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Francis

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CURRICULUM VITAE

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INTERNATIONAL PUBLICATIONS

1: Descamps FJ, Martens E, Proost P, Starckx S, Van den Steen PE, Van Damme J, Opdenakker G. Gelatinase B/matrix metalloproteinase-9 provokes cataract by cleaving lens
betaB1 crystallin. FASEB J. 2005;19:29-35.

2: Descamps FJ, Martens E, Ballaux F, Geboes K, Opdenakker G. In vivo activation of gelatinase B/MMP-9 by trypsin in acute pancreatitis is a permissive factor in streptozotocin-induced diabetes. J Pathol. 2004;204:555-61.

3: Abu El-Asrar AM, Struyf S, Descamps FJ, Al-Obeidan SA, Proost P, Van Damme J, Opdenakker G, Geboes K. Chemokines and gelatinases in the aqueous humor of patients with active
uveitis. Am J Ophthalmol. 2004 ;138:401-11.

4: Descamps FJ, Van den Steen PE, Nelissen I, Van Damme J, Opdenakker G. Remnant epitopes generate autoimmunity: from rheumatoid arthritis and multiple sclerosis to diabetes. Adv Exp Med Biol. 2003;535:69-77. Review.

5: Descamps FJ, Van den Steen PE, Martens E, Ballaux F, Geboes K, Opdenakker G. Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin. FASEB J. 2003;17:887-9. Epub 2003 Mar 05.

6: Descamps FJ, Martens E, Opdenakker G. Analysis of gelatinases in complex biological fluids and tissue extracts. Lab Invest. 2002;82:1607-8.

7: Descamps FJ, Martens E, Kangave D, Struyf S, Geboes K, Opdenakker G, Abu-El-Asrar A. Involvement of active gelatinase B/matrix metalloproteinase-9 in vitreous hemorrhagic transformation of proliferative diabetic retinopathy. Submitted.

ABBREVIATIONS

2D-GE	Two-dimensional gel electrophoresis
AGE	Advanced glycation end products
ANOVA	Analysis of variance
APMA	p-aminophenylmercuric acid
ATP	Adenosine triphosphate
CD	Cluster of differentiation
DCCT	The Diabetes Control and Complications Trial
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FADD	FAS-associating death domain-containing protein
GABA	γ-amino butyric acid
GAD	Glutamic acid decarboxylase
GCP-	Granulocyte chemotactic protein
HbA_{1c}	Glycated hemoglobin
HLA	Human leukocyte antigen
IA-2	Islet-antigen-2 (tyrosine phosphatase)
IDDM	Insulin-dependent diabetes mellitus
IFN-	Interferon-
Ig	Immunoglobulin
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit related protein
IL-	Interleukin-
IU/L	International units/Litre
kDa	Kilodalton
LPS	Lipopolysaccharide
m/z	Mass over charge
MALDI-TOF(-TOF) MS	Matrix-assisted laser desorption/ionization-time of flight – (time of flight) mass spectrometry
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MLD-STZ	Multiple low doses of streptozotocin
MMP-	Matrix metalloproteinase-
MS	Multiple sclerosis
NADH	Nicotinamide adenine- dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NeuroD	Neurogenic differentiation factor
NFκB	Nuclear factor κB

NGAL	Neutrophil gelatinase B-associated lipocalin
NIDDM	Non-insulin-dependent diabetes mellitus
NK	Natural killer
NO	Nitric oxide
NOD	Non-obese diabetic
PBS	Phosphate buffered saline
PDR	Proliferative diabetic retinopathy
PVR	Proliferative vitreoretinopathy
PKC	Protein kinase C
PLP	Proteolipid protein
RA	Rheumatoid arthritis
RAGE	Advanced glycation end products-specific receptor
RD	Retinal detachment
REGA	Remnant epitopes generate autoimmunity
rhMMP-9	Recombinant human MMP-9
rmMMP-9	Recombinant mouse MMP-9
RP-HPLC	Reverse phase-high performance liquid chromatography
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STZ	Streptozotocin
Th	T helper
TIMP	Tissue inhibitor of MMPs
TMB	Tetramethylbenzidine
TNF-	Tumor necrosis factor-
t-PA	Tissue-type plasminogen activator
UKPDS	UK Prospective Diabetes Study
VEGF	Vascular endothelial growth factor
VNTR	Variable number of tandem repeats

1 Diabetes mellitus

Diabetes mellitus is characterized by improper production or use of insulin. Insulin is produced by β-cells in the pancreatic islets of Langerhans and moves glucose into insulin-dependent cells (muscle cells, adipocytes, fibroblasts) as a sugar source of energy and for metabolism. Sugars are absorbed as monosaccharides from the intestine into the bloodstream after a meal. Blood glucose levels are sensed by pancreatic β-cells that proportionally secrete insulin. The binding of insulin to cell receptors activates mechanisms designed to absorb glucose into the cell.

Diabetes is subdivided in two major types. Type 1 diabetes occurs in 10% of diabetic patients and typically affects children and young adults (juvenile diabetes). In type 1 diabetes, the pancreas does not produce insulin since β-cells have been destructed by autoimmune reactions. Insulin must be administered via subcutaneous injection and therefore, this type of disease is also termed insulin-dependent diabetes mellitus (IDDM). In type 2 diabetes (80% of diabetic patients), cells are sluggish to respond to insulin (insulin resistance). This type of diabetes usually develops in middle age or later (adult onset diabetes) and symptoms often have a gradual onset. Insulin levels in these patients are usually normal or higher than average. Patients with type 2 diabetes are principally independent on insulin administration and the disease is therefore also termed non-insulin-dependent diabetes mellitus (NIDDM).

Both types of diabetes result in elevated blood glucose levels (hyperglycaemia), which may result in long-term complications.

1.1 Pathogenesis of type 1 diabetes

The overall progression of type 1 diabetes, as well as the polygenic and environmental influences that condition it, are well modeled in the non-obese diabetic (NOD) mouse. NOD mice spontaneously develop diabetes, due to genetic predisposition. In young NOD mice of less than 3 weeks, infiltration of islets with inflammatory cells is not observed. Loss of tolerance to islets antigens occurs after the age of 3 weeks and manifests by the massive influx of autoreactive T cells into the islet environment (insulitis). Insulin, glutamic acid decarboxylase (GAD), tyrosine phosphatase (IA-2) and islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) are among the most studied

islet autoantigens to which loss of tolerance occurs in type 1 diabetes. In spite of this massive T cell influx, insulitis is a relatively balanced condition since β-cells remain unaffected and glucose homeostasis is maintained. Some form of suppressor or regulatory cell populations may play a protective role here. Transition across the insulitis stage means that negative control mechanisms are perturbated, or that pathogenic autoimmune T cells have acquired aggressive effector functions. At this time point, insulitis becomes terminally aggressive and overt diabetes develops.

1.2 Genetic factors

Type 1 diabetes is a multifactorial disease associated with a strong genetic predisposition [22]. About 18 regions within the genome have been linked with type 1 diabetes susceptibility [58] and have been labelled IDDM1 to IDDM18. Over half of the inherited predisposition to type 1 diabetes maps to the region of chromosome 6 that contains the highly polymorphic human lymphocyte antigen (HLA) genes, which determine immune responsiveness [57;74;85]. Other susceptibility loci include genes that encode autoantigens like *INS* (encoding insulin)[9] and *GAD2* (GAD)[80] or corresponding transcription regulators (Neurod, a transcription regulator of the *INS*)[72], genes that encode immune response regulators like *IL12B* (interleukin-12 p40 subunit)[30], genes that encode apoptosis-related proteins like *FADD* (FAS-associating death domain-containing protein)[58] and genes that protect against agents that are toxic for β-cells (*SOD2*, encoding mitochondrial superoxide dismutase, which metabolizes harmful oxygen free radicals)[58]. All of these genes may play a role in the die-off or protection of pancreatic β-cells.

1.3 Environmental factors

Type 1 diabetes is associated with a strong genetic predisposition but only a small proportion (less than 10 %) of genetically susceptible individuals progresses to clinical disease. Concordance rates of type 1 diabetes in monozygotic twins are only 25 to 60% [42;45] and migration studies support a role for environmental agents in different geographical areas [64]. Viruses, dietary components, bacterial infections and drugs/toxins have all been considered as possible environmental triggers.

1.3.1 Dietary components

In NOD mice, the onset of insulitis coincides with weaning. At that time, the immune system is confronted with a novel array of antigens, originating from major shifts in food intake and intestinal flora. Oral antigen feeding typically results in tolerance induction [69], however, in particular circumstances this mode of immunization promotes the activation of cytotoxic autoreactive T cells [13]. In humans, accumulating data indicate that dysregulation of the gut immune system may play a fundamental role in the induction of type 1 diabetes. Triggering of mucosal immunity, such as by early introduction of cow milk formulas in infancy, may increase the risk of type 1 diabetes [4]. Especially for dietary insulin, it has been shown that primary immunization occurs in the gut by exposure to bovine insulin [76].

1.3.2 Viruses

Obviously, viruses that have an infectious tropism for β-cells have a direct effect on β-cell death. Alternatively, pancreatic β-cells are indirectly affected. On the one hand, the explicit cytokine profile of an antiviral response (IL1-β, TNF-α, IFN-γ) has a direct deleterious effect on *in vitro* cultured β-cells (bystander death) [15;65]. On the other hand, the pro-inflammatory cytokine milieu might improve antigen uptake, antigen processing and subsequent presentation of self-antigens by antigen-presenting cells and induce the overexpression of co-stimulatory molecules. This lowers the threshold for activation of autoreactive T cells. Furthermore, the release of sequestered self-antigens during virus-induced destruction of β-cells might provoke that antigens, which used to be ignored by autoreactive T cells, enter antigen-presentation pathways, favouring autoreactive T cell activation [52;53;81]. An initial immune response against viral proteins sharing similarity with self-proteins is believed to trigger autoreactive T and B cells, which may then destroy self-tissue, even after clearance of the viral infection. Perhaps the most striking sequence homology (also called “molecular mimicry”) is the one between the noncapsid protein P2-C of Coxsackie B virus and the islet autoantigen glutamic acid decarboxylase (GAD)[35].

1.3.3 Toxic compounds

Particular compounds, like the glucose analogue streptozotocin (STZ), are specifically toxic for β-cells. Intraperitoneal administration of STZ in mice or rats is widely used as an experimental model for diabetes. A single high dose of STZ

predominantly induces necrosis of β-cells, while multiple low doses of STZ (MLD-STZ) induce β-cell apoptosis and autoimmunity. For further information on mechanisms of necrosis induced by STZ, see **chapter 3**.

1.4 Players of the immune system

Within a specific genetic background and environmental setting, autoimmune diseases develop on the basis of dysregulation of fine balances of the immune system. These balances are provided by cells and molecules. We first describe the cellular players and then discuss a number of effector molecules, with special attention for those that are crucial within the framework of the presented research.

1.4.1 Cellular players of the immune system

1.4.1.1 Neutrophils, major players in innate immunity

Initially, the induction of inflammatory processes, including autoimmune processes, requires the participation of the innate immune system. This involves the binding of innate immunity players (neutrophils, NK cells, dendritic cells, macrophages) to well-defined molecular patterns, expressed on infectious agents, eliciting their activation. Neutrophils represent 50 to 60% of peripheral white blood cells. In addition to phagocytosis of infectious agents, which may involve killing and degradation, neutrophils produce inflammatory mediators and discharge the contents of their granules in the extracellular milieu. Doing so, they play important roles in the instigation of inflammation and ensuing adaptive immunity. In subjects at high risk of type 1 diabetes, IL-12 production by lipopolysacharide (LPS)-stimulated neutrophils was shown to be enhanced [31]. Increased IL-12 production amplifies the differentiation of naïve T helper cells (Th0) into Th1 cells, which are believed to be responsible for islet β-cell destruction in type 1 diabetes (*vide infra*). Furthermore, LPS has been shown to induce rapid release of gelatinase B from neutrophils [54]. The possible functions of gelatinase B as an inducer of autoimmunity will be discussed in **section 1.5**.

1.4.1.2 T and B lymphocytes, players of adaptive immunity

Autoimmune diseases are defined by the fact that a specific adaptive immune response evolves against own antigens. Both T and B lymphocytes play a role in islet β-cell insulitis and diabetes.

T lymphocytes Adoptive transfer experiments reveal that NOD mice-derived T helper cells ($CD4^+$) are sufficient to transfer diabetes to severe combined immunodeficient NOD mice (NOD.SCID), whereas cytotoxic T cells ($CD8^+$) can transfer insulitis, but not overt diabetes [18].
In recent years, the T helper cell population has been diversified into two subsets, Th1 and Th2, on the basis of functions and profiles of cytokine production. Th1-mediated immune responses include activation of cytotoxic T cells, NK cells, and delayed type of hypersensitivity reactions, whereas Th2 cells are principal stimulators of humoral immunity (antibody-producing B lymphocytes). It is now well established that especially the Th1 subset plays a pivotal role in islet β-cell destruction [33;44;70]. For example, administration of an antagonist against IL-12, a key cytokine driving Th1 cell development, results in a deviation of pancreas-infiltrating $CD4^+$ cells to the Th2 phenotype and reduction of spontaneous type 1 diabetes [75]. Th2 and Th1 responses may indeed inversely regulate each other, which has been exploited for immunostimulation therapies (triggering the Th2 subset) in the prevention of diabetes [20;73].
In apparent conflict with adoptive transfer experiments, which indicate either T helper or cytotoxic T cells alone to be sufficient for initiation of insulitis, other studies claim that cytotoxic T cells are essential. For example, depletion of cytotoxic T cells by treatment with anti-CD8 antibodies during an early stage of the disease was effective to protect against insulitis [82]. Along the same line, β2 microglobulin-deficient NOD mice, without functional MHC class I-molecules, do not develop insulitis nor diabetes [71]. Based upon these findings, it was hypothesized that cytotoxic T cells are required to infiltrate into the pancreatic β-cell islets and cause cell injury, which then leads to the priming of T helper cells.

B lymphocytes Autoantibodies do not seem to be sufficient to transfer diabetes directly. However, the detection of autoantibodies to insulin, GAD and other islet antigens indicates loss of tolerance in the B cell compartment and heralds the onset of the autoimmune process. Selective removal of B cells by genetic or serological techniques demonstrates an essential role for B cells in the development of both insulitis and diabetes in NOD mice, hypothetically through

their functioning as antigen-presenting cells [3]. It has been shown that B lymphocytes in the pancreas show a striking resemblance to B1 cell subsets populating the peritoneal cavity. Elimination of intraperitoneal B cells through hypotonic lysis indeed results in a redistribution of B1 cells away from insulitis lesions and at the same time, a delay of diabetes onset [43].

1.4.2 Molecular effector mechanisms

Molecular effector mechanisms may be operative at several phases in disease development, either in initial phases, e.g. the induction of loss of tolerance towards islets antigens, or in late phases -the ultimate killing of β-cells.
The major histocompatibility complex alleles of an individual decide whether particular epitopes are presented to autoreactive T cells or not. Accordingly, MHC proteins may be crucial for loss of tolerance towards specific autoantigens in autoimmune diseases.
Activated autoreactive Th1 cells may directly kill the β-cells through Fas/Fas ligand interactions, or kill via production of soluble mediators that induce β-cell death or through activation of cytocidal functions of macrophages in the vicinity. The nature of soluble mediators involved is controversial: IFN-γ, IL-1, TNF-α, IL-6 and NO. Molecular effector mechanisms of cytotoxic T cells include the perforin/granzyme, Fas/FasL and TNF/TNF-RII pathways.
Also, particular combinations of cytokines may directly induce β-cell death. Other cytokines, like IL-12 are crucial for directing the autoimmune response towards the Th1 phenotype [31].

1.5 Integration of extracellular proteolysis in models of diabetes

In the current research project on diabetes, we explore an innovative concept for autoimmune diseases, the REGA model, defining a mechanism how autoantigens may enter antigen-presentation pathways. In particular, the model indicates extracellular proteolysis of autoantigens to be crucial in these events. Gelatinase B or matrix metalloproteinase-9 (MMP-9) is incorporated in the model as a central extracellular proteinase.

Gelatinase B or MMP-9 is a member of the family of matrix metalloproteinases (MMPs). This family represents more than 20 proteolytic enzymes that contain a zinc ion in the active site and degrade components of the extracellular matrix. Gelatinase B is supposed to be involved in a number of physiological processes like embryonic development, leukocytosis, migration of leukocytes through the

extracellular matrix, angiogenesis and wound healing. These processes require fine-tuned proteolysis and accordingly, gelatinase B activity is tightly regulated, not only at the transcriptional level, but also posttranslationally. A first regulatory mechanism is the fact that gelatinase B is released in the extracellular milieu as an inactive 92 kDa precursor. Activation is often the final step of a proteolytic cascade [25;60] but eventually involves the removal of the amino-terminal pro-peptide, resulting in 82 kDa active gelatinase B. The removal of the pro-peptide leads to disruption of a coordination bond formed between a highly conserved unpaired cysteine in the pro-peptide and the zinc ion at the active site, which is denominated 'cysteine switch'[67]. A second regulatory mechanism is the binding of endogenous inhibitors, e.g. the family of tissue inhibitors of metalloproteinases (TIMPs) [79]. TIMP-1 efficiently binds gelatinase B and as such not only inhibits the activity of gelatinase B but also controls the activation of the pro-enzyme. Together, the environmental setting, which on the one hand is composed of proteases involved in a gelatinase B-activating cascade and on the other hand includes TIMP-1 or other endogenous gelatinase B-inhibitors, decides on the net gelatinase B activity. Furthermore, active gelatinase B has a high turn-over rate due to autocatalysis, which may be considered a negative feedback mechanism that controls gelatinase B activity [36].

In spite of its tight regulation, gelatinase B is involved in several pathological conditions, including cancer metastasis, sepsis and autoimmunity.

In autoimmunity, gelatinase B may have a dual function. First, it may be involved in the breakdown of *physical barriers* [48] that hide autoantigens in immunoprivileged organs like the brain, the eye and the testis. For example, autoantigens that are characteristic for MS like myelin basic protein (MBP) and proteolipid protein (PLP) reside behind the blood-brain barrier. Gelatinase B was shown to be involved in the degradation of the blood-brain barrier, which may allow autoreactive T cells to meet with their cognate presented antigens [48].
Second, gelatinase B may be involved in the breakdown of *molecular barriers*, defined by a protein's (autoantigen's) three-dimensional structure that may hide immunodominant epitopes. The implications of autoantigen breakdown through extracellular proteolysis are formulated in the so-called REGA model, in full Remnant Epitopes Generate Autoimmunity [55]. According to this model, remnant fragments that result from extracellular proteolysis of autoantigens may enter classical antigen-presentation pathways or alternatively, when they

constitute short immunodominant peptides, be extracellularly loaded on peptide-receptive MHC class II molecules. In both cases, this may favour autoreactive T cell activation.

In support of the REGA model, gelatinase B presents abundantly in MS lesions and cleaves the autoantigen MBP. Remnant peptides are encephalitogenic [59] and gelatinase B deficient mice are protected against experimental autoimmune encephalitis [26]. In rheumatoid arthritis, gelatinase B cleaves the collagen type II autoantigen after partial unwinding of the tightly folded triple helical structure by a single collagenase-catalysed cleavage, and generates more than 30 peptides including intact immunodominant epitopes [77;78]. **Chapter 6** reviews previously published data on the REGA model with regard to multiple sclerosis, rheumatoid arthritis and diabetes. The latter pathology is indeed the subject of the presented doctoral research.

2 Long-term complications of diabetes

The evolution of numerous long-term complications of diabetes mellitus correlates well, in most cases, with the severity and duration of hyperglycaemia. This has been convincingly demonstrated for both type 1 and type 2 diabetes [The Diabetes Control and Complications Trial (DCCT) Research Group, 1993; UK Prospective Diabetes Study (UKPDS) Group, 1998]. In the DCCT, every percentage increase in glycated hemoglobin (HbA_{1c}) was shown to be associated with an increase in the relative risk of developing complications. As accumulated from the data of the UKPDS, for each percent increase in HbA_{1c}, the relative risk of myocardial infarction increases by 18% and that of microvascular complications by 35%. Long-term complications in diabetic patients predominantly comprise macro- and microangiopathy. Here, I would like to focus on microangiopathy since it is principally relevant to the presented research in further paragraphs.

Microangiopathy includes retinopathy, nephropathy and neuropathy. It is histopathologically hallmarked by the thickening of capillary basement membranes and narrowing of the microvascular lumen, which subsequently induces occlusive angiopathy, tissue hypoxia and damage [32;47]. Biochemically, the progression of microangiopathy may be explained by an increased metabolic flux through the polyol pathway, the generation of advanced glycation end-products (AGE), the generation of reactive oxygen species and the inadequate regulation of protein kinase C activity (PKC).

The polyol pathway (sorbitol pathway) is an alternative glucose metabolising pathway that is particularly engaged under high glucose concentrations. As it uses reduced nicotinamide adenine dinucleotide phosphate (NADPH), it deprives the pathway for generation of glutathione (a powerful antioxidant) from NADPH. Therefore, an increased flow of metabolites through the sorbitol pathway may tilt the delicate balance of oxidants/antioxidants to the oxidative side. The synthesis of nitric oxide (NO) from arginine is another reaction that uses NADPH. NO is the key vasodilator in the microcirculation. Therefore, a shift in coenzyme availability might decrease NO synthesis and promote vasoconstriction and poor blood supply. Furthermore, downstream catabolism of sorbitol by sorbitol dehydrogenase yields NADH, which is sensed as ‘pseudohypoxia’.

Glycation of proteins was first discovered by Maillard in 1912. It is defined by a non-enzymatic reaction of sugars with a wide range of proteins to form early

glycation products that may react further, through a complex series of rearrangements and oxidative reactions, into various reactive species, collectively named advanced glycation end-products (AGEs)[1]. In humans, the formation of early glycation products was first demonstrated for hemoglobin. Currently, glycated hemoglobin (Hb_{1c}) is measured in patients to monitor disease development. [68]. Advanced glycation may affect virtually any protein [34]. In particular, AGEs may accumulate *in vivo* on vascular wall collagen and basement membranes as a function of blood glucose levels and form intermolecular bonds between collagen molecules [50], turning collagen fibers less elastic. Plasma proteins such as albumin, low-density lipoprotein and immunoglobulin G may get trapped in the subendothelium by covalently cross-linking to AGEs on collagen [14], which may further contribute to basement membrane thickening and luminal narrowing. Furthermore, AGEs may bind to cells in a receptor (RAGE)-mediated manner. Likewise, it has been shown that AGEs bind to endothelial cells, where they activate the free radical-sensitive transcription factor NFκB, a multifaceted coordinator of numerous "response-to-injury" genes [11]. These AGE-induced changes are involved in the modification of thrombomodulin and tissue factor production, favouring thrombus formation at sites of extracellular AGE accumulation [83].

Another adverse effect of hyperglycaemia is the modification of protein kinase C (PKC) activity through hyperglycaemia-induced increases in diacylglycerol. PKC regulates various vascular functions by modulating enzymatic activities, such as cytosolic phospholipase A_2 and Na^+/K^+-ATPase, or gene expressions e.g. of extracellular matrix components and intracellular contractile proteins. Changes in renal and retinal blood flow, contractility, permeability and cell proliferation are some of the resulting vascular abnormalities when PKC activity is poorly regulated.

[1] Incidentally, a similar reaction, though more complete and produced by harsher conditions, occurs between sugars and proteins in food- the final result being what is observed in bread or piecrusts, for instance.

AIMS

The general aim in the present doctoral research is to evaluate if gelatinase B is an effector molecule in the etiology of diabetes and its long-term complications.

- First, we would like to evaluate the applicability of the REGA model for the pathogenesis of diabetes (**Chapter 2**). We will test important autoantigens in diabetes for their susceptibility to cleavage by gelatinase B and see whether immunodominant epitopes are liberated. For assessments of gelatinase B in the pancreas, it will be a challenge to overcome problems with conventional zymography analysis that originate from abundant amounts of interfering neutral proteinases in that particular organ (**Chapter 1**). Then, we will perform gelatinase B assessments in pancreas extracts from mice with streptozotocin-induced diabetes. We will evaluate whether the expression of gelatinase B is enhanced in comparison with healthy mice and special attention will be given to the activation status of the enzyme. Gelatinase B knockout mice will be compared to wildtype mice. The disease status of the pancreas at particular time points will be investigated by histochemistry and biochemical analyses (**Chapter 3**).
- We would like to find out whether gelatinase B plays a role in proliferative diabetic retinopathy, a microangiopathic complication of diabetes. To that end, we will analyze vitreous fluids of diabetic patients with this diabetic complication (**Chapter 4**).
- Several groups have previously reported on the presence of gelatinase B in ocular fluids of diabetic patients and patients with specific ocular diseases. In this regard, we will perform a global analysis of ocular proteins for their susceptibility to cleavage by gelatinase B to evaluate what is the potential impact of gelatinase B presence in the eye (**Chapter 5**).

ARTICLES

Chapter 1 Analysis of gelatinases in complex biological fluids and tissue extracts.

Authors: Francis J. Descamps, Erik Martens and Ghislain Opdenakker.

Chapter 2 Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin.

Authors: Francis J. Descamps, Philippe E. Van den Steen, Erik Martens, Florence Ballaux, Karel Geboes and Ghislain Opdenakker.

Chapter 3 In vivo activation of gelatinase B/MMP-9 by trypsin in acute pancreatitis is a permissive factor in streptozotocin-induced diabetes.

Authors: Francis J. Descamps, Erik Martens, Florence Ballaux, Karel Geboes and Ghislain Opdenakker.

Chapter 4 Active gelatinase B is involved in vitreous hemorrhagic transformation of proliferative diabetic retinopathy.

Authors: Francis J. Descamps, Erik Martens, Dustan Kangave, Sofie Struyf, Karel Geboes, Ghislain Opdenakker and Ahmed Abu-El-Asrar.

Article submitted.

Chapter 5 Gelatinase B/matrix metalloproteinase-9 provokes cataract by cleaving lens betaB1 crystallin.

Authors: Francis J. Descamps, Erik Martens, Paul Proost, Sofie Starckx, Philippe E. Van den Steen, Jo van Damme and Ghislain Opdenakker.

Chapter 6 Remnant epitopes generate autoimmunity: from rheumatoid arthritis and multiple sclerosis to diabetes.

Authors: Francis J. Descamps, Philippe E. Van den Steen, Inge Nelissen and Ghislain Opdenakker.

REMNANT EPITOPES GENERATE AUTOIMMUNITY: FROM RHEUMATOID ARTHRITIS AND MULTIPLE SCLEROSIS TO DIABETES

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1. ABSTRACT

Autoimmune diseases are characterized by inflammation and by the development and maintenance of antibodies and T lymphocytes against "self" antigens. Although the etiology of these diseases is unknown, they have a number of cellular and molecular mechanisms in common. Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), are upregulated and activate the inflammatory process. Chemokines recruit and activate leukocytes to release proteases, including matrix metalloproteinases (MMPs). These proteases degrade proteins into remnant fragments, which often constitute immunodominant epitopes. Either by direct loading into major histocompatibility complex (MHC) molecules or after classical antigen uptake, processing and MHC presentation, these remnant epitopes are presented to autoreactive T lymphocytes. Also, posttranslationally modified remnant peptides may stimulate B cells to produce autoantibodies. This forms the basis of the "Remnant Epitopes Generate Autoimmunity" (REGA) model. We have documented evidences for this model in multiple sclerosis (MS), rheumatoid arthritis (RA) and diabetes, which are summarized here. Furthermore, three topics will be addressed to illustrate the importance of glycobiology in the pathogenesis of autoimmune diseases. In MS, gelatinase B or MMP-9 is a pathogenic glycoprotein of which the sugars contribute to the interactions with the tissue inhibitor of metalloproteinases (TIMP-1) and thus assist in the determination of the enzyme activity. In RA, gelatinase B cleaves denatured type II collagen into remnant epitopes, some of which constitute immunodominant glycopeptides. This implies that immunodominant epitope scanning experiments should preferably be done with the natural posttranslationally modified

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glycopeptides, rather than with unmodified (synthetic) peptides. Sugars can also be used as molecular probes to study autoimmune diseases. One of the best examples is the induction of acute pancreatitis, insulitits and diabetes by streptozotocin. In addition, gelatinase B is upregulated in pancreatitis and cleaves insulin. The most efficient cleavage by gelatinase B leads to a major insulin remnant epitope.

2. INTRODUCTION

Research on autoimmune diseases has been mainly focussed on lymphocyte functions. The knowledge of cellular and humoral immunity has progressed exponentially, but insufficiently to fully explain the pathogenesis of autoimmunity. In addition, specific immune mechanisms and various antigens, involved in different autoimmune disorders, have been studied in detail. Many components of the adaptive immune system [e.g., major histocompatibility complex (MHC) proteins, T cell receptors, antibodies] and the innate immune system (e.g., complement proteins, cytokines) are glycoproteins (Rudd et al., 2001). Although the protein functions have been studied extensively, knowledge of the functions of the attached oligosaccharides is still fragmentary. From our growing understanding of the working mechanisms of the adaptive immune system, it becomes clear that specific therapeutic strategies for autoimmune diseases (e.g., T cell vaccination, tolerance induction) will be patient-specific and perhaps not generally applicable.

We previously studied *aspecific* host defense or innate immune mechanisms and their role in autoimmunity. As a result of these investigations, we have proposed the REGA model (Remnant Epitopes Generate Autoimmunity) for the generation of autoantigens and their interaction with the T cell receptor-complex (Opdenakker and Van Damme, 1994; Van den Steen *et al.*, 2002). In this model, cytokine- and chemokine-regulated proteases play a central role in the generation of autoimmune antigens. As a consequence, disease-promoting cytokines and proteases are therapeutic targets, whereas disease-limiting cytokines and protease inhibitors may be products in treatment of autoimmune diseases. Most cytokines, proteases and protease inhibitors are glycoproteins, and their glycosylation often plays a role in molecular targeting or in fine-tuning their specific activity (Opdenakker *et al*., 1995; Van den Steen *et al.*, 1998a).

Here we will exemplify the glycoimmunology of gelatinase B/matrix metalloproteinase-9 (MMP-9). First, the oligosaccharides attached to MMP-9 influence its interaction with the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1). Second, gelatinase B as an enzyme cleaves collagen II into natural immunodominant glycopeptide antigens. Third, the sugar derivative streptozotocin is a molecular probe for the induction of acute pancreatic insulitis and diabetes. In pancreatitis, gelatinase B is shown to destroy insulin into remnant peptides, which may enhance the autoimmune process.

3. GLYCOSYLATION OF GELATINASE B

Gelatinase B is a complex multidomain glycoprotein (Opdenakker *et al.*, 2001 and Figure 1). Like all MMPs, gelatinase B contains a propeptide, an active domain and a Zn^{2+}-binding domain. These three domains together constitute the most essential functional unit of the enzyme. In addition, a hemopexin domain is present in most MMPs (except matrilysins) and is

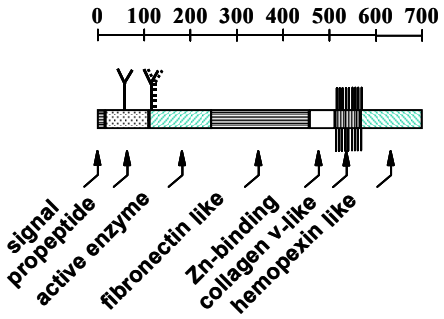

Figure 1. Domain structure and glycosylation of gelatinase B. Gelatinase B is constituted of several protein domains, some of which are common for most MMPs (signal peptide, propeptide, active enzyme and Zn^{2+}-binding domain, hemopexin domain), while the fibronectin domain is typical for gelatinases and the collagen type V domain is unique for gelatinase B. Three N-linked glycosylation consensus sequences are indicated (Y), one of which is conserved but not occupied (dotted Y). Since the collagen type V domain is likely to contain clustered O-linked glycans, these are indicated with vertical lines. Isolated O-linked sugars may be attached to other domains as well (not indicated).

located at the carboxyterminal part. A fibronectin domain, containing three type II fibronectin repeats, is inserted between the active domain and Zn^{2+}-binding domain of gelatinases and confers highly efficient gelatinolytic activity. Unique for gelatinase B is the so-called collagen type V domain, which is inserted between the Zn^{2+}-binding domain and the hemopexin domain.

In addition to these protein domains, gelatinase B contains also voluminous glycans. Two N-linked glycans are attached to N-glycosylation consensus sequences (Asn-Xaa-Ser/Thr, with Xaa any amino acid except Pro) in the prodomain and in the active domain. A third N-glycosylation site, located at only 4 amino acids from the sequon in the active domain, is conserved in most MMPs throughout different species. Recently, this site has been reported not to be occupied in recombinant gelatinase B (Kotra *et al.*, 2002). A large number of O-linked glycans are also attached to gelatinase B, but the exact attachment sites of these sugars remain to be determined. However, the so-called collagen type V domain is composed of 11 repeats of the sequence T/SXXP, which are putative attachment sites for O-linked glycans (Van den Steen et al., 1998a). The organisation of this gelatinase B domain is different from that of collagen type V, which is composed of repeats of the sequence Gly-Xxx-Xxx (with Xxx is often Pro). In gelatinase B, this domain rather resembles the sequence of mucins, which are glycoproteins known to contain a large proportion of clustered O-linked sugars. Therefore, this domain can better be named mucin-like domain (Van den Steen *et al.*, 2001). The presence of clustered O-linked oligosaccharides is expected to result in the extension of this glycoprotein domain, leading to a rigid bottle brush-like structure, which acts as a spacer between the Zn^{2+}-binding and the hemopexin domain (Mattu *et al.*, 2000). In gelatinase A, the hemopexin domain makes extensive contacts with the catalytic site of the enzyme (Morgunova *et al.*, 1999) and has a strong influence on the substrate-specificity by its binding to substrates, e.g., CC-chemokines (McQuibban *et al.*, 2000). To what extent the O-glycosylated mucin-type collagen type V domain modifies the substrate specificity of gelatinase B is not yet investigated, but it has been found that gelatinase B does not cleave CC-chemokines. In contrast, gelatinase B cleaves several CXC-chemokines, resulting in either potentiation or degradation of the chemokine (Van den Steen *et al.*, 2000).

The structures of the N-linked (Rudd *et al.*, 1999) and O-linked (Mattu *et al.*, 2000) sugars of natural gelatinase B from human neutrophils (after hydrazinolysis to separate the glycans from the protein) have been studied in great detail by NP-HPLC of the fluorescently labelled glycans and on-line mass spectrometry analysis. Complex bi- and tri-antennary N-

linked glycans were found with terminal sialylation and core fucosylation. The more abundant O-linked glycans have core 1 and core 2 structures with terminal sialylation, fucosylation and lactosamine-extensions. The sugars of recombinant gelatinase B, expressed in the yeast *Pichia pastoris* (Van den Steen *et al.*, 1998b) or in human HeLa cells (Kotra *et al.*, 2002) have also been studied.

The functions of the sugars, attached to gelatinase B are still unclear, and several hypotheses have been formulated. For instance, the sugars may be protective against autoproteolysis of gelatinase B or proteolysis by other proteases. In general, oligosaccharides may also influence the specific activity of enzymes (Rudd *et al*., 1994; Mori *et al.*, 1995). This might be particularly useful, since neutrophils secrete gelatinase B together with several other proteases. In addition, glycosylation is also important for the folding of and the recognition between glycoproteins (Rademacher et al., 1988; Varki, 1993; Rudd and Dwek, 1997)[32]. Finally, a limited effect of the sialic acids on the inhibition of gelatinase B by TIMP-1 has been found (Van den Steen *et al.*, 2001).

4. GELATINASE B IN MULTIPLE SCLEROSIS

Gelatinase B has been detected in the cerebrospinal fluid of patients with multiple sclerosis and in experimental animal models of multiple sclerosis, for example, in experimental autoimmune encephalomyelitis. Because all neurological pathologies, in which gelatinase B was detected, were characterized by demyelination, the effect of gelatinase B on myelin destruction was studied. It was found that human myelin basic protein (MBP) is a substrate for gelatinase B and that the cleavages in MBP resulted in peptide fragments which corresponded to the most important encephalitogenic autoantigens (Proost *et al*., 1993). These data led us to postulate the REGA model for autoimmune diseases (Opdenakker and Van Damme, 1994). Further proof of concept was obtained with the use of gelatinase B-deficient mice, young gelatinase B-deficient mice were found to be resistant against the development of experimental autoimmune encephalomyelitis and against an amputating form of tail necrosis (Dubois *et al*., 1999).

5. GELATINASE B AND ARTHRITIS

Production of gelatinase B is induced at sites of inflammation by cytokines for example TNF-α, interferon-γ (IFN-γ), IL-1 and chemokines. A typical example is the inflamed joint of patients with rheumatoid arthritis (Figure 2). In this autoimmune disease, inflammation is accompanied with the destruction of cartilage and an autoimmune reaction against the major cartilage constituent type II collagen. In the synovial fluids of these patients, the levels of the chemokine IL-8 are increased compared to controls (e.g., osteoarthritis patients) (Rampart *et al.*, 1992). IL-8 attracts neutrophils and stimulates these cells to release gelatinase B from their granules (Masure *et al.*, 1991). After activation by removal of the propeptide, the protease participates in the degradation of the cartilage. A single cleavage of type II collagen by collagenases (MMP-1, MMP-8, MMP-13) induces the partial unwinding of the triple helix structure (Gioia *et al.*, 2002), so that it becomes accessible to gelatinase B. Gelatinase B then cleaves the type II collagen into a large number of fragments. Determination of the cleavage sites by Edman degradation and mass spectrometry of the fragments yielded interesting data

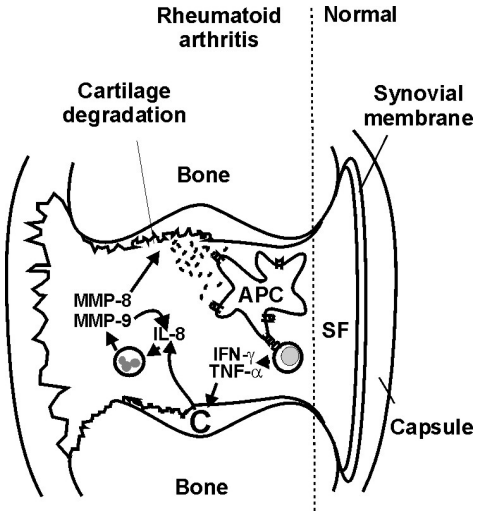

Figure 2. Role of gelatinase B in rheumatoid arthritis. Under influence of proinflammatory cytokines, IL-8 is produced and released in the synovial fluid. As a consequence, neutrophils are chemoattracted and stimulated to release gelatinase B and neutrophil collagenase (MMP-8). A positive feedback loop occurs as gelatinase B potentiates IL-8 10-fold by aminoterminal processing. Gelatinase B and collagenases degrade the cartilage, in particular type II collagen, of which the immunodominant fragments activate autoreactive T-lymphocytes after presentation by antigen presenting cells (APC). APC may act locally or transfer the antigens to stimulate T-cells in lymph nodes and follicles. This results in a second positive feedback loop, since the activated T cells produce pro-inflammatory cytokines.

about the substrate specificity of the enzyme and the posttranslational modifications of the substrate (Van den Steen *et al.*, 2002). Thus it was observed that prolines in type II collagen are often hydroxylated, in particular when occurring at the third position of the $(Gly\text{-}Xxx\text{-}Xxx)_n$ repeat. Lysine at the same position is also often hydroxylated, and hydroxylysine can be glycosylated with a single galactose (Galβ1-O-Lys) or with a galactose and glucose (Glcα1-2Galβ1-O-Lys). These posttranslational modifications enhance the solubility and stability of the collagen, and their exact localisation has been found to influence the interactions with gelatinase B. In addition to known substrate sequence preferences (a hydrophobic residue at P1', a small amino acid at P1 and proline at P3), it was found that gelatinase B has a preference for hydroxyprolines at the position P5', while hydroxyprolines are not well tolerated at P2'. In addition, one of the two immunodominant epitopes of collagen II has been found to be modified by Lys-hydroxylation, and possibly also by glycosylation. Such modifications play a major role in the binding of the peptides to the MHC (Haurum *et al.*, 1995) and in the subsequent recognition of such an MHC-peptide complex by T-cells (Haurum et al., 1994). In addition, it was shown that the severity of arthritis, induced in mice by immunization with type II collagen, correlates with the extent of modifications of type II collagen (Michaëlsson *et al.*, 1994). Recently, most of the autoreactive T-cell clones from rheumatoid arthritis patients have been shown to react preferentially with the glycosylated form of the immunodominant type II collagen epitope (Bäcklund *et al.*, 2002). These findings imply that posttranslational modifications may play an essential role in autoimmune diseases.

From the positions of the cleavage sites in collagen II it can be inferred that gelatinase B does not destroy the immunodominant epitopes, but rather induces their release from the whole protein. This is also the case with other major auto-antigens in other autoimmune diseases (Descamps *et al.*, 2003; Proost *et al.*, 1993). Gelatinase B thus participates in the generation of the autoantigenic peptides, according to the REGA model (Opdenakker and Van Damme, 1994).

6. ACUTE PANCREATITIS, DIABETES AND ROLES OF GELATINASE B

Until recently, only correlative data existed about the role of gelatinase B in diabetes (Ebihara *et al.*, 1998, Maxwell *et al.*, 2001, Tomita and Iwata, 1997). We demonstrated by immunohistochemistry high expression levels of gelatinase B by neutrophils in acute pancreatitis and by ductular epithelial cells in chronic pancreatitis (Descamps *et al.*, 2003). With the use of double immunostainings for insulin and gelatinase B, it was shown that high expression levels of gelatinase B are maintained in the immediate proximity of insulin-secreting beta cells. After secretion of insulin by beta cells and gelatinase B by neighbouring (ductular) cells, both molecules may interact.

Ongoing research is directed to elucidate the role of gelatinase B *in vivo*. For this purpose, we chose to use an animal model in which diabetes is chemically induced with multiple low doses of streptozotocin (Like and Rossini, 1976). As shown in Figure 3, streptozotocin is a glucose-derivative, which can be used as a molecular probe to induce diabetes. The immunology of the streptozotocin-induced diabetes model is well documented and is similar to human insulin-dependent diabetes mellitus (Lukic *et al.*, 1998). Another advantage is that the induction is suitable to establish diabetes in a variety of mouse strains, allowing the use of specific knockout mice, without the need of backcrossing to naturally sensitive strains such as the non-obese diabetic mice.

In the development of autoimmune diabetes, insulin is probably the most important autoantigen (Wegmann and Eisenbarth, 2000). The fragment of the insulin beta-chain, containing residues 9 to 23, constitutes the immunodominant epitope (Wegmann *et al.*, 1994). Loss of tolerance to beta cell antigens can be mediated by genetic, endocrine and environmental factors. In some particular cases, diabetes may result from pancreatitis (Wakasugi *et al.*, 1998, Koizumi *et al*., 1998). Autoimmunity then arises from the uptake of insulin or other beta cell antigens and their ferrying to the pancreatic lymph nodes, permitting presentation to naive circulating beta cell-reactive T lymphocytes.

We recently documented that gelatinase B degrades insulin into fragments and may assist in the generation of immunodominant insulin epitopes (Descamps *et al*., 2003). Human insulin

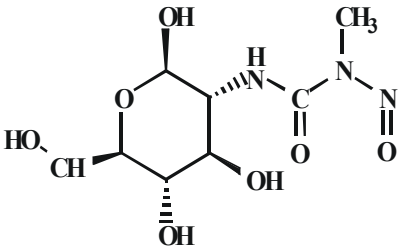

Figure 3. Structure of streptozotocin.

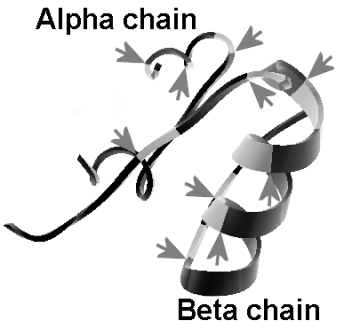

Figure 4. Three-dimensional view of the crystal structure of insulin. Cleavage sites by gelatinase B are indicated by means of arrows to demonstrate the accessibility to the enzyme and the resulting complete degradation of insulin.

and gelatinase B were incubated at a substrate:enzyme ratio of 6.5 or more. The reaction mixture of a complete digestion was separated by RP-HPLC on a C18 column and analyzed by on-line mass spectrometry. Exact molecular masses of peptide fragments were derived from the spectra and were fitted to those of virtual insulin fragments. Predicted amino-acid sequences were confirmed by tandem analysis of the corresponding peptides. Identification of all degradation products denoted 10 cleavage sites as illustrated on a three-dimensional model of insulin (Figure 4) (Adams *et al.*, 1969; Smith *et al.*, 2001).

We determined that the cleavage in front of residue 24 is the most efficient one. The beta chain fragment from residues 24 to 30 was the first appearing degradation product as a function of time. This implies that the remnant fragment, containing residues 1 to 23 of the beta chain, may be presented in a complex with MHC class II, either after processing into the immunodominant epitope [residues 9 to 23] or after direct uptake by antigen-presenting cells (Pu *et al.*, 2002). However, if the fragment 1 to 23 persists in the extracellular milieu for prolonged time intervals, then it will be further degraded by the action of gelatinase B. In both cases, the REGA model is applicable to understand the development of the diabetogenic role of gelatinase B.

7. CONCLUSIONS AND PERSPECTIVES

The role gelatinase B in the REGA model has been validated in three different autoimmune diseases: multiple sclerosis, rheumatoid arthritis and diabetes. Gelatinase B is a disease-promoting glycoprotein and forms a target for therapy. This implies that inhibitors of gelatinase B may become novel drugs for the treatment of autoimmune diseases.

8. ACKNOWLEDGEMENTS

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9. REFERENCES

Adams, M. J., Baker, E. N., Blundell, T. L., Harding, M. M., Dodson, E. J., Hodgkin, D. C., Dodson, G. G., Rimmer, B., Vijayan, M., and Sheats, S, 1969, Structure of rhombohedral 2 zinc insulin crystals, Nature 224: 491-495.

Bäcklund, J., Carlsen, S., Hoger, T., Holm, B., Fugger, L., Kihlberg, J., Burkhardt, H., and Holmdahl, R., 2002, Predominant selection of T cells specific for the glycosylated collagen type II epitope (263-270) in humanized transgenic mice and in rheumatoid arthritis., Proc.Natl.Acad.Sci.U.S.A. 99: 9960-9965.

Descamps, F.J., Van den Steen, P.E., Martens, E., Ballaux, F., Geboes, K., and Opdenakker G., 2003, Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin, FASEB J. 17:887-889.

Dubois, B., Masure, S., Hurtenbach, U., Paemen, L., Heremans, H., van den Oord, J., Sciot, R., Meinhardt, T., Hämmerling G., Opdenakker, G., and Arnold, B., 1999, Resistance of young gelatinase B-deficient mice to experimental autoimmune encephalomyelitis and necrotising tail lesions, J. Clin. Invest. 104: 1507-1515.

Ebihara, I., Nakamura, T., Shimada, N., and Koide, H., 1998, Increased plasma metalloproteinase-9 concentrations precede development of microalbuminuria in non-insulin-dependent diabetes mellitus, Am. J. Kidney Dis. 32: 544-550.

Gioia, M., Fasciglione, G. F., Marini, S., D'Alessio, S., De Sanctis, G., Diekmann, O., Pieper, M., Politi, V., Tschesche, H., and Coletta, M., 2002, Modulation of the catalytic activity of neutrophil collagenase MMP-8 on bovine collagen I. Role of the activation cleavage and of the hemopexin-like domain, J. Biol. Chem. 277: 23123-23130.

Haurum, J. S., Arsequell, G., Lellouch, A. C., Wong, S. Y., Dwek, R. A., McMichael, A. J., and Elliott, T., 1994, Recognition of carbohydrate by major histocompatibility complex class I-restricted, glycopeptide-specific cytotoxic T lymphocytes, J. Exp. Med. 180: 739-744.

Haurum, J. S., Tan, L., Arsequell, G., Frodsham, P., Lellouch, A. C., Moss, P. A., Dwek, R. A., McMichael, A. J., and Elliott, T., 1995, Peptide anchor residue glycosylation: effect on class I major histocompatibility complex binding and cytotoxic T lymphocyte recognition, Eur. J. Immunol. 25: 270-3276.

Koizumi, M., Yoshida, Y., Abe, N., Shimosegawa, T., and Toyota, T., 1998, Pancreatic diabetes in Japan, Pancreas 16: 385-391.

Kotra, L. P., Zhang, L., Fridman, R., Orlando, R., and Mobashery, S., 2002, N-Glycosylation pattern of the zymogenic form of human matrix metalloproteinase-9, Bioorg.Chem. 30: 356-370.

Like, A.A., and Rossini, A.A., 1976 Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus, Science 30: 415-417.

Lukic, ML, Stosic-Grujicic, S, and Shahin, A., 1998, Effector mechanisms in low-dose streptozotocin-induced diabetes, Dev. Immuno., 6: 119-128.

Masure, S., Proost, P., Van Damme, J., and Opdenakker, G., 1991, Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8, Eur. J. Biochem. 198: 391-398.

Mattu, T. S., Royle, L., Langridge, J., Wormald, M. R., Van den Steen, P. E., Van Damme, J., Opdenakker, G., Harvey, D. J., Dwek, R. A., and Rudd, P. M., 2000, O-Glycan analysis of natural human neutrophil gelatinase B using a combination of normal phase- HPLC and online tandem mass spectrometry: implications for the domain organization of the enzyme, Biochemistry 39: 15695-15704.

Maxwell, P. R., Timms, P.M., Chandran, S., and Gordon, D., 2001, Peripheral blood level alterations of TIMP-1, MMP-2 and MMP-9 in patients with type I diabetes, Diabetic Med. 18: 777-780.

McQuibban, G. A., Gong, J. H., Tam, E. M., McCulloch, C. A., Clark-Lewis, I., Overall, C. M., 2000, Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3, Science 289: 1202-1206.

Michaëlsson, E., Malmstrom, V., Reis, S., Engstrom, A., Burkhardt, H., and Holmdahl, R., 1994, T cell recognition of carbohydrates on type II collagen, J. Exp. Med. 180:745-749.

Morgunova, E., Tuuttila, A., Bergmann, U., Isupov, M., Lindqvist, Y., Schneider, G., and Tryggvason, K., 1999, Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed, Science 284: 1667-1670.

Mori, K., Dwek, R.A., Downing, A.K., Opdenakker, G., and Rudd, P.M., 1995, The activation of type 1 and type 2 plasminogen by type 1 and type 2 tissue plasminogen activator, J. Biol. Chem. 270:3261-3267.

Opdenakker, G., Rudd, P.M., Wormald, M., Dwek, R.A., and Van Damme, J., 1995, Cells regulate the activities of cytokines by glycosylation, FASEB J. 9:453-457.

Opdenakker, G., and Van Damme J., 1994, Cytokine-induced proteolysis in autoimmune diseases, Immunol. Today 15:104-107.

Opdenakker, G., Van den Steen P.E., and Van Damme, J., 2001, Gelatinase B: a tuner and amplifier of immune functions, Trends Immunol. 22: 571-579.

Proost, P., Van Damme, J., and Opdenakker, G., 1993, Leukocyte gelatinase B cleavage releases encephalitogens from human myelin basic protein, Biochem. Biophys. Res. Commun. 192:1175-1181.

Pu, Z., Carrero, J. A., and Unanue, E. R., 2002, Distinct recognition by two subsets of T cells of an MHC class II- peptide complex, Proc. Natl. Acad. Sci. U.S.A. 99: 8844-8849.

Rademacher, T. W., Parekh, R. B., and Dwek, R. A., 1988, Glycobiology, Annu. Rev. Biochem. 57: 785-838.

Rampart, M., Herman, A. G., Grillet, B., Opdenakker, G., and Van Damme, J., 1992, Development and application of a radioimmunoassay for interleukin-8: detection of interleukin-8 in synovial fluids from patients with inflammatory joint disease. Lab. Invest. 66: 512-518.

Rudd, P. M., and Dwek, R. A., 1997, Glycosylation: heterogeneity and the 3D structure of proteins., Crit. Rev. Biochem. Mol. Biol., 32: 1-100.

Rudd, P.M., Elliott, T., Cresswell, P., Wilson, I.A., and Dwek, R.A., 2001, Glycosylation and the immune system, Science 291:2370-2376.

Rudd, P.M., Joao, H.C., Coghill, E., Fiten, P., Saunders, M.R., Opdenakker, G., and Dwek, R.A., 1994, Glycoforms modify the dynamic stability and functional activity af an enzyme, Biochemistry 33:17-22.

Rudd, P. M., Mattu, T. S., Masure, S., Bratt, T., Van den Steen, P. E., Wormald, M. R., Küster, B., Harvey, D. J., Borregaard, N., Van Damme, J., Dwek, R. A., and Opdenakker, G., 1999, Glycosylation of natural human neutrophil gelatinase B and neutrophil gelatinase B-associated lipocalin, Biochemistry 38: 13937-13950.

Smith, G. D., Pangborn, W. A., and Blessing, R. H., 2001, Phase changes in T(3)R(3)(f) human insulin: temperature or pressure induced?, Acta Crystallogr. D. Biol. Crystallogr, 57: 1091-1100.

Tomita, T., and Iwata, K., 1997, Gelatinases and inhibitors of gelatinases in pancreatic islets and islet cell tumors, Mod. Pathol. 10: 47-54.

Van den Steen, P. E., Opdenakker, G., Wormald, M. R., Dwek, R. A., and Rudd, P. M., 2001, Matrix remodelling enzymes, the protease cascade and glycosylation, Biochim. Biophys. Acta. 1528: 61-73.

Van den Steen, P. E., Proost, P., Grillet, B., Brand, D. D., Kang, A. H., Van Damme, J., and Opdenakker, G., 2002, Cleavage of denatured natural collagen type II by neutrophil gelatinase B reveals enzyme specificity, post-translational modifications in the substrate, and the formation of remnant epitopes in rheumatoid arthritis, FASEB J, 16: 379-389.

Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J., and Opdenakker, G., 2000, Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact, Blood 96: 2673-2681.

Van den Steen, P., Rudd, P. M., Dwek, R. A., and Opdenakker, G., 1998a, Concepts and principles of O-linked glycosylation, Crit. Rev. Biochem. Mol. Biol. 33: 151-208.

Van den Steen, P., Rudd, P. M., Proost, P., Martens, E., Paemen, L., Küster, B., Van Damme, J., Dwek, R. A., and Opdenakker, G., 1998b, Oligosaccharides of recombinant mouse gelatinase B variants, Biochim. Biophys. Acta,.1425: 587-598.

Varki, A., 1993, Biological roles of oligosaccharides: all of the theories are correct, Glycobiology 3: 97-130.

Wakasugi, H., Funakoshi, A., and Igushi, H., 1998, Clinical assessment of pancreatic diabetes caused by chronic pancreatitis, J. Gastroenterol. 33: 254-259.

Wegmann, D. R., and Eisenbarth, G. S., 2000, It's insulin, J. Autoimmun. 15: 286-291.

Wegmann, D. R., Norbury-Glaser, M., and Daniel, D., 1994, Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice, Eur. J. Immunol. 24: 1853-1857.

DISCUSSIONS AND PERSPECTIVES

In addition to my experimental work that is outlined in five research reports, which are incorporated in the previous chapters (1 to 5), I would like to evaluate the results here in view of the aims that were outlined in the introduction. In the first paragraph, I will estimate the applicability of the REGA model with regard to insulin autoimmunity and delineate perspectives for the study of other autoantigens. Furthermore, I will evaluate the *in vivo* study on the role of gelatinase B in diabetes and highlight the importance of pancreatitis as a trigger for 'secondary' diabetes.

A second paragraph concerns the study on the role of gelatinase B in a microangiopathic complication of diabetes with reference to proliferative diabetic retinopathy. The significance of the results was discussed in chapter 4. Here, I will provide additional information and illustrations on diagnostic hallmarks of the pathology.

The last paragraph complements on the detrimental role of gelatinase B in cataract. This study is not directly related to the aims of my doctoral program, but evolved from the efforts to develop new techniques to identify functions of gelatinase B activity. The relevance of the results was discussed in chapter 5. Here, I will focus on technical aspects and the innovative design of this study.

1 Autoimmune Diabetes

1.1 The insulin autoantigen

1.1.1 B and T cell responses against insulin

Insulin was the first known β-cell protein to which an autoimmune response was documented in type 1 diabetic patients [56]. More recently, the use of improved assays revealed that nearly 70 % of new-onset patients have circulating antibodies to insulin [88]. Anti-insulin antibodies are highly predictive for future development of disease [29].

Immune complexes between autoantibodies and their targets may accumulate as deposits and activate the complement cascade with severe inflammation as a consequence. Such mechanisms are characteristic of the non-organ autoimmune disease systemic lupus erythematosus, in which anti-nuclear immune complexes accumulate, typically in the glomeruli, and cause severe glomerulonephritis. In organ-specific autoimmune diseases like multiple sclerosis and rheumatoid

arthritis, complement-mediated inflammation usually results in a more severe and acute form of disease. In diabetes, anti-insulin immune complexes are not basically involved in β-cell destruction but are related to late-diabetic complications in distant organs [6;24].

Autoreactive T cells -rather than autoantibodies- are believed to be responsible for destruction of β-cells. A potential role for insulin-reactive T cells in the development of type 1 diabetes has extensively been studied in the past decades. A first proof for their involvement comes from genetic studies. In humans, the strongest susceptibility locus to type 1 diabetes other than the MHC locus, is the insulin (*Ins*) upstream variable number of tandem repeats (VNTR) regulatory region. This VNTR region controls thymic expression of insulin. Class I alleles of this promoter region, which contain approximately 30-60 repeats of the 14-base pair sequence ACAGGGGTCTGGGG, are correlated with reduced thymic expression of the *Ins* gene and predispose to diabetes. On the contrary, class III alleles, which have more than 100 repeats, are dominantly protective. Studies in NOD mice are consistent with this view. Mice have two genes encoding the insulin protein. In the mouse; both *Ins1* and *Ins2* are expressed in the β cell, yet only *Ins2* is expressed in the thymus. *Ins2*$^{-/-}$ NOD mice show accelerated diabetes and insulitis, suggesting that, as in humans, reduced thymic expression and impaired induction of central tolerance do indeed account, at least in part, for why insulin becomes target for the immune system.

In line with these genetic experimental approaches, the majority of islet infiltrating CD4^{+} T cell clones in NOD mice were found to recognize insulin [84]. More than 90 % of such T cells react with amino acids 9-23 of the insulin B chain ($B_{(9-23)}$)[19]. Unexpectedly, several studies in man could not demonstrate a pronounced cellular response to insulin [27;28]. In contrast, it was shown in a more recent study that T cell responses to the single insulin peptide $B_{(9-23)}$ were about complete in patients with type 1 diabetes [5]. This dichotomy between T cell responses against a whole molecule (insulin) and an epitope ($B_{(9-23)}$-peptide) within a molecule rises the question what mechanisms can turn this particular epitope into an MHC-presented peptide *in vivo*. The REGA-model presents a potential mechanism in this regard (**Introduction, section 1.5.2 and Chapter 6**).

1.1.2 The REGA-model and insulin autoimmunity

In contrast to several autoantigens that are hidden behind a *physical barrier* (**Introduction, section 1.5.2**), insulin is freely accessible. On the other hand, we questioned if conditions for the REGA-model were fulfilled, referring to the liberation of immunodominant epitopes that are hidden behind *molecular barriers*.

1.1.2.1 Issues from the literature

Observations in NOD mice support the potential importance of the REGA model in diabetes. It was reported that the short insulin peptide $B_{(9\text{-}23)}$ induces a strong humoral anti-insulin response in NOD mice after subcutaneous administration in incomplete Freund's adjuvant or without adjuvant [1]. Cognate epitopes of activated B cells were scattered throughout the whole insulin molecule. These data imply that the small $B_{(9\text{-}23)}$ peptide had entered MHC class II antigen-presentation pathways. Uptake of such a small peptide by antigen-presenting cells is not favourable[2]. Therefore, it is more likely that $B_{(9\text{-}23)}$ peptide-loading executed extracellularly, e.g. by immature dendritic cells that carry high amounts of (peptide-receptive) MHC class II molecules at their cell surface[3] [62]. In this respect, accidental generation of a peptide like $B_{(9\text{-}23)}$ would be an unsafe situation with regard to induction of loss of tolerance. Extracellular loading of $B_{(9\text{-}23)}$ on class II MHC molecules would then represent a clear-cut example of how the REGA-model may be operative *in vivo*.

Ensuing from MHC-presentation of the $B_{(9\text{-}23)}$ peptide, a whole range of B cells with dispersed immunoglobulin receptors against insulin epitopes are activated. This implies that all these B cell clones at least present the insulin peptide $B_{(9\text{-}23)}$ on their MHC class II molecules. It must therefore be assumed that they take up the intact insulin molecule via their membrane-bound Ig-receptors and present the particular peptide after intracellular processing.

The affinity of the $B_{(9\text{-}23)}$ epitope for the presenting MHC type II molecules in a particular subject is crucial. Also here, the susceptibility to anti-insulin antibody induction after immunisation with the $B_{(9\text{-}23)}$ peptide mapped strongly to the

[2] Related to this, peptide immunisations are generally carried out after coupling peptides onto large carrier proteins.

[3] A physiological role was suggested for these molecules in a new pathway through which secreted DC proteases act extracellularly to process intact proteins into antigenic peptides that are loaded onto empty cell surface class II MHC molecules.

major histocompatibility complex[4] [1]. As previously discussed, this genetic region is the most important in type 1 diabetes.

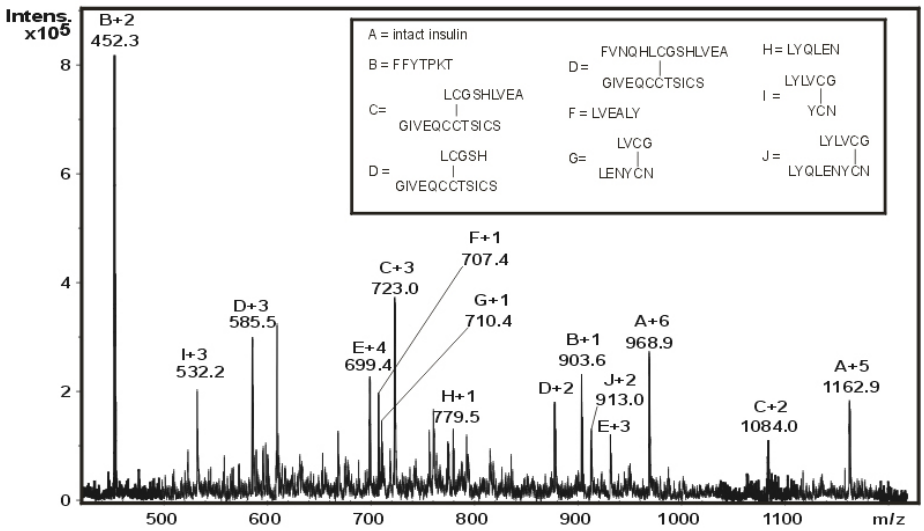


Figure 1: Average profile mass spectrum of an incubation mixture of insulin with gelatinase B. The sample was collected after 4 h incubation.

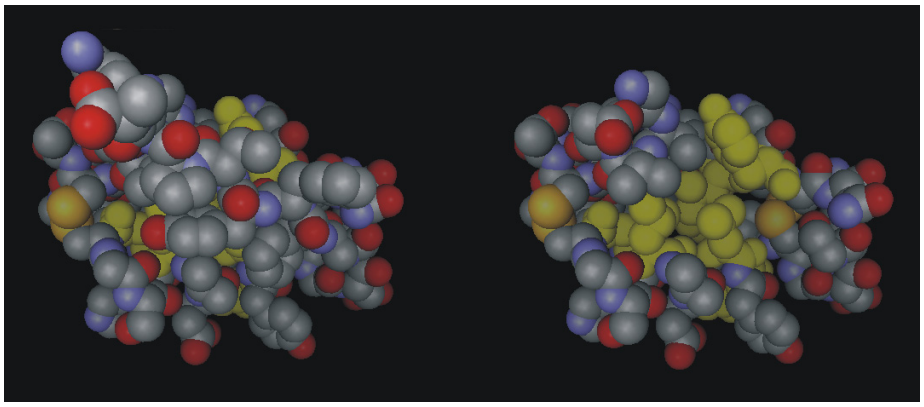


Figure 2: Space filling representations of the insulin molecule. Hydrophobic leucine residues in the inner core are indicated in yellow. The left panel represents intact insulin; the right panel represents insulin after removal of B24-30.

[4] NOD mice and Balb/c mice with related MHC genes were susceptible; C57BL6 mice with divergent MHC genes were not.

1.1.2.2 Role of gelatinase B in the breakdown of insulin's molecular barrier

Insulin is cleaved at 10 different sites by gelatinase B. Mass spectra that were taken during the incubation experiment revealed that a [m/z = 452.1]-peak, representing residues $B_{(24-30)}$, was the first appearing peak in time (t < 30 min). Cleavage between B_{23} and B_{24} (P_1-P_1') was therefore graded as the most efficient. Nonetheless, the complementary fragment $A_{(1-21)}$-$B_{(1-23)}$ was not detected at any time point, suggesting that the latter had instantly been processed further. Also, partially digested insulin (t = 4 h) exposes the same arsenal of fragments as completely digested insulin (t = 10 h) (**Figure 1**). Thus, once insulin has become a target of gelatinase B, it is at once processed to completion.

The obtained cleavage pattern of insulin has six times a hydrophobic leucine residue at position P1', which is in line with the cleavage preference of gelatinase B [78]. Nonetheless, such cleavage pattern would not be expected on the basis of insulin's three-dimensional structure. Indeed, the monomer structure constitutes a well-ordered hydrophobic core that is stabilised by three disulfide bridges and close packing of aliphatic and aromatic side chains [39]. As such, the identified P1' cleavage positions are oriented inwards to the molecule. It is thus questionable how gelatinase B can reach these residues. In search of an answer to this question, we considered the structure-function relationship of insulin. Insulin residues that are functionally important because they bind to the insulin receptor are situated in the inner core (like the P1' cleavage positions). Binding of these residues to the insulin receptor involves the separation (but not removal) of the carboxy-terminal residues of the B-chain, $B_{(26-30)}$[5] [40]. The absence of residues B_{26}-B_{30} indeed implies significant exposure of binding residues. Taking this into account, we propose that the proteolytic removal of $B_{(24-30)}$ may similarly expose P1' cleavage positions to gelatinase B (**Figure 2**).

The *in vitro* action of gelatinase B on insulin does not result in the generation of intact $B_{(9-23)}$ peptide, but generates smaller fragments that are perhaps too small to be loaded on MHC class II molecules. Hence, these data do not support liberation of the immunodominant T cell epitope. Nevertheless, these data do not necessarily argue against the importance of remnant insulin fragments for the generation of autoimmunity (the REGA model). Indeed, degraded insulin

[5] This model has been investigated through crystallographic studies of the truncated insulin analogue (*des*-pentapeptide[B26-B30]-insulin; DPI) [10].

randomly exposes B cell epitopes that used to be hidden in the inner core (*molecular barrier*). Eventually, B cell epitopes in diabetic patients are scattered throughout the whole molecule, but initially those from inside the molecule may be crucial for loss of tolerance. Indeed, negative selection mechanisms in the bone marrow are based on the binding of naïve B cells to native insulin [49] so that B cells with a buried cognate epitope are not likely to be deleted. Peripheral degradation of insulin by gelatinase B may liberate these cryptic B cell epitopes. After specific binding and internalization, these B cells may act as antigen-presenting cells and present these epitopes on MHC class II molecules [21]. In this manner, T helper cells are activated, by which the immune response may spread to the whole insulin molecule. The early appearance of anti-insulin autoantibodies suggests that loss of tolerance in the B cell compartment occurs quickly after disease onset. In these initial stages, one can imagine the involvement of innate immunity players, like neutrophils as a source of gelatinase B (**Introduction, section 1.4.1.1**).

Apart from the generation of autoimmunity, a pathological relevance of insulin breakdown may be expected from the fact that insulin is no longer functional after cleavage. Loss of activity may intuitively be assumed, since the molecule is broken at 10 cleavage sites. The fact that the functional surface residues Ile_2, Val_3, and Tyr_{19} of the A chain are physically separated [8;23;41] by the cleavages scientifically supports this assumption. Also, residue Leu_{16} of the A chain was shown to be critical for the structure and stability of the entire molecule, suggesting that only a cleavage at this site would be sufficient to destroy insulin's stability and function.

1.1.3 Perspectives

1.1.3.1 Pro-insulin autoimmunity

A first perspective is related to an additional observation of our experiments, namely the generation of the stable peptide $B_{(24-30)}$. Dislocation of this peptide from the insulin molecule occurs first and the peptide seems to be stable at least until the end of kinetic follow-up of *in vitro* cleavage of intact insulin by gelatinase B. Not $B_{(24-30)}$ itself has been reported to be an immunodominant insulin epitope but recent research indicates the pro-insulin fragment B_{24}-C_{33} as immunodominant, of which $B_{(24-30)}$ forms a part [34;61]. Therefore, we plan

further experiments to test if the latter epitope might be liberated from pro-insulin upon cleavage by gelatinase B.

For a good understanding, it is important to note that insulin is processed from the single-chain precursor pre-pro-insulin by removal of the leader peptide, generating pro-insulin (**Figure 3**) and subsequent removal of the C-peptide region by two endoproteolytic cleavages and trimming of the B chain by carboxypeptidase E. The concept of pro-insulin rather than mature insulin to be an autoantigen in type 1 diabetes is relatively new. Initially, there was some controversy about which autoantigen in type 1 diabetes is the earliest target. T cell reactivity to GAD65 was reported to precede reactivity to insulin, indicating that insulin would not be an early target [86]. Meanwhile, the hypothesis of pro-insulin being an early autoantigen gained more attention. In prenatal NOD mice, T cells from peripheral lymphoid tissues did not react with $B_{(9\text{-}23)}$, but responded strongly to pro-insulin with the same kinetics as the GAD65-specific T cell response. The decamer peptide B_{24}–C_{33} of mouse pro-insulin was found to be an immunodominant epitope in these NOD mouse T cell responses [16]. In newly diagnosed patients with type 1 diabetes, the magnitude of the peripheral T cell response to pro-insulin was not particularly higher in comparison with islet autoantibody-negative relatives [28] but strong pro-insulin B_{24}-C_{36} T cell reactivity was found in subjects at high risk to develop diabetes (i.e. in a very early stage of development) [61].

Considering gelatinase B‘s cleavage preference with regard to insulin’s C-peptide (**Figure 3)**, it can be expected that the B_{24}-C_{36} epitope remains intact. First, it is plausible that the known cleavages within the insulin A and B chains will occur when pro-insulin is targeted instead of insulin. Second, when we speculate on one or more cleavages further upstream in the C-peptide, e.g. before L_{37}, L_{53}, L_{56}, L_{58} or L_{62}, an immunodominant peptide would be generated that may directly be loaded on MHC class II molecules.

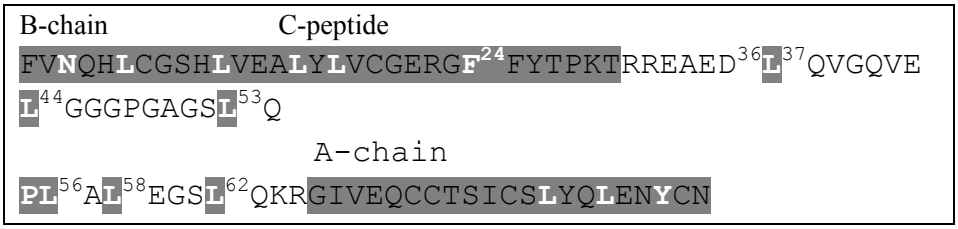


Figure 3: Sequence of human pro-insulin. The insulin B and the A chain are marked grey; amino acids in white font indicate P1' positions of gelatinase B cleavage sites. In the C-peptide, preferred (hydrophobic) gelatinase B P1' positions are marked in grey. A pro-insulin immunodominant epitope spreads from amino acid F^{24} to D^{36}.

1.1.3.2 Glutamic acid decarboxylase autoimmunity

The γ-aminobutyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase (GAD) is another major autoantigen, involved in the initial stages of autoimmune diabetes in humans and mice. GAD is expressed at comparable levels in GABA-secreting neurons and in pancreatic β-cells in the islets of Langerhans. GABA is an inhibitory neurotransmitter in the central nervous system. Its function in islets remains elusive, though the expression of GABA receptors on glucagon-producing α-cells in islets suggests a role in paracrine signalling within the islet. GAD has been identified as the 64-kDa autoantigen of the β cells, to which autoantibodies arise concomitantly with β-cell destruction and the development of type 1 diabetes [7]. Autoantibodies against GAD are detected up to several years before the clinical diagnosis of disease and can persist for many years following the clinical onset of diabetes. GAD in neurons is an autoantigen in stiff-man syndrome, a rare neurological disease characterized by a high coincidence with type 1 diabetes [66]. Strikingly, GAD suppression in the pancreas with antisense transgene constructs under the control of the rat insulin promoter prevented development of autoimmune diabetes in NOD mice [87]. The reasons for the unusual susceptibility of the GAD molecule to be targeted as an autoantigen are unclear.

In view of the possible role of remnant epitopes in the onset and maintenance of autoimmune diseases and the results obtained by analysis of insulin degradation, we evaluated susceptibility of GAD65 to gelatinase B degradation. We gathered preliminary data from analyses in crude mouse brain extracts with the use of Western blot analysis with an anti-GAD-65 monoclonal antibody and revealed

GAD65 indeed to be susceptible to cleavage by gelatinase B. Proteolytic cleavage results in a remnant 55 kDa fragment that is marginally cleaved further into a 46 kDa fragment in the presence of higher gelatinase B concentrations. Active mouse gelatinase B catalysed similar cleavages (**Figure 4**). To determine the exact cleavage sites, it will be essential to purify GAD65 and identify gelatinase B degradation products with the use of mass spectrometry or NH_2-terminal sequencing analysis (*vide infra*).

Notably, the proteolytic generation of a 55 kDa remnant GAD65 fragment was previously described by Christgau *et al.* who identified a proteolytic 'hot spot' around residue 70 [17]. Cleavage at this site was suggested to have pathophysiological relevance with regard to immunogenicity. A cleavage at this site deprives the molecule from the membrane-anchoring residues, enabling its release into the extracellular environment. In the extracellular space, the native molecule is prone to be taken up by antigen-presenting cells [63].

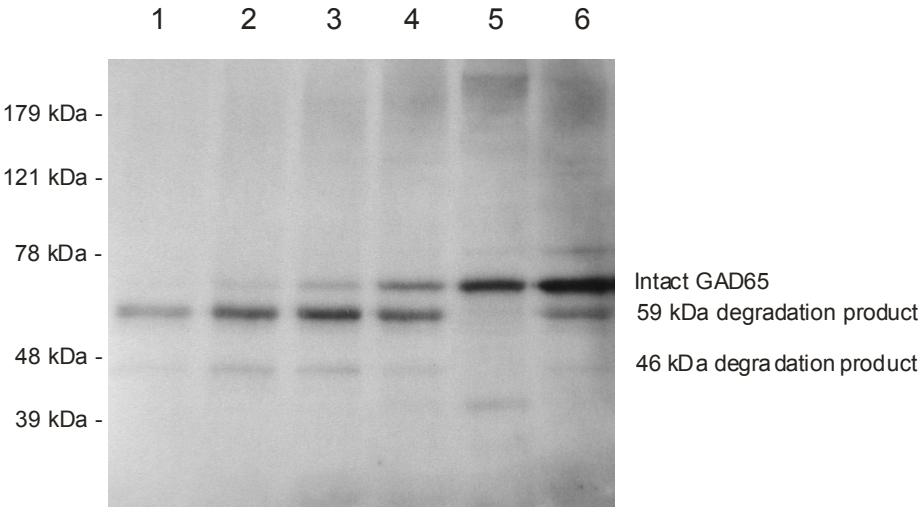


Figure 4: GAD65 Western blot analysis of brain extract (80 µg total protein), incubated with a dilution series of activated recombinant gelatinase B (lane 1: 5 µg; lane 2: 1.25 µg; lane 3: 312 ng; lane 4: 78 ng; lane 5: 0 ng) or with 100 ng activated mouse gelatinase B (lane 6).

1.2 *In* vivo significance of insulin breakdown by gelatinase B

1.2.1 Analysis of gelatinase B in pancreas tissue

Zymography analysis is widely used to examine proteinases in biological samples. The method combines molecular weight separation of proteins with the use of sodium dodecyl sulphate-polyacrylamide gelelectrophoresis (SDS-PAGE) and detection of specific proteolytic activities. When gelatinases are at issue (gelatin zymography), the substrate gelatin is co-polymerised with acrylamide in SDS-PAGE separation gels. After protein separation, denaturing SDS detergent is replaced by non-denaturing Triton X100[6], which allows partial renaturation of separated proteins. The gels are then incubated to allow gelatinases to degrade co-polymerised gelatin. Afterwards, gelatin is homogenously stained throughout the gel whereas transparent zones represent gelatinase activity. The term zymography refers to the fact that both zymogens, although inactive under physiological conditions, are detected alongside active enzymes, because of the denaturation-renaturation step that is intrinsically linked to the method. Eventually, the technique allows sensitive and individual semi-quantitative grading of pro-gelatinase A, active gelatinase A, pro-gelatinase B, active gelatinase B, neutrophil gelatinase associated lipocalin (NGAL)-complexed neutrophil gelatinase B and gelatinase B dimers and multimers.

Unfortunately, the measurement of gelatinase B in pancreas extracts is hampered due to the presence of excessive amounts of digestive enzymes, like trypsin, that non-specifically degrade gelatin. Trypsin is a compact molecule, stabilised by five intramolecular disulfide bridges and designed to be active in the gut in the presence of denaturing biliary acids. Its activity is not blocked by the presence of denaturing sodium dodecyl sulphate (SDS) during SDS-PAGE analysis. So, pancreatic trypsin fatally interferes with zymography analysis since it digests co-polymerised gelatin already during electrophoresis, resulting in complete gelatinolysis from the top of the zymogram up to the position of the molecule after electrophoresis. Another major analytical problem is the observation that gelatinase B levels in complex biological fluids may be below the detection limit of zymography analysis, since a maximum protein load of ~50 µg needs to be respected. Alternatively, Western blot analysis with an antibody against gelatinase B is not hampered by the presence of interfering enzymes. However,

[6] The ultra-low critical micellar concentration of Triton X-100 in comparison to SDS allows efficient uni-directional exchange.

despite the use of high affinity monoclonal antibodies, this method is still 10 times less sensitive compared to zymography analysis.
To overcome these problems, we needed to develop a technique in which gelatinases were pre-purified from the complex pancreas extract to eliminate interfering proteases and which meanwhile allowed concentration of gelatinases to meet detection limits. To that end, a high-throughput pre-purification system was designed with gelatin-Sepharose mini-columns (**Chapter 1**). The technique was based on the affinity of gelatinases for their substrate gelatin. With appropriate control tests, we established a new method that was quantitative and enabled us to measure gelatinase B in pancreas extracts. Moreover, it improved considerably the analysis of gelatinases in plasma and other biological fluids.

1.2.2 Active gelatinase B generation in pancreatitis

Pancreas surgery is only rarely executed because traumatic or surgical damage to the pancreas often leads to fistulization and other complications. As a consequence, pancreas biopsies of diabetic patients were not available for scientific research. As an alternative, we investigated the role of gelatinase B in animal models of type 1 diabetes.
Human type 1 diabetes is experimentally simulated in two widely used mouse models. In non-obese diabetic (NOD) mice, diabetes develops spontaneously due to genetic predisposition. Chemical induction of diabetes with the glucose analogue streptozotocin (STZ) is a model that can be applied to several mouse strains. Both models incompletely mimic the human diabetic pathology. Since we planned to use gelatinase B deficient mice, which have a C57BL6 genetic background, it was required to use the STZ-induced diabetes model. The data are represented in **Chapter 3**. We could not unequivocally indicate a role for gelatinase B in STZ-induced diabetes. Nonetheless, we do neither exclude a potential role of gelatinase B in human type 1 diabetes since we believe that STZ-induced diabetes is unrepresentative for human type 1 diabetes. The rapid onset of disease e.g., excludes any involvement of antigen-specific immunity. Instead, hyperglycaemia basically originated from a combination of apoptosis and necrosis of pancreatic β-cells. These mechanisms may have been totally overwhelming and may have interfered with the detection of more fine-tuned events.

Despite the fact that the STZ-induced diabetes model did not reveal a role of gelatinase B in diabetes development, activation of pro-gelatinase B was induced

in particular animals and was found to be related to pancreatitis in these animals. Pancreatitis refers to inflammation of the exocrine part of the pancreas, representing the acinar cells and ductular cells, where several digestive enzymes are produced (trypsinogen, chymotrypsinogen, amylase, lipase…) and secreted by the pancreatic duct into the duodenum. In addition to our findings in mice with STZ-induced diabetes, we found high expression levels of gelatinase B in pancreatic sections of patients with acute and chronic pancreatitis (Courtesy of Prof. K. Geboes) (**Chapter 2**). Furthermore, the activation of gelatinase B in pancreatitis was consolidated in a true pancreatitis model (caerulein-induced pancreatitis).

Pancreatitis is hallmarked by the presence of active trypsin in the pancreas. The concomitant presence of active gelatinase B in pancreatitis and the fact that activation of pro-gelatinase B by trypsin was previously described *in vitro* hint to potential *in vivo* relevance of pro-gelatinase B activation by trypsin. The fact that gelatinase B was completely converted to the active form in particular samples supports such hypothesis. The abundance of trypsin in the pancreas is indeed in line with the observed uncontrolled activation of pro-gelatinase B.

Patients with pancreatitis often suffer from diabetes, which is referred to as 'secondary' diabetes. We propose that the cleavage of insulin by gelatinase B may particularly have diabetogenic consequences in such setting. Apart from this, the activation of pro-gelatinase B by trypsin may constitute a mechanism how exocrine pancreas destruction may be reinforced in pancreatitis.

1.2.3 Perspectives

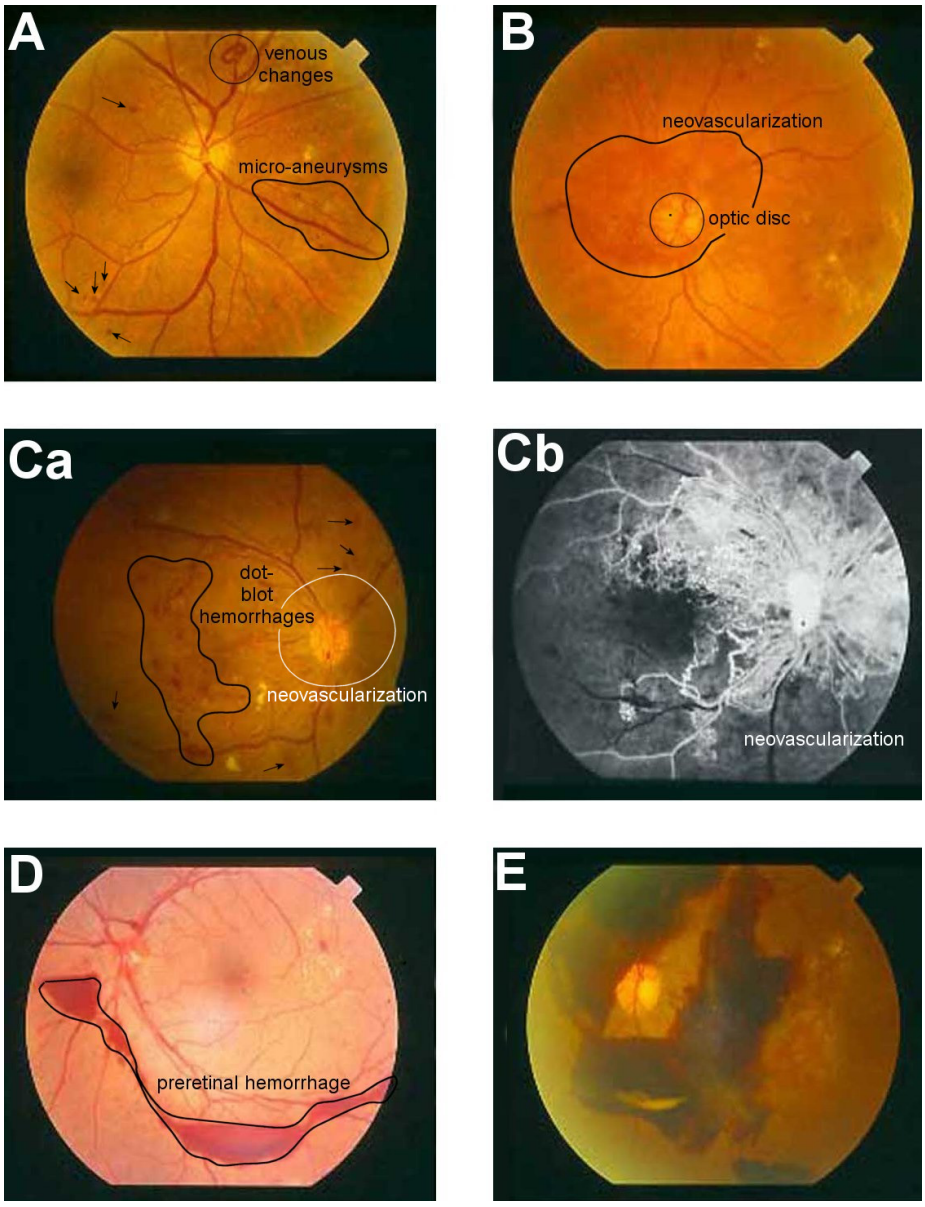
We will attempt to find further *in vivo* evidence for a role of gelatinase B in diabetes development in a pancreatitis-unrelated setting using the NOD mouse model. One possible study design could be the follow-up of the presence and activation status of gelatinase B in the pancreas (which can be evaluated through zymography analysis) in function of disease progression (which can be evaluated with the use of histochemistry).

2 Proliferative diabetic retinopathy

As a long-term microangiopathic complication of diabetes, we studied proliferative diabetic retinopathy (PDR). This pathology develops through early and late-stage phases with respective ophthalmoloscopic changes (**Figure 5**). Pre-proliferative retinopathy is characterized by microaneurysms, dot-blot hemorrhages, cotton wool spots and hard exudates. Retinal microaneurysms are focal dilatations of retinal capillaries, 10 to 100 microns in diameter, and appear as red dots. When the wall of a capillary or microaneurysm is sufficiently weakened, it may rupture, giving rise to an intraretinal haemorrhage. If the hemorrhage is deep (i.e., in the inner nuclear layer or outer plexiform layer), it usually is round or oval ("dot or blot"). If the hemorrhage is more superficial, it takes a flame shape. Cotton wool spots are white, fluffy lesions in the nerve fiber layer that result from occlusion of arterioles supplying the nerve fiber layer. Hard exudates are yellow deposits of lipid and protein within the sensory retina. As the retina becomes more ischemic, new blood vessels may arise from the optic disc or in the periphery of the retina (clearly visible with the use of fluorescein angiography). The neovessels usually originate from the venous side of the retinal circulation along the major vascular arcades and are initially seen as fine tufts on the surface of the optic disc. The neovessels also grow outside the retina into the vitreous humour and are in turn prone to bleeding. Vitreous hemorrhage (bleeding) may result in profound loss of vision if the macula is obscured. Only a small amount of blood is required since dissolved blood in the vitreous produces a haze effect which impairs vision. As the new vessels mature, connective tissue and fibrosis occurs allowing the vitreous to exert traction, which may cause retinal detachment (RD). If the detachment extends across the fovea, vision will be lost.

Our study of vitreous samples from PDR, PVR or RD patients resulted in two important findings (**Chapter 4**). First, it was shown that specifically gelatinase B and not gelatinase A was elevated in the vitreous of patients with PDR. In fact, this observation might not be surprising, in view of the finding that gelatinase B is an inducible enzyme whereas gelatinase A is produced constitutively [12]. Notably, total vitreous protein content was mainly composed of serum proteins (data not shown) that must have been trickled through the blood-retinal barrier. Together with the observation that gelatinase A correlated well with total vitreous

protein levels, this implies that gelatinase A in the vitreous most likely originated from serum.



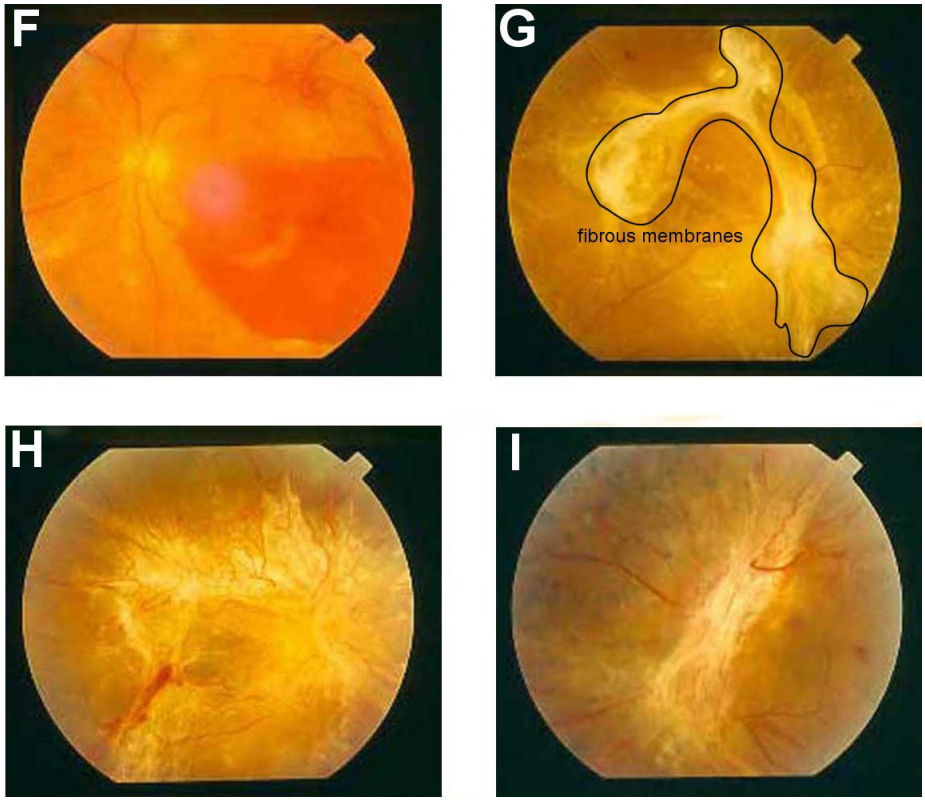


Figure 5: (A) Severe non-proliferative diabetic retinopathy showing venous changes, micro-aneurysms and dot-blot hemorrhages (arrows). (B) Proliferative diabetic retinopathy (PDR) showing optic nerve head neovessels. (C) PDR showing optic nerve head neo-vessels, venous changes and dot-blot hemorrhages (Ca). Fundus fluorescein angiography showing extensive nonperfusion of the retina (Cb). (D-E) PDR with preretinal hemorrhage. (F). PDR showing neovessels in the periphery of the retina and preretinal hemorrhage. (G-I) End-stage PDR showing extensive fibrous membranes causing traction retinal detachment (Courtesy of Prof. Dr. A. Abu-El-Asrar, King Saud University, Riyadh, Saudi-Arabia).

A second important finding in this study was the discovery of active gelatinase B in relation with the presence and degree of vitreous hemorrhage. We titrated hemoglobin in the vitreous as a quantitative measure for hemorrhage and we found a strong correlation with active MMP-9. In **Chapter 4**, we came to the interpretation that active gelatinase B had been released in the vitreous together with blood during hemorrhage. The actual bleeding (or hemorrhage) was suggested to be based on similar processes as described for cerebral hemorrhage

after ischemic stroke. We were not the first who measured gelatinase B in vitreous fluids of PDR patients. A number of other groups previously reported on the presence of pro-gelatinase B, but not the activated form (cited in **Chapter 4**). Neither of these groups specified if patients had vitreous hemorrhage, nor paid attention to the presence of the activated form of gelatinase B.

Perspectives

Apart from active gelatinase B that may be released during vitreous hemorrhage, gelatinase B conspicuously appears as 'pro'-gelatinase B in the vitreous. In that regard, it is hypothesized that the co-localization of TIMP-1 guarantees the prevention of gelatinase B activation in the vitreous (**Chapter 5**). The vitreous would then have a similar status as cartilage, in which TIMP was originally discovered [51]. Testing this hypothesis by experiment may be a perspective for the future.

In practice, we would assay the gelatinase B-buffering capacity of the vitreous, i.e. titrate the amount of activated gelatinase B that must be added to a specified volume of vitreous fluid in order to detect gelatinase B activity. As a general consideration, such assay would elegantly characterize vitreous or other tissues with regard to their protective potential against gelatinase B activity, either in physiological or pathological conditions.

3 Cataract

3.1 Technical aspects

To identify possible roles of gelatinase B in diabetes, we used different strategies throughout this doctoral research. In one approach, we evaluated the presence, up-regulation and activation status of the enzyme in relevant biological samples. To obtain a view on the pathophysiological context, we assessed additional parameters or samples that were subjected to histochemical analysis. This strategy yielded important information on the role of gelatinase B in pancreatitis (**Chapter 3**) and vitreous hemorrhagic transformation in PDR (**Chapter 4**).
In a second approach, we performed *in vitro* proteolysis assays with purified gelatinase B. We selected particular substrates (on the basis of their autoantigenic nature) and tested these for their susceptibility to cleavage. In doing so, we found insulin to be a new gelatinase B substrate (**Chapter 1**).
In a third approach, -with reference to **Chapter 5** and the current section- potential novel substrates were no longer artificially selected. Instead, protein extracts were prepared from pancreas and eye tissue and submitted to limiting concentrations of active gelatinase B in order to randomly identify novel substrates. After incubation, samples with addition of gelatinase B and control samples were subjected to SDS-PAGE analysis and protein profiles were compared. Next, selected protein bands that disappeared or bands from degradation products that appeared in mixtures with added gelatinase B, were identified. A global study of proteolytic degradation with the use of proteomics has previously been denominated "degradomics"[46]. By this approach, a 31 kDa gelatinase substrate was selected in a murine eye extract on the basis of its efficient degradation (**Chapter 5**).
Identification of proteins from biological fluids is routinely performed in our laboratory facilities by Edman degradation (courtesy of Prof P. Proost). The technique involves the cyclic degradation of proteins or peptides based on the reaction of phenylisothiocyanate with the free amino group of the NH_2-terminal residue such that amino acids are removed one at a time and identified as their phenylthiohydantoin derivatives. Unfortunately, the 31 kDa protein appeared to be NH_2-terminally blocked, i.e. the NH_2-terminal reactive group was occupied, so that the first reaction could not be executed. Therefore, we decided to purify the protein from the crude extract and characterize it afterwards with the use of mass

spectrometry[7]. Neither anion- nor cation exchange chromatography, nor RP-HPLC, nor combinations of these separation techniques were effective to purify the protein. Only the use of preparative SDS-PAGE, basically under denaturing conditions, yielded a pure 31 kDa protein fraction (**Figure 6**).

Because of the presence of SDS in the elution fractions, mass spectrometry was not an option with regard to protein identification. As an alternative, we generated a fragment from the 31 kDa protein that was NH_2-terminally 'free'. To that end, fractions were extensively dialysed and exposed to active gelatinase B. As such, the protein was identified as βB1 crystallin and at the same time, it was revealed that gelatinase B cleavage occurred in front of position P1'= Lys^{47}. In retrospect, we realize that the technical problems encountered for the purification of βB1 crystallin were rational, seeing that lens crystallins form a heteropolymeric network.

Subsequent evaluation of βB1 crystallin cleavage *ex vivo* and *in vivo* occurred with the use of two-dimensional electrophoresis, which enabled the detection of subtle shifts of the 31 kDa intact βB1 crystallin to the 24 kDa degradation product. The identity of 24 kDa protein spots was systematically confirmed with the use of an Ultraflex MALDI-TOF-TOF mass spectrometer (Bruker, Bremen, Germany, courtesy of Dr. P.E. Van den Steen and Prof. P. Proost).

Figure 6: SDS-PAGE analysis of preparative electrophoresis fractions. The 31 kDa protein of interest is indicated in red. The first lane represents the crude eye extract before preparative electrophoresis; the last lane is a molecular weight marker with proteins of respectively 116, 96, 66, 55, 31, 21, 14 and 6 kDa.

[7] Mass spectrometry may also be used to identify SDS-PAGE separated proteins after in-gel tryptic digest. However, for such techniques, MALDI-TOF-TOF MS/MS equipment is required, while the in-house mass spectrometer loads samples via electron-spray.

3.2 Proteolytic truncation of lens crystallins as a mechanism for cataractogenesis

After identification of βB1 crystallin as an efficient gelatinase B substrate *in vitro*, it was verified if such cleavage could be relevant *in vivo*. The finding of a lens crystallin to be the most efficient substrate in the eye hinted to a role of this particular cleavage in cataract formation. We gathered *ex vivo* and *in vivo* data that supported this presumption (**Chapter 5**). The cleavage of βB1 crystallin was observed *in vivo* after endogenous gelatinase B induction, and, incubations of lenses with gelatinase B *ex vivo* immediately led to cataract.

Unexpectedly, we found that direct injection of relative high amounts of active recombinant gelatinase B (1 µg/eye) did not result in more radical cleavage of βB1 crystallin than endogenously induced gelatinase B, which represented lower amounts. An explanation could be the high turn-over rate of the injected material. Only a small fraction of the enzyme could be recovered shortly after injection. We hypothesize therefore that gelatinase B, which is delivered by host immune cells (neutrophils, attracted by chemokines such as GCP-2), is released in a more directed fashion and may accumulate locally to high concentrations. The cleavage of substrates probably occurs immediately after release of the enzyme, before it is drained away by ocular clearing mechanisms.

The methodology of this study was not directed to identify a role of gelatinase B in diabetes. Hence, the fact that cataract may be a complication of diabetes is a coincidence in the framework of this study on diabetes. Cataract may develop through several mechanisms. With regard to diabetic cataract, osmotic stress imposed by sorbitol accumulation in the ocular lens has long been suggested to be the major culprit. Besides, also oxidative stress and direct glycation of crystallin proteins may contribute to crystallin insolubilization and lens opacification. The clipping of βB1 crystallin by gelatinase B is related to yet another mechanism of cataract formation, namely the unregulated proteolysis of crystallins. In diabetes, the long-term elevated gelatinase B levels in the vitreous of patients with PDR (**Chapter 5**) may gradually account for crystallin cleavage and lead to slowly progressive cataract. However, it is questionable if gelatinase B is active in the vitreous of PDR patients regarding the fact that TIMP-1 is also elevated in these patients (cfr. **Chapter 4**). In fact, we argue that our findings may rather be relevant in situations of intraocular inflammation (uveitis), e.g. in patients with Behçet's disease which is characterized by high levels of intraocular gelatinase B [2].

4 Clinical practice

At present, diabetes is predominantly treated by insulinotherapy, controlling blood glucose levels. insulinotherapy prevents short-term complications of hyperglycaemia that are directly related to a disbalance in energy metabolism as like polyuria, polydipsia and ketoacidosis. However, the DCCT and UKPDS studies indicate that tight controlled insulinotherapy also retards the development of long-term complications. Curative therapy implies replacement of a patient's islets of Langerhans, either by pancreas organ transplantation or by isolated islet transplantation. It is the only treatment to achieve a constant normoglycemic state and avoid hypoglycaemic episodes, a typical adverse event of multiple daily insulin injections. However, the expense of the benefit is still the need for immunosuppressive treatment of the recipient with all its potential risks. Stem-cell therapy implies the replacement of diseased or lost cells from progeny of pluripotent or multipotent cells. Both embryonic stem cells and adult stem cells have been used to generate surrogate β-cells or otherwise restore β-cell functioning.

The studies in the current doctoral research hint to new therapeutic avenues, using MMP inhibitors in the treatment of secondary diabetes and diabetic complications. Small molecular weight inhibitors like peptide hydroxamates act by chelation of the catalytic Zn^{2+} but are unselective between different MMPs and may therefore cause side-effects. To achieve higher selectivity, we are currently designing longer peptidomimetics that mimic a consensus sequence of a substrate binding site but are uncleavable [37]. Recently, the use of the peptidomimetic inhibitor Regasepin1 has convincingly been shown to protect against endotoxin shock, either when delivered intraperitoneally or intravenously [38]. Also the antibody inhibitor REGA-3G12, which has recently been discovered to bind to the active site domain of gelatinase B is a potential therapeutic drug. A less immunogenic single chain Fv derivative has been synthesized [89]. The effectiveness of this molecule is under investigation.

SAMENVATTING

Gelatinase B behoort tot de matrix metalloproteinasen, een familie van meer dan 20 enzymen. Matrix metalloproteinasen dragen een zinkion in het actief centrum en degraderen efficiënt componenten van de extracellulaire matrix. Gelatinase B heeft, naast zijn fysiologische functie, ook een pathologische impact in processen zoals invasie en metastasering van kankercellen en antigeenbewerking bij autoimmuniteit. Een van de mogelijke verklaringen hiervoor is het feit dat gelatinase B naast extracellulaire matrixeiwitten ook atypische substraten klieft. Het REGA-model -een letterwoord dat staat voor 'restepitopen genereren autoimmuniteit'- stelt in het licht dat extracellulaire proteolyse van autoantigenen cruciaal kan zijn voor het induceren van een tolerantiebreuk tegenover lichaamseigen antigenen, meer bepaald door de vrijzetting van immunodominante epitopen die normaal 'verborgen' zijn in de drie-dimensionale structuur van het intacte molecule. Dit doctoraatsproject is gegroeid vanuit onze belangstelling naar een mogelijke rol van gelatinase B bij de ontwikkeling van de autoimmuunziekte (juveniele) type 1 diabetes.

Type 1 diabetes ontstaat door autoimmuun-gemedieerde destructie van de β cellen van de eilandjes van Langerhans, die instaan voor de aanmaak van insuline. Insuline is een sleutelmolecule voor het handhaven van evenwichtige bloedglucosewaarden en is tegelijkertijd een belangrijk autoantigeen. In type 1 diabetes ontwikkelt het immuunsysteem dus een immuunrespons tegen lichaamseigen insuline. Wij ontdekten dat insuline wordt afgebroken door gelatinase B tot welgedefinieerde, inactieve fragmenten. Logischerwijze kan deze inactivatie rechtstreeks diabetogene consequenties hebben. De aard van de gevormde klievingsfragmenten levert echter geen sluitende argumenten voor een eventuele implementering van het REGA-model als verklaring voor de ontwikkeling van autoimmuniteit tegen insuline.

Om de rol van gelatinase B verder te substantiëren *in vivo*, werd het eiwit gemeten in pancreasweefsels van proefdieren. Daarbij was het uiterst belangrijk om een onderscheid te kunnen maken tussen inactief 92 kDa 'pro'-gelatinase B en de 82 kDa actieve vorm. Gelatinezymografie is een techniek die dit toelaat. Om zymografische analysen te kunnen uitvoeren van complexe biologische stalen ontwikkelden we een elegante prezuiveringstechniek voor gelatinasen. Zonder deze techniek zijn analysen van gelatinase B in de pancreas onmogelijk door de overweldigende interferentie van verteringsenzymen. In muizen waarin diabetes werd geïnduceerd door toediening van de chemische component

streptozotocine toonden we op deze wijze aan dat gelatinase B verhoogd tot expressie komt in pancreasweefsel, kort na de inductie van diabetes. Het enzym is echter vooral aanwezig in zijn inactieve vorm en gelatinase B-knockout muizen zijn niet beschermd tegen de ontwikkeling van diabetes.
Immunohistochemische analyse van gelatinase B in pancreasweefsel van pancreatitispatiënten duidt op een sterke expressie van het enzym in infiltrerende leukocyten en ductulaire cellen. Pancreatitis is een pathologie die refereert naar het exocriene deel van de pancreas, maar pancreatitispatiënten ontwikkelen opvallend vaak secundaire diabetes. Dubbelkleuringen voor insuline en gelatinase B toonden de expressie van de respectievelijke eiwitten aan in cellen die rechtstreeks contact maken. Immunohistochemische analysen geven echter geen uitsluitsel over de activatietoestand van het enzym. Om activatie van gelatinase B te bestuderen in pancreasweefsel bij ontstekingen induceerden we pancreatitis in muizen. Hierbij toonden we aan dat de aanwezigheid van actief trypsine in de pancreas, een diagnostisch kenmerk van pancreatitis, gerelateerd is met de aanwezigheid van actief gelatinase B. Bij deze studie ontdekten en beschreven wij als eersten de *in vivo* activatie van gelatinase B door trypsine, een mechanisme dat tot hiertoe enkel *in vitro* werd gedocumenteerd. Deze resultaten, samen met de klieving van insuline door gelatinase B, suggereren dat een diabetogeen effect door insuline-afbraak in het bijzonder van toepassing kan zijn bij pancreatitiden. Dit kan van belang zijn voor de ontwikkeling van secundaire diabetes. Bovendien kan de activatie van gelatinase B in de pancreas bij pancreatitis een mechanisme zijn die de verdere afbraak van de exocriene pancreas in de hand werkt.
Daarnaast onderzochten we de rol van gelatinase in een diabetes-gerelateerde pathologie. Proliferatieve diabetische retinopathie is een microangiopathische aandoening van de retina bij diabetespatiënten waarbij de hyperglycemische toestand van het bloed leidt tot verdikking van de basale membraan van de retinale haarvaatjes en een versmalling van het lumen. Dit leidt tot een ischemische toestand die op zijn beurt de ontwikkeling van nieuwe bloedvaatjes stimuleert (neovascularizatie). Deze nieuwe haarvaatjes groeien roekeloos vanuit de retina tot in het glasachtig lichaam (corpus vitreum). Deze bloedvaatjes breken makkelijk waarbij bloed in het glasachtig lichaam terecht komt en een troebel effect veroorzaakt. Wij detecteerden geactiveerd gelatinase B in het glasachtig lichaam van patiënten met proliferatieve diabetische retinopathie, meer bepaald en quasi exlusief in patiënten met intravitreale bloedingen. Waarschijnlijk speelt

gelatinase B hier een rol in de afbraak van de basale membraan van de 'nieuwe' haarvaatjes, hetgeen uiteraard rechtstreeks bloedingen tot gevolg kan hebben.
Eveneens in het oog, maar niet noodzakelijk in diabetici, toonden we aan dat gelatinase B nog een ander schadelijk effect kan hebben, namelijk het onstaan van cataract. We vonden dat lenzen van muizen, die in cultuur worden gebracht, snel cataract ontwikkelen na toevoeging van gelatinase B aan het cultuurmedium. De klieving van βB1 crystalline, een structurele component van de lens- ligt aan de basis van de geobserveerde vertroebeling. We demonstreren dat inductie van endogeen gelatinase B in het oog van muizen voldoende is om dezelfde klieving van βB1 crystalline te induceren *in vivo*. Bij controle-inducties in gelatinase B knockoutmuizen blijft het eiwit immers intact.

Onze resultaten duiden op een diabetogene rol van gelatinase B, niet zozeer als verwekker van autoimmuniteit, maar door directe vernietiging van insuline. Daarnaast tonen we aan dat gelatinase B schadelijk kan zijn bij proliferatieve diabetische retinopathie en bij het ontstaan van cataract.

SUMMARY

Gelatinase B is a member of the family of matrix metalloproteinases. This family represents more than 20 proteolytic enzymes that contain a zinc ion in the active site and degrade components of the extracellular matrix. In addition to its function in a variety of physiological processes, gelatinase B has detrimental effects in several pathological conditions. This may partially be explained by an enzymatic bystander effect by which it affects a number of serendipitous substrates. The REGA model for autoimmune diseases –abbreviated remnant epitopes generate autoimmunity- refers to the possibility that extracellular proteolysis of autoantigens may be crucial for the induction of loss of tolerance to 'own' antigens, more specifically through the release of immunodominant epitopes that used to be hidden in the molecule's three-dimensional structure. My doctoral program started from the curiosity to study the potential role of gelatinase B in the development of diabetes, specifically autoimmune juvenile type 1 diabetes.

Type 1 diabetes progresses from autoimmune-mediated destruction of the insulin-producing pancreatic β-cells. Insulin is a key molecule in glucose homeostasis and at the same time, a most important autoantigen. We found that gelatinase B efficiently processes insulin into well-defined inactive fragments. The cleavage of insulin may have a direct diabetogenic effect but from the observed insulin clippings, we have at present no argumentation for the concept of immunodominant epitope generation.

For *in vivo* assessments of gelatinase B, it was crucial to distinguish between inactive 92 kDa pro-gelatinase B and the active 82 kDa form. To that end, we have refined the methodology of classical zymography analysis into a method applicable to complex biological samples and/or samples with interfering proteases (*in casu* trypsin). With this method, we could demonstrate the induction of gelatinase B expression in the pancreas of mice with acute streptozotocin-induced diabetes. However, the activation status of the enzyme was low and gelatinase B deficient mice were not protected against diabetes progression.

Human pancreas tissue was not available. Instead, we analysed pancreatic sections from patients with pancreatitis, a condition which mainly refers to the exocrine part of the pancreas. Immunohistochemical analysis of gelatinase B revealed intense positive staining of infiltrating cells and pancreatic ducts. Double immunostainings for insulin and gelatinase B showed the expression of both molecules by cells in close or direct contact. However, the presence of the

enzyme did not necessarily imply that the enzyme was proteolytically active. In that regard, we gathered data from two mouse models showing that active trypsin in the pancreas, which is a hallmark of pancreatitis, catalyzes the activation of pro-gelatinase B, a mechanism that was hitherto only demonstrated *in vitro*. In line with the fact that patients with pancreatitis often suffer from 'secondary' diabetes, we propose that the cleavage of insulin by gelatinase B may particularly have diabetogenic consequences in this setting. Besides, we note that the activation of gelatinase B in the pancreas of pancreatitis patients may catalyze the further destruction of the exocrine pancreas.

Two other diabetes-related pathologies were investigated with regard to a possible function of gelatinase B. Proliferative diabetic retinopathy is a microangiopathic complication in diabetic patients in which long-lasting hyperglycaemia induces the thickening of retinal basement membranes and concomitant luminal narrowing. This results in an ischemic state, which in turn stimulates the growing of new vessels (neovascularization). New blood vessels grow rashly from the retina into the vitreous chamber and are prone to bleeding (vitreous hemorrhage). We found activated gelatinase B in the vitreous of patients with proliferative diabetic retinopathy, more specifically and exclusively in those with vitreous hemorrhage. Gelatinase B probably plays a role here in the breakdown of the basal membrane of the new vessels, which may directly result in vitreous bleedings.

Also in the eye, but not exclusively in the diabetic eye, a detrimental effect of gelatinase B was discovered with regard to cataract formation. Opacification of *in vitro* cultured lenses occurred quickly after exposure to active gelatinase B. The cleavage of βB1 crystallin was discovered as a possible biochemical basis that explained the opacification. Moreover, we demonstrated that induction of endogenous gelatinase B was sufficient to trigger the same cleavage of βB1 crystallin *in vivo*. Control inductions in gelatinase B deficient mice had no effect on βB1 crystallin.

In conclusion, our data are in support of a role of gelatinase B in diabetes, not primarily as an inducer of autoimmunity, but as a direct inactivator of insulin. In addition, gelatinase B is a detrimental agent in proliferative diabetic retinopathy and in the development of cataract.

REFERENCE LIST

1. Abiru N, Maniatis AK, Yu L, Miao D *et al*. 2001. Peptide and major histocompatibility complex-specific breaking of humoral tolerance to native insulin with the B9-23 peptide in diabetes-prone and normal mice *Diabetes* **50**:1274-1281.

2. Abu El-Asrar AM, Struyf S, Descamps FJ, Al Obeidan SA *et al*. 2004. Chemokines and gelatinases in the aqueous humor of patients with active uveitis *Am J Ophthalmol* **138**:401-411.

3. Akashi T, Nagafuchi S, Anzai K, Kondo S *et al*. 1997. Direct evidence for the contribution of B cells to the progression of insulitis and the development of diabetes in non-obese diabetic mice *Int Immunol* **9**:1159-1164.

4. Akerblom HK and Knip M 1998. Putative environmental factors in Type 1 diabetes *Diabetes Metab Rev* **14**:31-67.

5. Alleva DG, Crowe PD, Jin LP, Kwok WW *et al*. 2001. A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin *J Clin Invest* **107**:173-180.

6. Andersen OO 1976. Anti-insulin-antibodies and late diabetic complications *Acta Endocrinol (Copenh)* **83**:329-340.

7. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A *et al*. 1990. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase *Nature* **347**:151-156.

8. Baker EN, Blundell TL, Cutfield JF, Cutfield SM *et al*. 1988. The Structure of 2Zn Pig Insulin Crystals at 1.5-A Resolution *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **319**:369.

9. Bennett ST, Lucassen AM, Gough SCL, Powell EE *et al*. 1995. Susceptibility to Human Type-1 Diabetes at Iddm2 Is Determined by Tandem Repeat Variation at the Insulin Gene Minisatellite Locus *Nat Genet* **9**:284-292.

10. Bi RC, Dauter Z, Dodson E, Dodson G *et al*. 1984. Insulin structure as a modified and monomeric molecule *Biopolymers* **23**:391-395.

11. Bierhaus A, Schiekofer S, Schwaninger M, Andrassy M *et al*. 2001. Diabetes-associated sustained activation of the transcription factor nuclear factor-kappa B *Diabetes* **50**:2792-2808.

12. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ *et al*. 1993. Matrix metalloproteinases: a review *Crit Rev Oral Biol Med* **4**:197-250.

13. Blanas E, Carbone FR, Allison J, Miller JF *et al*. 1996. Induction of autoimmune diabetes by oral administration of autoantigen *Science* **274**:1707-1709.

14. Cerami A, Vlassara H, and Brownlee M 1986. Role of Nonenzymatic Glycosylation in Atherogenesis *J Cell Biochem* **30**:111-120.

15. Cetkovic-Cvrlje M and Eizirik DL 1994. TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly via generation of nitric oxide *Cytokine* **6**:399-406.

16. Chen W, Bergerot I, Elliott JF, Harrison LC *et al*. 2001. Evidence that a peptide spanning the B-C junction of proinsulin is an early autoantigen epitope in the pathogenesis of type 1 diabetes *The Journal of Immunology* **167**:4926-4935.

17. Christgau S, Aanstoot HJ, Schierbeck H, Begley K *et al*. 1992. Membrane anchoring of the autoantigen GAD65 to microvesicles in pancreatic beta-cells by palmitoylation in the NH2-terminal domain *J Cell Biol* **118**:309-320.

18. Christianson SW, Shultz LD, and Leiter EH 1993. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors *Diabetes* **42**:44-55.

19. Daniel D, Gill RG, Schloot N, and Wegmann D 1995. Epitope Specificity, Cytokine Production Profile and Diabetogenic Activity of Insulin-Specific T-Cell Clones Isolated from Nod Mice *Eur J Immunol* **25**:1056-1062.

20. Daniel D and Wegmann DR 1996. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23) *Proc Natl Acad Sci USA* **93**:956-960.

21. Davidson HW and Watts C 1989. Epitope-Directed Processing of Specific Antigen by Lymphocyte-B *J Cell Biol* **109**:85-92.

22. Davies JL, Kawaguchi Y, Bennett ST, Copeman JB *et al*. 1994. A genome-wide search for human type 1 diabetes susceptibility genes *Nature* **371**:130-136.

23. Derewenda U, Derewenda Z, Dodson EJ, Dodson GG *et al*. 1991. X-Ray-Analysis of the Single Chain-B29-A1 Peptide-Linked Insulin Molecule - A Completely Inactive Analog *J Mol Biol* **220**:425-433.

24. Di Mario U, Borsey DQ, Contreas G, Prowse CV *et al*. 1986. The relationship of soluble immune complexes, insulin antibodies and insulin-anti-insulin

complexes to platelet and coagulation factors in type 1 diabetic patients with and without proliferative retinopathy *Clin Exp Immunol* **65**:57-65.

25. Dreier R, Grassel S, Fuchs S, Schaumburger J *et al*. 2004. Pro-MMP-9 is a specific macrophage product and is activated by osteoarthritic chondrocytes via MMP-3 or a MT1-MMP/MMP-13 cascade *Exp Cell Res* **297**:303-312.

26. Dubois B, Masure S, Hurtenbach U, Paemen L *et al*. 1999. Resistance of young gelatinase B-deficient mice to experimental autoimmune encephalomyelitis and necrotizing tail lesions *J Clin Invest* **104**:1507-1515.

27. DurinovicBello I, Hummel M, and Ziegler AG 1996. Cellular immune response to diverse islet cell antigens in IDDM *Diabetes* **45**:795-800.

28. Ellis T, Jodoin E, Ottendorfer E, Salisbury P *et al*. 1999. Cellular immune responses against proinsulin - No evidence for enhanced reactivity in individuals with IDDM *Diabetes* **48**:299-303.

29. Gardner SG, Gale EAM, Williams AJK, Gillespie KM *et al*. 1999. Progression to diabetes in relatives with islet autoantibodies - Is it inevitable *Diabetes Care* **22**:2049-2054.

30. Gately MK, Renzetti LM, Magram J, Stern AS *et al*. 1998. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses *Annu Rev Immunol* **16**:495-521.

31. Glowacka E, Banasik M, Lewkowicz P, and Tchorzewski H 2002. The Effect of LPS on Neutrophils from Patients with High Risk of Type 1 Diabetes Mellitus in relation to IL-8, IL-10 and IL-12 Production and Apoptosis In Vitro *Scand J Immunol* **55**:210-217.

32. Gugliucci A 2000. Glycation as the glucose link to diabetic complications *J Am Osteopath Assoc* **100**:621-634.

33. Haskins K and Wegmann D 1996. Diabetogenic T-cell clones *Diabetes* **45**:1299-1305.

34. Hayase F, Nagaraj RH, Miyata S, Njoroge FG *et al*. 1989. Aging of Proteins - Immunological Detection of A Glucose-Derived Pyrrole Formed During Maillard Reaction In vivo *J Biol Chem* **264**:3758-3764.

35. Hou J, Said C, Franchi D, Dockstader P *et al*. 1994. Antibodies to glutamic acid decarboxylase and P2-C peptides in sera from coxsackie virus B4-infected mice and IDDM patients *Diabetes* **43**:1260-1266.

36. Howard EW, Bullen EC, and Banda MJ 1991. Regulation of the autoactivation of human 72-kDa progelatinase by tissue inhibitor of metalloproteinases-2 *J Biol Chem* **266**:13064-13069.

37. Hu J, Fiten P, Van den Steen PE, Chaltin P *et al*. 2005. Simulation of evolution-selected propeptide by high-throughput selection of a peptidomimetic inhibitor on a capillary DNA sequencer platform *Anal Chem* **77**:2116-2124.

38. Hu J, Van den Steen PE, Dillen C, and Opdenakker G 2005. Targeting neutrophil collagenase/matrix metalloproteinase-8 and gelatinase B/matrix metalloproteinase-9 with a peptidomimetic inhibitor protects against endotoxin shock *Biochem Pharmacol* in press.

39. Hua QX, Hu SQ, Frank BH, Jia WH *et al*. 1996. Mapping the functional surface of insulin by design: Structure and function of a novel A-chain analogue *J Mol Biol* **264**:390-403.

40. Hua QX, Shoelson SE, Kochoyan M, and Weiss MA 1991. Receptor-Binding Redefined by A Structural Switch in A Mutant Human Insulin *Nature* **354**:238-241.

41. Hua QX and Weiss MA 1991. Comparative 2D-Nmr Studies of Human Insulin and Des-Pentapeptide Insulin - Sequential Resonance Assignment and Implications for Protein Dynamics and Receptor Recognition *Biochemistry* **30**:5505-5515.

42. Kaprio J, Tuomilehto J, Koskenvuo M, Romanov K *et al*. 1992. Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland *Diabetologia* **35**:1060-1067.

43. Kendall PL, Woodward EJ, Hulbert C, and Thomas JW 2004. Peritoneal B cells govern the outcome of diabetes in non-obese diabetic mice *Eur J Immunol* **34**:2387-2395.

44. Koarada S, Wu Y, and Ridgway WM 2001. Increased entry into the IFN-gamma effector pathway by CD4+ T cells selected by I-Ag7 on a nonobese diabetic versus C57BL/6 genetic background *J Immunol* **167**:1693-1702.

45. Kyvik KO, Green A, and Beck-Nielsen H 1995. Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins *BMJ* **311**:913-917.

46. Lopez-Otin C and Overall CM 2002. Protease degradomics: A new challenge for proteomics *Nat Rev Mol Cell Biol* **3**:509-519.

47. Lorenzi M 1992. Glucose Toxicity in the Vascular Complications of Diabetes - the Cellular Perspective *Diabetes Metab Rev* **8**:85-103.

48. Maeda A and Sobel RA 1996. Matrix metalloproteinases in the normal human central nervous system, microglial nodules, and multiple sclerosis lesions *J Neuropathol Exp Neurol* **55**:300-309.

49. Marshall AJ, Niiro H, Yun TJ, and Clark EA 2000. Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase Cgamma pathway *Immunol Rev* **176**:30-46.

50. Monnier VM, Kohn RR, and Cerami A 1984. Accelerated Age-Related Browning of Human Collagen in Diabetes-Mellitus *Proc Natl Acad Sci USA* **81**:583-587.

51. Moses MA, Sudhalter J, and Langer R 1990. identification of an inhibitor of neovascularization from cartilage *Science* **248**:1408-1410.

52. Ohashi PS, Oehen S, Buerki K, Pircher H *et al*. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice *Cell* **65**:305-317.

53. Oldstone MB, Nerenberg M, Southern P, Price J *et al*. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response *Cell* **65**:319-331.

54. Opdenakker G, Masure S, Grillet B, and Van Damme J 1991. Cytokine-mediated regulation of human leukocyte gelatinases and role in arthritis *Lymphokine Cytokine Res* **10**:317-324.

55. Opdenakker G and Van Damme J 1994. Cytokine-regulated proteases in autoimmune diseases *Immunol Today* **15**:103-107.

56. Palmer JP, Asplin CM, Clemons P, Lyen K *et al*. 1983. Insulin-Antibodies in Insulin-Dependent Diabetics Before Insulin-Treatment *Science* **222**:1337-1339.

57. Platz P, Jakobsen BK, Morling N, Ryder LP *et al*. 1981. HLA-D and -DR antigens in genetic analysis of insulin dependent diabetes mellitus *Diabetologia* **21**:108-115.

58. Pociot F and McDermott MF 2002. Genetics of type 1 diabetes mellitus *Genes Immun* **3**:235-249.

59. Proost P, Van Damme J, and Opdenakker G 1993. Leukocyte gelatinase B cleavage releases encephalitogens from human myelin basic protein *Biochem Biophys Res Commun* **192**:1175-1181.

60. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H *et al*. 1999. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion *J Biol Chem* **274**:13066-13076.

61. Rudy G, Stone N, Harrison LC, Colman PG *et al*. 1995. Similar Peptides from 2 Beta-Cell Autoantigens, Proinsulin and Glutamic-Acid Decarboxylase, Stimulate T-Cells of Individuals at Risk for Insulin-Dependent Diabetes *Mol Med* **1**:625-633.

62. Santambrogio L, Sato AK, Carven GJ, Belyanskaya SL *et al*. 1999. Extracellular antigen processing and presentation by immature dendritic cells *Proc Natl Acad Sci USA* **96**:15056-15061.

63. Schwartz HL, Chandonia JM, Kash SF, Kanaani J *et al*. 1999. High-resolution autoreactive epitope mapping and structural modeling of the 65 kDa form of human glutamic acid decarboxylase *J Mol Biol* **287**:983-999.

64. Serrano-Rios M, Goday A, and Martinez LT 1999. Migrant populations and the incidence of type 1 diabetes mellitus: an overview of the literature with a focus on the Spanish-heritage countries in Latin America *Diabetes Metab Res Rev* **15**:113-132.

65. Soldevila G, Buscema M, Doshi M, James RF *et al*. 1991. Cytotoxic effect of IFN-gamma plus TNF-alpha on human islet cells *J Autoimmun* **4**:291-306.

66. Solimena M and De Camilli P 1991. Autoimmunity to glutamic acid decarboxylase (GAD) in Stiff-Man syndrome and insulin-dependent diabetes mellitus *Trends Neurosci* **14**:452-457.

67. Springman EB, Angleton EL, Birkedalhansen H, and Van Wart HE 1990. Multiple-Modes of Activation of Latent Human Fibroblast Collagenase - Evidence for the Role of A Cys-73 Active-Site Zinc Complex in Latency and A Cysteine Switch Mechanism for Activation *Proc Natl Acad Sci USA* **87**:364-368.

68. Stevens VJ, Vlassara H, Abati A, and Cerami A 1977. Nonenzymatic Glycosylation of Hemoglobin *J Biol Chem* **252**:2998-3002.

69. Strobel S and Mowat AM 1998. Immune responses to dietary antigens: oral tolerance *Immunol Today* **19**:173-181.

70. Suarez-Pinzon W, Rajotte RV, Mosmann TR, and Rabinovitch A 1996. Both CD4+ and CD8+ T-cells in syngeneic islet grafts in NOD mice produce interferon-gamma during beta-cell destruction *Diabetes* **45**:1350-1357.

71. Sumida T, Furukawa M, Sakamoto A, Namekawa T *et al*. 1994. Prevention of insulitis and diabetes in beta 2-microglobulin-deficient non-obese diabetic mice *Int Immunol* **6**:1445-1449.

72. Tamimi R, Steingrimsson E, Copeland NG, Dyer-Montgomery K *et al*. 1996. The NEUROD gene maps to human chromosome 2q32 and mouse chromosome 2 *Genomics* **34**:418-421.

73. Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A *et al*. 1996. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes *J Exp Med* **183**:1561-1567.

74. Todd JA, Bell JI, and McDevitt HO 1987. HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus *Nature* **329**:599-604.

75. Trembleau S, Penna G, Gregori S, Gately MK *et al*. 1997. Deviation of pancreas-infiltrating cells to Th2 by interleukin-12 antagonist administration inhibits autoimmune diabetes *Eur J Immunol* **27**:2330-2339.

76. Vaarala O, Paronen J, Otonkoski T, and Akerblom HK 1998. Cow milk feeding induces antibodies to insulin in children--a link between cow milk and insulin-dependent diabetes mellitus? *Scand J Immunol* **47**:131-135.

77. Van den Steen PE, Proost P, Brand DD, Kang AH *et al*. 2004. Generation of glycosylated remnant epitopes from human collagen type II by gelatinase B *Biochemistry* **43**:10809-10816.

78. Van den Steen PE, Proost P, Grillet B, Brand DD *et al*. 2002. Cleavage of denatured natural collagen type II by neutrophil gelatinase B reveals enzyme specificity, post-translational modifications in the substrate, and the formation of remnant epitopes in rheumatoid arthritis *FASEB J* **16**:379-389.

79. Visse R and Nagase H 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry *Circ Res* **92**:827-839.

80. von Boehmer H and Sarukhan A 1999. GAD, a single autoantigen for diabetes *Science* **284**:1135, 1137.

81. von Herrath MG, Dockter J, and Oldstone MB 1994. How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model *Immunity* **1**:231-242.

82. Wang B, Gonzalez A, Benoist C, and Mathis D 1996. The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus *Eur J Immunol* **26**:1762-1769.

83. Wautier JL, Zoukourian C, Chappey O, Wautier MP *et al*. 1996. Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy - Soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats *J Clin Invest* **97**:238-243.

84. Wegmann DR, Norburyglaser M, and Daniel D 1994. Insulin-Specific T-Cells Are A Predominant Component of Islet Infiltrates in Prediabetic Nod Mice *Eur J Immunol* **24**:1853-1857.

85. Wolf E, Spencer KM, and Cudworth AG 1983. The genetic susceptibility to type 1 (insulin-dependent) diabetes: analysis of the HLA-DR association *Diabetologia* **24**:224-230.

86. Wong FS, Karttunen J, Dumont C, Wen L *et al*. 1999. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library *Nat Med* **5**:1026-1031.

87. Yoon JW, Yoon CS, Lim HW, Huang QQ *et al*. 1999. Control of autoimmune diabetes in NOD mice by GAD expression or suppression in beta cells *Science* **284**:1183-1187.

88. Yu LP, Robles DT, Abiru N, Kaur P *et al*. 2000. Early expression of antiinsulin autoantibodies of humans and the NOD mouse: Evidence for early determination of subsequent diabetes *Proc Natl Acad Sci USA* **97**:1701-1706.

89. Zhou N, Paemen L, Opdenakker G, and Froyen G 1997. Cloning and expression in Escherichia coli of a human gelatinase B-inhibitory single-chain immunoglobulin variable fragment (scFv) *FEBS Lett* **414**:562-566.