IL-2, IFN-γ & TNF-α) and higher levels of Th2 cytokines (IL-4 & IL10). In addition anti-apoptotic genes expression (Bcl-2 and Bcl-x) was increased in the CTLA4Ig treated group, and the pro-apoptotic genes (CPP-32 and Bad) decreased. The animals treated at 16 weeks with CTLA4Ig also showed significantly reduced disease on light microscopy of their kidneys.

Conclusions: This study indicates that the progression of chronic allograft dysfunction is T cell dependent, (at least in this model) and that strategies targeted at T cell costimulatory blockade may provide a novel approach to prevent and interrupt this.

Infection of human and mouse tissues by porcine endogenous retrovirus after pig islet xenotransplantation

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Purpose: The purpose of our study was to determine the risk of infecting human cells by porcine endogenous retroviruses (PERV) after pig islet xenotransplantation. Islet transplantation is a promising new approach for treatment of diabetes mellitus, however, the number of potential human pancreas donors will never provide enough islet tissue to treat the millions of diabetic patients worldwide. Xenotransplantation of pig islets might help overcome this shortage. However, the promise of xenotransplantation is offset by the public health risk of a cross-species infection. All strains of pig contain multiple copies of PERV which have been shown to infect human cell lines in culture. Thus, if xenotransplantation of pig tissues results in PERV viral replication, there is a risk of spreading this retrovirus to the human host.

Methods: The potential of adult pig islets to produce infectious PERV was determined using in vitro coculture assays with the human kidney epithelial cell line U293 as target. Infection was detected by PCR for viral-specific DNA sequences, RT-PCR for viral (subgenomic) mRNA, quanti-

tative RT assay and immunohistochemistry. Using immunodeficient NOD/SCID mice as a model for immunosuppressed human patients we studied the risk of PERV infection after pig islet xenotransplantation. For this we combined transplantation of pig islets and human target cells after which PERV infection of human and mouse tissues was determined.

Results: Pig islets were shown to release infectious PERV virus which can productively infect U293 *in vitro*. After transplantation in NOD/SCID mice PERV mRNA and p30 gag expression was observed in islet cells. Moreover, we demonstrated productive PERV infection of transplanted human cells as well as infection of mouse cells in multiple tissue compartments. Furthermore, extensive chimerism of pig cells was observed in mice after transplantation, indicating that the exposure to PERV will exceed the transplant site.

Conclusions: Pig islet xenotransplantation to human patients can result in long-term exposure to replication competent endogenous retrovirus. Therefore, a concern for PERV infection associated with xenotransplantation in human patients is justified.

Differential effects of TNF α expression under control the TTA-system in pancreatic β -cells on the incidence of Type 1 diabetes in RIP-LCMV-mice

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Immunology—II

The role of TNF α in type I diabetes pathogenesis is controversial. Results obtained with NOD mice or other models point towards a dual role, whereby the exact time of TNF α expression appeared important. Therefore, we generated double transgenic mice expressing (i) the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV) under the control of a rat insulin promoter (RIP) and (ii) TNF α under control of a tetracycline regulated promoter system (tTA) in the pancreatic β -cells. Using these RIP-GP/tet-TNF mice we can first, induce diabetes by LCMV infection and second, switch on TNF α expression specifically in β -cells by removal of doxycycline (Dox) from the diet at precise times before, during and after triggering of diabetes. Kinetic studies by

RNase protection assays revealed TNF α mRNA in 50% of uninfected mice as early as 4 days after removal of Dox; at day 14 TNF α mRNA was upregulated by a factor of 4 in 85% of mice. TNF α expression was restricted to the islets of Langerhans. Diabetes incidence was reduced by 80% when TNF α was induced 14 days before or 10 days after viral infection. In contrast, when induced at the time of infection the incidence was slightly increased. Furthermore, when Dox was removed 14 days post-infection 50% of those mice that were diabetic at days 14 and 21 post-infection recovered in the following weeks. Our data show completely opposite effects of TNF α on the incidence of type 1 diabetes depending of the time of its expression.

Generation and modulation of diabetogenic T cells in DR-BB rat adult thymus organcultures (ATOC)

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Immunology—II

Purpose: We have shown that cultured diabetes-resistant (DR) and diabetes-prone (DP) BB rat thymi, respectively, recapitulate the developmental kinetics and the normal and abnormal T cell phenotypes observed in these animals *in vivo*. The aims of the present studies were to determine whether DR-BB rat adult thymic organ cultures (ATOC) can generate functional diabetogenic T cells, and to determine if the addition of candidate autoantigens to DR-BB rat ATOC would modify the generation of diabetogenic T cells.

Methods: DR-BB rat ATOC were cultured in the presence or absence of candidate autoantigens. Diabetogenic T cell function was assessed by measuring the ability of cells from the ATOC to adoptively transfer diabetes into histocompatible athymic recipients. Recipients were treated with anti-RT6 antibody to deplete regulatory T cells and with poly I:C to stimulate the RT6– diabetogenic cells.

Results: We found that cells from DR-BB rat ATOC adoptively transferred autoimmune diabetes to athymic recipients. Co-culture with pancreatic islets, but not thyrocytes, abrogated the ability of cells from DR-BB rat ATOC to transfer diabetes. Metabolically active islets were not required, and streptozotocin (STZ) treated islets with very low insulin content also abrogated the adoptive transfer of diabetes. Histologic analysis of pancreata revealed little insulinitis in recipients of cells from ATOC co-cultured with islets.

Conclusions: Diabetogenic T cells are generated in DR-BB rat ATOC, and are functionally silenced by exposure to pancreatic islets but not to thyrocytes. We interpret these data to suggest that tissue specific tolerance can be induced *in vitro* in DR-BB rat ATOC. Successful tolerance induction by insulin-depleted STZ-treated islets suggests that islet antigens other than insulin may be able to induce tolerance in the BB rat ATOC system. We plan to use the ATOC system to screen for candidate initiating autoantigens in the BB rat model of human type 1 diabetes.

CD8 T cells in the pathogenesis of type 1 diabetes in the NOD mouse

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Immunology—II

Purpose: The NOD (Non-Obese Diabetic) mouse is a model for human type 1 diabetes in which there is lymphocyte infiltration into the islets of Langerhans and later destruction of islet cells leading to glycosuria and diabetes. Cloned CD8 T cells, G9C8, from the lymphocytic infiltrate in the pancreatic islets of Langerhans of young NOD mice can cause diabetes in 5 days upon adoptive transfer into irradiated NOD mice. The identification of the autoantigen recognised by the cells was of considerable importance as it may then be possible to use the information to learn how to tolerise these cells.

Methods: Using an expression cloning system and indicator hybrid cells generated from the cloned CD8 T cells, an islet cDNA library was screened.

Results: We identified the antigen to be a peptide of preproinsulin 1, restricted by H-2K^d. This was confirmed to be a 9-mer in the insulin B chain by screening all the possible peptides in the preproinsulin 1 sequence. The cloned CD8 T cells proliferated in response to the peptide and produced interferon- γ and killed target cells coated with the peptide in a ⁵¹Chromium release assay.

To assess the frequency of cells recognising this peptide specificity, we used tetrameric insulin peptide-K^d complexes and showed that a large proportion of the cells in the early infiltrate in NOD mice consists of cells which recognise this complex and this decreases with age. This is of particular interest as the cloned T cells were generated from the infiltrate of young mice, and may represent a population of cells involved in the early events in pathogenesis of disease.

Preliminary studies have shown that the peptide binds with low affinity to the MHC and that mutant peptides bind with greater affinity and stimulate the cells considerably better.

Conclusions: This is the first identification of an autoantigen recognised by a pathogenic, islet reactive CD8 T cell clone. Moreover, our data suggests that insulin is recognised as an antigen early in disease and reaffirms the importance of insulin as an autoantigen in type 1 diabetes.

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