COXSACKIEVIRUS INFECTIONS AND OXIDATIVE STRESS AS MEDIATORS OF BETA CELL DAMAGE

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To all close Friends and Relatives

ABSTRACT

Type I diabetes (T1D) is an autoimmune disease resulting in gradual cell-mediated destruction of insulin producing beta cells in the pancreatic islets of Langerhans. The most plausible environmental triggers to launch and/or accelerate this process in a genetically predisposed organism include enterovirus infections and oxidative stress. Among other enteroviruses the group B of coxsackieviruses is associated with potential beta cell toxicity. Beta cells are weak in antioxidative defense, which makes them hypersensitive to oxidative stress. The viral and oxidative hazards may interact in an additive fashion in suitable conditions and result in cumulative damage. However, prior to the present study the mechanisms of the resulting beta cell damage were poorly characterized. Thus, this study was set to clarify the patterns and consequences of coxsackie B virus infections and the effects of experimental oxidative stress in various cell culture models to understand the mechanistic pattern of beta cell damage and death.

The performed experiments were planned with the particular intention to detect different forms of beta cell death due to varying viral and oxidative conditions. We chose the coxsackie B strain (CVB) because of its aggressive nature in cell systems and the previously characterized epidemiologic link to T1D. Acknowledging the inhibitory potential of *in vivo* conditions we developed two models resembling a slowly progressing coxsackievirus infection first, by restricting the production of viral progeny with a selective inhibitor of viral RNA replication and second, by means of lowering the multiplicity of infection (comparable to the amount of infective viral particles per cell). Hydrogen peroxide was selected to serve as the oxidative stressor because it represents a physiological agent present during most oxidative processes *in vivo*. L-cysteine, the precursor of the most important intracellular antioxidant glutathione, was chosen to counteract hydrogen peroxide.

This study shows that a productive CVB-infection results in lytic beta cell death. When pharmacologically restricted by guanidine-HCl, the viability increases dramatically through decreased necrosis and associates with simultaneous stimulation of apoptotic death. A similar pattern of host cell death characterizes a CVB5 infection of low multiplicity. L-cysteine protects dose-dependently against hydrogen peroxide

induced damage. Also apoptosis provoked by a moderate level of hydrogen peroxide is reversed by L-cysteine. The intracellular glutathione content transiently decreases during a low multiplicity CVB5 infection, but shows full recovery at later timepoints. This might imply a more physiological type of damage, which has previously been linked to induction of apoptotic cell death. Furthermore, the glutathione machinery might have a protective role against damage induced by coxsackievirus infection.

In conclusion, this study introduces potential mechanistic models for enterovirus infections in beta cells. It points to apoptosis as a favored outcome during a slowly progressing prototype coxsackievirus B5 infection, which is in contrast to the lytic beta cell death that dominates a freely progressing, high multiplicity coxsackievirus B5 infection. Moreover, when correctly administered, substituent antioxidants efficiently protect insulin-producing cells against oxidative damage. Intracellular glutathione system may also contribute to the survival of CVB-infected beta cells independent of the pace of progression of infection.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on these four original publications, referred in the text by their Roman numerals:

I   Roivainen M, Rasilainen S, Ylipaasto P, Nissinen R, Ustinov J, Bouwens L, Eizirik DL, Hovi T and Otonkoski T. (2000) Mechanisms of Coxsackievirus-induced damage to human pancreatic beta cells. The Journal of Clinical Endocrinology & Metabolism 85, 432-440.

II   Rasilainen S, Nieminen JM, Levonen A-L, Otonkoski T and Lapatto R. (2002) Dose-dependent cysteine-mediated protection of insulin-producing cells from damage by hydrogen peroxide. Biochemical Pharmacology 63, 1297-1304.

III   Rasilainen S, Ylipaasto P, Roivainen M, Bouwens L, Lapatto R, Hovi T and Otonkoski T. (2004) Mechanisms of beta cell death during restricted and unrestricted enterovirus infection. Journal of Medical Virology 72, 451-461.

IV   Rasilainen S, Ylipaasto P, Roivainen M, Lapatto R, Hovi T and Otonkoski T. Mechanisms of coxsackievirus B5 mediated beta-cell death depend on the multiplicity of infection. Journal of Medical Virology, *in press*

ABBREVIATIONS

AA	ascorbic acid
alpha-TOH	alpha-tocopherol
AP-1	activator protein-1
APC	antigen presenting cell
BB rat	biobreeding rat
CAR	coxsackie/adenoviral receptor
CAT	catalase
CVA	coxsackievirus A strain
CVB	coxsackievirus B strain
DC	dendritic cell
ERK 1/2	extracellular signal-regulated kinase 1 and 2
EthD-1	ethidium homodimer
G-HCl	guanidine hydrochloride
GAD	glutamic acid decarboxylase
Glut-2	glucose transporter 2
Gpx	glutathione peroxidase
GSH	glutathione
GSSG	oxidized glutathione
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HO	Hoechst dye 33342
HO-1	heme oxygenase-1
T1D	type I, insulin dependent, diabetes mellitus
IFN	interferon
IL-1ß	interleukine 1 beta
iNOS	inducible nitric oxide synthase
IRES	internal ribosome entry site
Isl-1	islet-1 transcription factor
KRB	Krebs-Ringer bicarbonate buffer
LA	R-alpha-Lipoic acid
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
M.O.I.	multiplicity of infection
MTT	C,N-diphenyl-N'-4,5-dimethyl-thiazol-2-yl-tetrazolium bromide
M∅	macrophage
NAC	N-acetylcysteine
NF-kB	nuclear factor-kappaB transcription factor
NK	natural killer cell
NO	nitric oxide
NOD mouse	spontaneously diabetic non-obese
Pdx-1	pancreatic duodenal homeobox
ROS	reactive oxygen species
SOD	superoxide dismutase
STZ	streptozotocin
TNF-α	tumor necrosis factor alpha
TNF-R	tumor necrosis factor receptor
Trx	thioredoxin
VSV	vesicular stomatitis virus

INTRODUCTION

T1D is considered to represent a multifactorial disorder based on genetic predisposition triggered by an environmental factor e.g. virus infection. The effector stage, insulitis, is characterized by gradual invasion of macrophages, T-cells and the produced cytokines and oxidative radicals into the islets. The prediabetic period is usually long and characterized by formation of islet cell autoantibodies even years before the development of diabetic symptoms. Enterovirus infections are epidemiologically linked to the pathogenesis of T1D. Beta cells exhibit poor intracellular antioxidative capacity, which renders them vulnerable to oxidative stress. Thus, both enterovirus infections and oxdative stress are considered to represent particularly potential triggers and/or accelerators of beta cell destruction.

It is generally known that the amount of viral particles needed to establish a local infection is low and all viruses invading a physiological environment face natural resistance by factors of the hosting immune system. Considering that coxsackievirus infections most often remain subclinical *in vivo*, the progression of infection and amount of virus are possibly limited and submaximal. Thus, this study was set to first create experimental models of slowly progressing enterovirus infection and second, to study the pattern of mechanisms of virus induced beta cell damage. Further aims included characterizing the mechanisms of oxidative beta cell injury, counteracting it by means of antioxidative agents and studying the effects of virus infection on intracellular redox balance.

REVIEW OF THE LITERATURE

1. Type I Diabetes (T1D)

1.1 Definition and pathogenesis

Juvenile, insulin-dependent diabetes mellitus (type I diabetes, T1D) is a consequence of selective autoimmune destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans (Bach, 1994; Schranz and Lernmark, 1998). The resulting insulin deficiency leads to a chronic metabolic derangement associated with significant secondary morbidity. According to the traditional opinion consistent within most autoimmune diseases, both a genetically predisposed individual and a suitable environmental trigger are needed for the destructive process, in the case of T1D against beta cells, to begin. Today, the group of human leukocyte antigen (HLA) genes is considered to account for the major genetic risk and virus infections to represent prime environmental triggers.

Multiple lines of evidence suggest that the autoimmune destruction is launched by a local insult to islet(s) exciting a pool of immune cells, dominantly T-cells and macrophages, to invade the islets resulting in insulitis (Gepts, 1965; Horwitz et al. 2002). Considering the pathogenesis, most results are obtained from studies in spontaneously diabetic non-obese (NOD) mice, whose pathogenesis mimics the human type I diabetes (Makino et al. 1980). Most probably both cytotoxic (CD8^{+}) and helper (CD4^{+}) T-cells are required for both the primary attack and the later overt destruction, respectively, to progress (Wong et al. 1996; Jarpe et al. 1990-1991; Wicker et al. 1994; Sumida et al. 1994; Miller et al. 1988; Hanafusa et al. 1988; Bendelac et al. 1987). Additionally, a pool of antigen presenting cells (APC) is indispensable in the vicinity of pancreatic islets to introduce the islet antigen to the autoreactive T-cells. According to current opinion, programmed cell death (apoptosis) represents the dominant mechanism of beta cell death during immune mediated T1D (Eizirik and Mandrup-Poulsen, 2001). The immune reaction related proinflammatory cytokines, especially interferon gamma (IFN-γ), interleukine 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), are thought to play a primary role in activating signal transduction pathways leading to beta cell dysfunction and apoptosis (Mandrup-

Poulsen, 1996; Rabinovitch, 1998; Delaney et al. 1997). The direct killing through granzyme-perforin pathway by cytotoxic T cells is also believed to be an important mechanism leading to apoptotic beta cell death.

1.2 Genetics and epidemiology

Finland has the highest incidence of T1D in under 15-year-old children worldwide. It has gradually increased during the last 43 years from 12/100000 (1953) to 45/100000 (1996) the average relative annual increase thus being 3.4 % between 1965 and 1996 (Somersalo O, 1955; Tuomilehto et al. 1999). The incidence is not only geographically but also seasonally distributed peaking in winter (Levy-Marchal et al. 1995). Finland and some other Nordic high-incidence countries have also shown gender-influence with a slight male predominance (Padaiga et al. 1997).

Approximately 10 % of newly diagnosed T1D patients have an affected first degree-relative (Dahlquist et al. 1985; Tuomilehto et al. 1992). The genetic risk and familial clustering of T1D has traditionally been concidered to originate from the class II major histocompatibility complex (MHC) genes on chromosome 6p21, including HLA DP, DQ and DR (Davies et al. 1994; Campbell and Trowsdale, 1993). These genes encode heterodimeric proteins expressed on APC-cells and have a function in presenting antigenic peptides to CD4+ T-cells. HLA class II genes have a high degree of polymorphism and marked linkage disequilibrium, which potentiate the risk to form certain high-risk allele-pairs. Furthermore, these susceptibility alleles may then select potential autoreactive T-cells during thymic selection, and increase the disease probability. Many recent studies have demonstrated the DQ-locus to primarily harbor the susceptibility for T1D (Thorsby and Ronningen, 1993; Heimberg et al. 1992; Dorman and Bunker, 2000). Specifically, worldwide approximately 30% of T1D patients are heterozygous for the high risk HLA-DQA1*0501-DQB1*0201 / DQA1*0301-DQB1*0302 alleles (previously referred to as HLA-DR3/4 or HLA-DQ2/DQ8). Also lack of the protective HLA-DQA1*0102-DQB1*0602 allele (HLA-DR2 or HLA-DQ6) increases T1D susceptibility through potentiating the effects of predisposing alleles (Pociot and McDermott, 2002). Genes located in the HLA-region, including genes important for structure and function of both the HLA-DQ and DR, are considered capable of accounting for less than 50% of the inherited disease risk (Pociot and McDermott, 2002; Thomson et al. 1988). Largely by means of genome

scan based linkage studies of affected sib-pair families at least 17 non-HLA T1D susceptibility loci have also been identified (Pociot and McDermott, 2002). Out of these, the insulin gene locus (IDDM2) is the strongest and most consistent (Bennett et al. 1995).

2. Description of cell death mechanisms and their association to T1D

Several routes and mechanisms have been described for a cell to die. The particular pathway chosen depends mostly on the trigger, but the end result may vary according to the cellular capacity of defense and the overall status of the host (age, inflammation etc.). The rough division is traditionally done by the resulting morphological characteristics: apoptotic, pyknotic, necrotic. (Figure 1)

| VIABLE | PYKNOSIS | APOPTOSIS | NECROSIS |
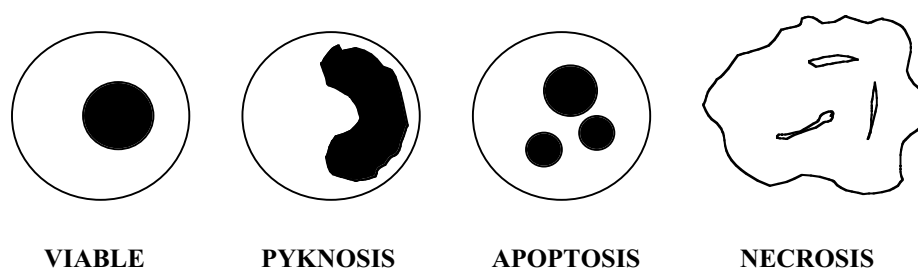
Figure 1. Illustration of nuclear morphology associated with different forms
of cell death

2.1. Necrosis
Necrotic cell death is an accidental, traumatic event always associated with some pathology. It is an unregulated process and follows no specific pattern. The key features include cytoplasmic and mitochondrial swelling and breakdown of cell to cell junctions and communication which lead to early rupture of cellular membranes and leakage of the contents to the extracellular space provoking inflammation (Lemasters, 1999; Buja et al. 1993).

Considering beta cells, detailed studies on cytokine induced stress in rat islets have revealed nitric oxide (NO) to mediate functional deterioration and mostly necrotic beta cell death (Hoorens et al. 2001). More recently, necrotic beta cell damage was

shown to dominate the pattern of spontaneous, NO-induced and STZ-induced diabetes in diabetes prone BB rats (Fehsel et al. 2003). Particularly considering cell culture models, environmental factors such as high cell density may also direct the outcome of cell death towards necrosis.

2.2. Apoptosis

The term apoptosis descends from the *Greek* words: *'apo'* (away) + *'ptosis'* (drop) and characterizes a cellular suicide (Ueda and Shah, 1994). Apoptosis is a physiological, energy-requiring, orderly phenomenon used by multicellular organisms to control the size of cell populations and to eliminate damaged, infected or mutated cells. It is thus crucial for proper embryonic morphogenesis, development, and adult cellular homeostasis. The apoptotic machinery is constitutively expressed in all nucleated animal cells and is strictly regulated by a set of evolutionarily conserved genes (Horvitz, 1999; Vaux et al. 1992). The apoptotic process can be divided in three stages: 1) initiation: a cell receives an apoptotic stimulus, 2) execution: enzymatic events, and 3) degeneration: disintegration and elimination of the cell. In general, apoptotic stimuli may activate two different pathways: an extrinsic death-receptor pathway or an intrinsic pathway based on mitochondrial dysfunction (Hengartner, 2000). The extrinsic trail is launched by triggering of a death receptor on cell surface, the most common of which belong to the family of tumor necrosis factor receptors (TNF-R) (Armitage, 1994; Baker and Reddy, 1998). These receptors include a death domain (Tartaglia et al. 1993), which is responsible for the activation of the downstream cascade: the recruitment of intracellular adapter proteins (FADD, TRADD, RAIDD) and the following activation of caspase (cysteine aspartic acid-specific proteases) 8 and possibly caspase 2 (Ashkenazi and Dixit, 1998; Alnemri et al. 1996). T-cells use alternative extrinsic routes by activating either Fas (discussed later) or granzyme B leading to stimulation of either caspase 8 or the intrinsic pathway (Pinkoski et al. 2001). Commonly the intrinsic route is activated by stress factors including DNA damage, cell cycle perturbation or growth factor deprivation. They engage pro-apoptotic members of the Bcl-2-family (Bak, Bax) to translocate to mitochondria and form pores to permit cytochrome c to release from the mitochondrial intermembrane space and to compose the apoptosome with Apaf-1 and procaspase 9 (Zou et al. 1999; Rodriguez and Lazebnik, 1999). Recently, a new mitochondrial flavoprotein (apoptosis inducing factor; AIF) was found to translocate

to nucleus in response to e.g. oxidative apoptotic stimuli and launch caspase-(in)dependent chromatin condensation and DNA cleavage (Susin et al. 1999). At the final stage, both routes activate the effector caspases (3, 6 and 7), that proteolytically activate other downstream caspases (Slee et al. 2001; Nicholson and Thornberry, 1997). Caspase 3 activates DFF/CAD, which results in DNA cleavage to n x 180 bp fragments (Liu et al. 1997; Enari et al. 1998). This step is called 'point of no return', since the proteolytic cascade irreversibly leads to cellular collapse featured by the characteristic morphology: cell shrinkage, chromatin condensation, blebbing of the plasmamembrane and formation of apoptotic bodies, which become eliminated by neighboring phagocytes (Kerr et al. 1972; Wyllie, 1981).

Apoptotic death mediated by immune effector cells is considered to dominate beta cell death. Several molecular pathways may be activated for this outcome to occur. Macrophages and activated T-cells produce and secrete proinflammatory cytokines, IL-6, IL-2, IL-10, IL-1β, TNF-α and IFN-γ, the last three of which are known for their potential to induce functional and structural beta cell damage and to provoke apoptosis (Marselli et al. 2000; Liu et al. 2000a; Saldeen, 2000; Zumsteg et al. 2000). A recent microarray survey on cytokine-stressed rat islets revealed the transcription factor NF-κB to be a core regulator of cytokine induced signaling pathways (Cardozo et al. 2001). By inhibiting its activity the cytokine induced beta cell apoptosis could be prevented (Heimberg et al. 2001)

Attracted T-cells express Fas-ligand (FasL) on cell surface, which by binding to the target cell Fas receptor results in apoptosis (Kagi et al. 1994). In spontaneously diabetic NOD mice the proinflammatory cytokines may induce Fas expression in beta cells and thus contribute to beta cell death through interaction with FasL on the surface of the neighboring CD4+ T-cells (Nakayama et al. 2002; Suarez-Pinzon et al. 1999; Suarez-Pinzon et al. 2000; Augstein et al. 2003; Petrovsky et al. 2002). Also human beta cells in pancreatic biopsies from newly diagnosed diabetics have been reported to express Fas and to contain apoptotic beta cells (Moriwaki et al. 1999). As mentioned before, CD8+ T-cells, suggested to have a critical role in the early stages of beta cell destruction, may mediate apoptosis also through the perforin-granzyme pathway, based on perforin-built channels on target cell membrane allowing

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granzyme entry and caspase activation (Kagi et al. 1994; Garcia-Sanz et al. 1987; Yoon and Jun, 1999).

2.3. Nuclear pyknosis

Beside the well-known apoptotic and necrotic pathways, a pyknotic form of cell death has also been distinguished. It morphologically resembles apoptosis with intact plasma membrane, but is characterized by condensed but intact chromatin, unlike the apoptotic fragmented DNA, demarcated at the margins of the nucleus (Tolskaya et al. 1995; Agol et al. 1998). Pyknotic morphology is typically seen in the early phase of a cytocidal virus infection when the first virus-induced alterations in host cell membrane organization and cytoskeleton have occurred (Koch and Koch, 1985). This change is often irreversible and leads to lytic cell death. A comparable phenomenon has also been observed during an apoptotic process, which due to a sudden ATP-depletion or some other disability was interrupted and turned into secondary necrosis (Hirsch et al. 1997; McCarthy et al. 1997).

3.   Enteroviruses

3.1. Family of picornaviridae

Picornaviruses are a diverse group of small, non-enveloped animal viruses with single-stranded RNA genome of positive polarity ('pico' *Greek*: very small - RNA viruses) (Racaniello, 2001). The first infections, later pinpointed to have been caused by a specific picornavirus, were described over 3000 years ago (poliomyelitis in a temple record from Egypt ca. 1400 BC). Nowadays they comprise the most common infections of humans in the developed world (Rotbart, 2002). Picornaviruses were previously classified according to their physicochemical properties (particle density, pH-sensitivity) and serological relatedness. More recently, the classification has been based on nucleotide sequence comparisons. The picornavirus genera infecting man include enteroviruses, hepatoviruses, kobuviruses, parechoviruses and rhinoviruses. The enterovirus genus is further divided into five species, which are listed in Table 1 together with the previous subgroups (based on pathogenesis in experimental animals) and serotypes (Hyypiä et al. 1997; King et al. 1999)

Species	Subgroups and serotypes
Poliovirus	Polioviruses 1-3
Human enterovirus A	Coxsackievirus A 2-8, 10, 12, 14, 16
Human enterovirus B	Coxsackievirus A9
	Coxsackievirus B 1-6
	Echovirus 1-7, 9, 11-21, 24-27, 29-33
	Enterovirus 69
Human enterovirus C	Coxsackievirus A 1, 11, 13, 15, 17-22, 24
Human enterovirus D	Enterovirus 68, 70

Table 1. Species and subgroup division of enteroviruses

3.2. Enteroviral genome and viral replication cycle

In order to multiply, the virus must first bind to its specific receptor on the surface of the target cell. Several receptors have been demonstrated for enteroviruses: coxsackie-adenovirus receptor (CAR) is recognized by CVB 1-6 and decay-accelerating factor (DAF, CD55) by CVB 1,3,5 (Bergelson et al. 1995; Bergelson et al. 1997; Bergelson et al. 1998). In addition, CVA 9 has been shown to use alpha v beta 3 integrin, the vitronectin receptor, to enter the host cells (Roivainen et al. 1994). In studies using neutralizing antibodies, both alpha and beta cells in human islets were found to express CAR (Chehadeh et al. 2000). After contacting the receptor, the virus should successfully enter the cell, which commonly requires some conformational changes of the viral capsid. During entry or immediately afterwards the viral genomic material is released into the host's cytoplasm in a process called uncoating. Once released in the cytoplasm, the viral genomic RNA, about 7500 nucleotides in length, functions directly as a template for the production of viral proteins. The single open reading frame encodes a large precursor polyprotein. This precursor includes two proteolytic sequences (2A and 3C), which cleave the precursor into intermediate products P1, P2 and P3 (Kitamura et al. 1981). P1 is further digested into capsid (structural) proteins VP0, VP3 and VP1 (Korant, 1973). P2 and P3 are in the sequential steps cleaved into seven non-structural proteins functioning in the replication and encapsidation of the viral RNA and in the processing of the proteins. One specific product is the virus RNA-dependent RNA polymerase (3D). It copies the genomic RNA into a negative-sense strand, which then acts as a template for positive-strand RNA synthesis. Finally the newly produced genomic material is packaged into the procapsids formed by twelve pentameric structures, each of which contains five protomers (VP0-VP3-VP1

–heterotrimers) (Korant, 1973). During this RNA-encapsidation, the VP0-protein is further cleaved into VP2 and VP4 (maturation cleavage; relevant for enteroviruses, but not for all picornaviruses (Hyypiä et al. 1992; Yamashita et al. 1998)) stabilizing the procapsid.
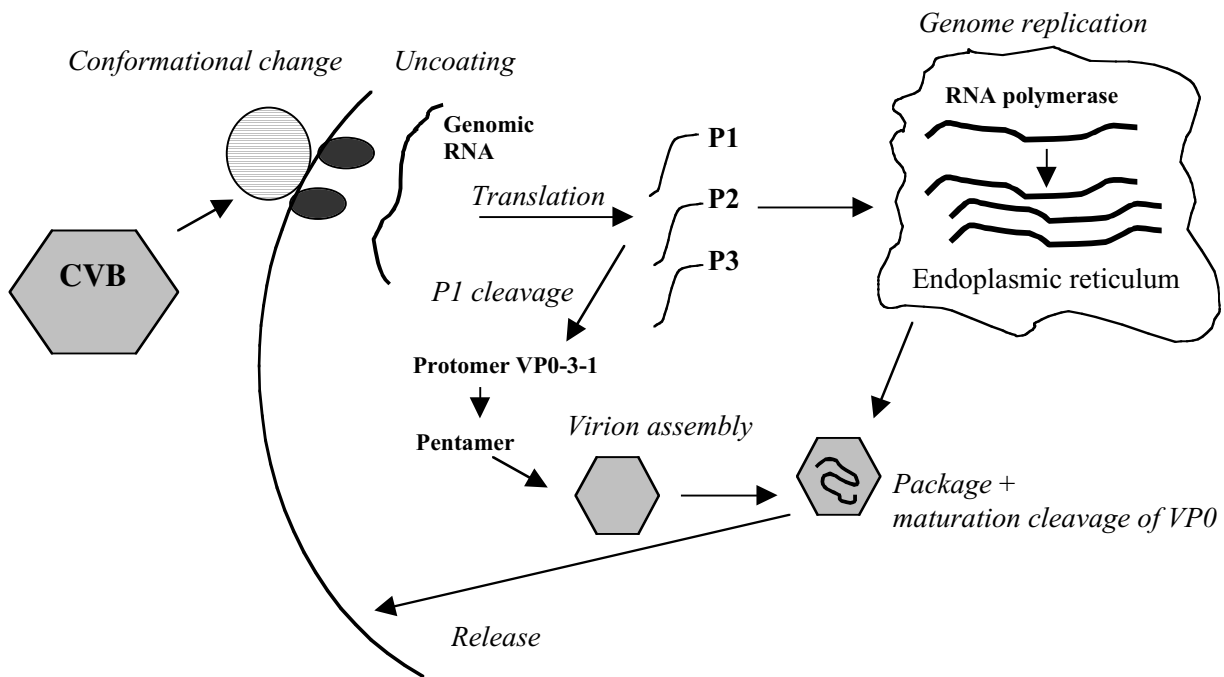

Figure 2. The replication cycle of enteroviruses.

3.3. Host response to virus infection

As suggested earlier, the outcome of an enterovirus infection may vary between aggressive fulminant and acute subclinical (Ramsingh, 1997b; Yoon et al. 1979). In suitable conditions persistence may develop (Chehadeh et al. 2000; Tam and Messner, 1999; Klingel et al. 1992; Adachi et al. 1996; Conaldi et al. 1997a; Conaldi et al. 1997b). In general, a viral invasion into an organism supplied with a properly functioning immune system always attracts it to respond. The innate arm of immunity composed of natural killer cells, natural (nonspecific) antibodies, the complement system, phagocytes, antimicrobial peptides and interferons (IFNs) together constitute the first line of defense. IFNs may mediate the antiviral response through many routes. Firstly, through binding to type I IFN receptor (Platanias et al. 1996) they stimulate protein tyrosine kinases (JAK 1, TYK2) which further act to stimulate STAT-, Crk- and IRS- pathways (Darnell et al. 1994; Uddin et al. 2000;

Ahmad et al. 1997). These routes finally lead to the induction of anti-viral interferon stimulated genes (ISG) (Bose and Banerjee, 2003). Interferons are also able to induce the production of MHC-molecules on virus-infected cells and on antigen presenting cells required for CD4+ T-cell action. Furthermore, they may initiate or contribute to the production of antigen specific antibodies and thus activate the second line of antiviral host defense, the adaptive or cell-mediated arm of immunity (Kadowaki et al. 2000). Recent observation in human islets concluded CVB-infection to stimulate INF-α production selectively in beta cells, which by inhibiting the efficiency of viral replication prevents the infection from disseminating and maintains it persistent (Chehadeh et al. 2000). Furthermore, infection–induced IFN-α has been speculated to participate in the initiation of beta cell autoimmunity (Chehadeh et al. 2000; Chakrabarti et al. 1996; Stewart et al. 1993). It was recently observed that efforts of the host to inhibit viral dissemination and persistence often involve induction of apoptosis through either perforin-granzyme or death receptor mediated pathways (Froelich et al. 1998; Ashkenazi and Dixit, 1998). IFNs are reported to sensitize infected cells to apoptosis (Tanaka et al. 1998; Balachandran et al, 2000). When beta cells are stimulated with IFNs in combination with double-stranded viral RNA or the cytokine IL-1ß, a synergistic apoptotic effect is seen (Liu et al. 2002).

To defend themselves, many viruses have evolved to counteract the host responses. To secure the replicative potential and production of progeny virions, it is favorable for the virus to maintain the host cell viable and for this purpose many routes of the host signaling machinery may be manipulated. For example, various steps of the IFN cascade may be blocked by several different viruses (Ronco et al. 1998; Komatsu et al. 2000; Katze, 1995; Munoz-Jordan JL et al. 2003). Also cleavage of p21(ras) GTPase-activating protein (RasGAP) and activation of the MAPK family members ERK 1/2, observed essential for CVB3 replication, could be affected (Opavsky et al. 2002; Huber et al. 1999a; Luo et al. 2002).

After stimulation of the innate arm of immunity a cell-mediated immune response is launched. The dendritic cells, stimulated during the innate response by cytokines and the pathogen itself, present antigen to naive T-cells, which start to develop differential markers. Specifically the Th1 type CD4+ T-cells secrete proinflammatory cytokines, which enhance virus-specific host cell lysis by CD8+ T-cells and stimulate the MCH-upregulation on APC cells, which further helps in activating antibody production by

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B-cells. Other interleukines secreted by Th2 type CD4+ T-cells promote this B-cell activation (Salusto et al. 1998).

The host responsiveness to virus and the outcome of the infection have been observed to furthermore depend on the status of the host cell cycle. Several studies have revealed actively dividing cells, like T-cells, to be more susceptible to virus and to efficiently produce viral progeny (Molina et al. 1992; Liu et al. 2000b). Quiescent cells (in G_0 phase) on the other hand do not support viral multiplication, but may harbor infective viruses thus creating persistence (Feuer et al. 2002). These theories may partially explain the individual susceptibility of cells to infection. Some studies further indicate several viruses capable of forcing the host cell into a favored phase of cell cycle (Op De Beeck and Caillet-Fauquet, 1997; Swanton and Jones, 2001). Viral mRNA, containing an internal ribosome entry site (IRES) instead of the eukaryotic 5' cap, may also redirect the cellular translation pattern and favor viral protein synthesis, particularly in enterovirus-infected cells where cap-dependent translation is specifically inhibited (Kuyumcu-Martinez et al. 2002; Marissen et al. 2000).

3.4. Infections and clinical manifestations

Enteroviruses are predominantly transmitted by the fecal-oral route into the human body, although transmission via either upper respiratory tract or the conjunctiva of the eye is also possible. They normally replicate in the respiratory and gastrointestinal mucosa, where the infection may remain subclinical or result in mild symptoms. In a proportion of cases, the virus spreads through the lymphatics into the circulation, and after a brief viraemic phase may establish secondary replication sites in specific tissues and organs. Polioviruses may proverbially enter the central nervous system, replicate in the motor neurons and in about 1% of cases with the most virulent strains result in flaccid paralysis (Melnick, 1996). Coxsackie A viruses typically induce diseases with mucosal and skin lesions (e.g. herpangina, hand, foot, and mouth disease) (Itagaki et al. 1983; Seddon and Duff, 1971; Bendig and Fleming, 1996). In addition to the milder disorders, coxsackie B viruses are also associated with more severe and possibly chronic diseases including meningitis, myopericarditis, epidemic pleurodynia and T1D (Gauntt and Huber, 2003; Beck et al. 1990; Muir and van Loon, 1997; Rotbart, 1995; Tracy et al. 2000). They are together with echoviruses responsible for severe neonatal viral infections (Chiou et al. 1998; Sawyer, 1999).

3.5. Enteroviruses and T1D

Enterovirus infections could initiate or accelerate beta cell damage many years before the clinical onset of T1D. Seroepidemiological studies exist to support this at least after early CVB and rubella exposures (Hyöty et al. 1995; Lönnrot et al. 1998; Lönnrot et al. 2000, Salminen et al, 2003). Some small case-control reports show evidence for intrauterine viral exposure, a maternal enterovirus infection, as a risk factor for future development of T1D (Dahlquist et al. 1995, Hyöty et al. 1995). In a larger birth cohort study (Viskari et al. 2002) and in another, particularly CVB-directed, smaller study (Fuchtenbusch et al. 2001) though contradictory results were obtained. A recent survey reveals that while the incidence of T1D has doubled within the last 40 years in Finland, the incidence of enterovirus infections has simultaneously decreased (Karvonen et al. 1999; Viskari et al. 2002). Also the maternal enterovirus antibody levels have decreased (Viskari et al. 2002). The low maternal antibody status could boost the viral exposure of the fetus/newborn and the rarer incidence furthermore exposes the child to catch the infection later, often in the absence of protecting maternal antibodies (Viskari et al. 2000). This phenomenon could partially explain the increasing number of type I diabetics and supports the role of enteroviruses as possible triggers. Coxsackie B viruses have long been associated with the pathogenesis of T1D (Andreoletti et al. 1997; Banatvala et al. 1985; Yoon, 1990). Groundbreaking findings were made in 1979 (Yoon et al.) when the autopsy specimens of a 10-year-old diabetic showed lymphocytic infiltration of the islets and beta cell necrosis which lead to the detection of a diabetogenic variant of CVB-4 from the pancreatic cultures (Yoon et al. 1979). This very serotype was further observed to induce T1D in mouse. However, in a series of 88 pancreas-specimens from patients who died soon after onset of T1D, coxsackieviruses were not detected in the islets (Foulis et al. 1990). Therefore, it should be considered unlikely that an acute CVB infection in pancreatic islets would frequently associate with the onset of T1D. However, this does not exclude the possibility that CVB could have been present in the islets at an earlier stage of T1D pathogenesis. In recent seroepidemiological studies several enterovirus serotypes have been shown to share the association with T1D (Frisk et al. 1992; Helfand et al. 1995; Roivainen et al. 1998; Otonkoski et al. 2000; Vreugdenhil et al. 2000). In fact, it has been proposed that any serotype could potentially possess diabetogenicity. Furthermore, aggressive, cytolytic isolates can develop from originally benign enterovirus serotypes (Roivainen et al. 2002).

3.6. Mechanisms of beta cell destruction

T1D is an immune-mediated disease in which a specific immune response to islet beta cells is induced. In the animal model of T1D, the NOD mouse, diabetes may be transferred only with cells of the immune system. Also in humans T1D has been adoptively transferred via bone-marrow transplantation from a diabetic donor. However, there are several mechanisms through which a virus could induce immune-mediated destruction of islet beta cells, as shown in figure 3 beneath.
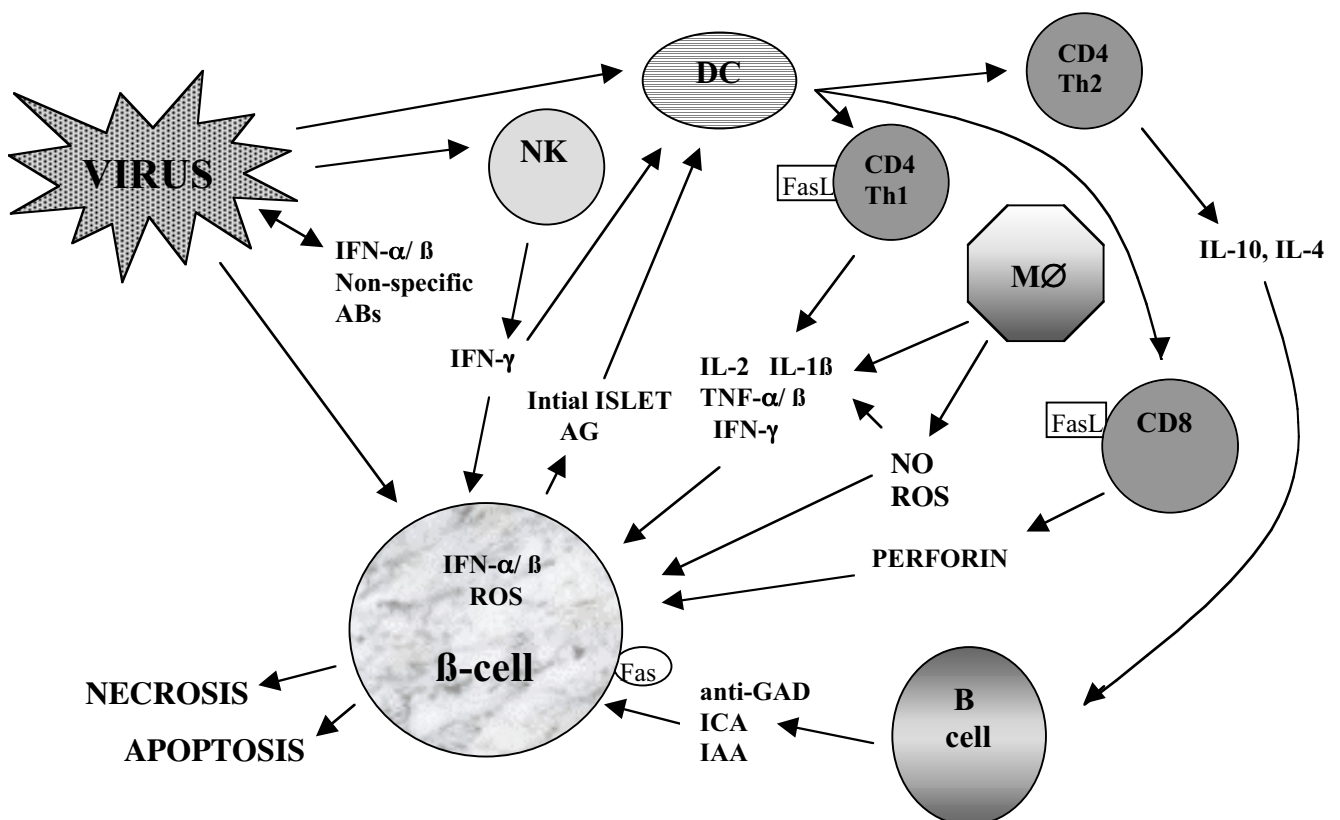
Figure 3. The putative pathways involved in virus induced beta cell death.

A potential course of the process: Virus infects the beta cell and stimulates intracellular IFNα/ ß and MHC I production and the innate arm (IFN, non-specific antibodies and NK cells) of the hosting immune system. The resulting initial beta cell damage provokes the activation and infiltration of macrophages and T cells, which secrete cytokines and finally stimulate antibody production. The produced cytokines, interferons, nitric oxide, ROS and perforin contribute to beta cell death through apoptosis or necrosis.

3.6.1. Lysis

Lytic cell death is an aggressive, rapid process featured by breakdown of the cell's structural integrity. It equals necrosis, which is used generally to describe cell death of aggressive nature. As mentioned earlier viruses often induce host cell pyknosis in the initial phase of infection (Koch and Koch, 1985), which may in later stages turn into lysis. In a recent study (Roivainen et al. 2002) a large group of prototype enteroviruses from different genetic subgroups were characterized according to their cytolytic activity. Echovirus 6, 7, 11, CVA 13, CVB 1, 3, 4, 5, 6 and poliovirus type 1/Mahoney were detected to cause beta cell lysis. Furthermore an echovirus 9 isolate, from a 6-week-old baby with acute onset T1D was found with destructive features unlike its corresponding prototype virus. These variations of outcomes might have to do with donor-related characteristics, thus the possibility that a certain HLA-type or antibody-positivity could confer resistance or susceptibility for destructive beta cell death remains.

3.6.2 Apoptosis

In order to maintain an efficient multiplication environment, viruses may prevent or delay host cell death through various routes. Especially the abilities to block p53 tumor suppressor -dependent and Bax/Bak/Bik -dependent apoptosis are well characterized (Bargonetti et al. 1992; Dobner et al. 1996; Scheffner et al. 1993; Chen et al. 1996; Afonso et al. 1996; Henderson et al. 1993). On the other hand the same pathways may be used in the opposite direction to induce host cell apoptosis and safely release new viral particles in enveloped bodies to be engulfed by other target cells (Debbas and White, 1993; Sastry et al. 1996; Westendorp et al. 1995). Additionally, by this mechanism the inflammatory/immune reactions following lytic cell death or free virus exposure are avoided and contact with neutralizing antibodies is prevented, thus potentiating the survival of the virus and uninterrupted dissemination of infection (Jeurissen et al. 1992; Gliedman et al. 1975). Concerning non-enveloped viruses, into which category enteroviruses belong, the mechanisms of exit from a dying host cell are still unknown. Although cell lysis is thought to play a major role, a recent report presents host cell apoptosis as a potential new explanation. It would result in formation of membrane-bound bodies enabling viral exit (Teodoro and Branton, 1997). In the group of picornaviruses, apoptosis has been extensively studied by the model of CVB3 induced myocarditis, during which persistence often

develops and apoptotic myocardial death is evident (Carthy et al. 1998; Huber et al. 1999b). Also poliovirus infection or individual poliovirus proteases alone are reported to be able to induce apoptotic death in selected cell types (Lopez-Guerrero et al. 2000; Goldstaub et al. 2000; Barco et al. 2000; Girard et al. 1999; Tolskaya et al. 1995).

Considering development of T1D, virus infections are regarded as the leading environmental triggers and apoptosis the leading form of beta cell death (Eizirik and Mandrup-Poulsen, 2001). Their possible association is difficult to study in clinical materials. Experimentally, recent studies on rat islets stressed with synthetic double stranded RNA, a general product of viral replication, reveal enhanced susceptibility to cytokine induced islet cell apoptosis by Fas-FasL interaction (Liu et al. 2002). Virus infections generally induce an inflammatory reaction mediated in part by proinflammatory cytokines. In addition to their direct proapoptotic potential, cytokines may also launch the expression of MHC class I molecules in beta cells followed by their appearance on plasma membranes, which exposes beta cells to T-cell mediated death through Fas or perforin dependent pathways (Kim et al. 2002; Seewaldt et al. 2000).

3.7. Enteroviruses as triggers of autoimmunity in T1D

3.7.1. Molecular mimicry

Molecular mimicry is based on a sequential and structural homology between a foreign antigen and a host protein enabling an immunologic attack against the pathogen to cross-react with the host molecule (Davies, 1997). Glutamic acid decarboxylase GAD_{65}, an enzyme synthesizing the inhibitory neurotransmitter gamma amino butyric acid (GABA), is one of the important islet cell autoantigens in T1D. In 1992 the sequences of GAD_{65} and the non-structural protein 2C of CVB-like enteroviruses were shown to share a similar motif (PEVKEK) and to bind the same groove in a MHC-molecule in an exactly similar position (Kaufman et al. 1992). In later studies with synthetic peptides, the binding was restricted to HLA-DR3 molecule (Vreugdenhil et al. 1998). Furthermore, GAD_{65}-reactive T-cells are present and circulating in both T1D patients and healthy normal people, with the difference of functioning like pre-activated memory cells in patients and thus being highly more

susceptible to enter clonal expansion. These data have given evidence for molecular mimicry between enteroviral and self-factors as a possible mechanism of beta cell destruction, although contradictory observations have also been made (Schloot et al. 2001; Horwitz et al. 1998; Marttila et al. 2001; Atkinson and Maclaren, 1994). Accordingly, molecular mimicry could also exist between GAD and cytomegalovirus on T-cell level (Roep et al. 2002).

3.7.2. Local inflammatory damage and bystander activation

The model of locally (intra-pancreatically) launched beta cell autoimmunity and destruction has been widely studied by Horwitz et al. (1999). Their recent report concluded that the primary role of an enterovirus infection (pancreatrophic CVB4) in the pathogenesis of T1D is to specifically infect and damage beta cells leading to release of sequestered islet antigen and stimulation of a local inflammatory response. The intra-pancreatic antigen presenting cells introduce the antigen to the population of resting beta-cell-autoreactive T-cells resulting in initiation of the disease process. Unless a critical threshold level of these precursor autoreactive T-cells exist, the destructive process will not be triggered (Horwitz et al. 1998). They also observed the critical role of beta cell damage as the driving force of virus induced T1D, since a non-specific cytokine-attack did not precipitate T1D in mice with a diabetogenic T-cell repertoire, while exposure to the beta-cell toxin streptozotozin was able to do it (Horwitz et al. 2002). CVB infections have previously been reported to lead to necrotic cell death in both rodent and human exocrine pancreatic tissue (Lansdown, 1976; Vella et al. 1992; Arnesjo et al. 1976; Ozsvar et al. 1992; Ramsingh, 1997a). The inflammatory mediators produced during this infective process have been speculated to activate the bystanding beta-cell-autoreactive T-cell pool and thus possibly accelerate T1D development (Serreze et al. 2000).

4. Oxidative stress

4.1. Oxidative metabolites and cell death

Oxygen derived reactive metabolites (reactive oxygen species, ROS) appear abundantly in the human body. They form as a consequence of incomplete reduction of molecular oxygen by the mitochondrial respiratory (electron-transport) chain

(Fernandez-Checa et al. 1998) or through some reactions of cellular metabolism by oxidizing enzymes including xanthine oxidase, P450 mono-oxygenase, cyclo-oxygenase, lipoxygenase, monoamine oxidase etc (Forman, 1982; Boveris and Chance, 1973; Maeda et al. 1999; Siraki et al. 2002). The most common intracellular ROS molecules include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (^-OH) (Fridovich, 1998; DiGuiseppi and Fridovich, 1984). ROS react with biological molecules such as proteins, lipids, carbohydrates and DNA threatening their integrity and exposing the organism to toxic, mutagenic and carcinogenic assaults (Stadtman and Levine, 2000; Steinberg, 1997; Marnett, 2000). Small GTPases Ras and Rac1, for example, have been shown to be ROS targets leading to altered signal transduction (Sundaresan et al. 1996; Irani et al. 1997). Except for ^-OH, which is mostly noxious, ROS also have beneficial functions mainly as stimulators and mediators of intracellular signaling cascades (Ohba et al. 1994; Lo and Cruz, 1995; Krieger-Brauer and Kather, 1992; Sundaresan et al. 1995). They also function in microbial killing during infection, for example through inactivating viral enzymes by nitrosylation (Colasanti et al. 1999; Babior, 1978a; Babior, 1978b; Adams et al. 1990). Their properties often shift into pathologic in response to increasing concentrations, which might be due to the metabolic state (Di Meo and Venditti, 2001; Gambelunghe et al. 2001), pH, oxygen partial pressure (pO2) and ADP availability. Very commonly overproduction of ROS is triggered by non-physiological states like inflammation or exogenous toxins, which stimulate phagocytic cells to produce ROS. In a variety of disease processes associated with either acute or chronic inflammation, increased oxidative stress appears to be involved. Depending on the surrounding milieu, ROS molecules may also react together. Specifically, in a reaction between O_2^- and NO highly cytotoxic peroxynitrite, $ONOO^-$ is formed (Beckman and Koppenol, 1996), which may further react with carbon dioxide (CO_2) to form peroxocarboxylate ($ONOOCO_2^-$). H_2O_2 may react with $H^+ + Cl^-$ or Cu^+/Fe^{2+} forming either highly reactive HOCl (hypochlorus acid) or a hydroxyl radical, respectively (Babior 2000).

Highlighting the dual role of ROS in both physiological and disease states, recent data show concentration dependent stimulation of either cell growth or death. Moderate levels of pro-oxidants may promote mitogenic stimuli (Burdon and Rice-Evans, 1989) e.g. by affecting kinase or proto-oncogene activities (Larsson and Cerutti, 1988;

Cerutti and Trump, 1991). At slightly increased levels ROS are reported at various different experimental conditions to be able to induce apoptotic cell death either directly or in combination with antioxidant, mainly glutathione (GSH), depletion (Hampton and Orrenius, 1997; Macho et al. 1997; Lennon et al. 1991). The stimulatory mechanisms often involve exposing to or triggering the mitochondrial membrane permeability transition which leads to the release of apoptogenic factors and thus stimulation of the downstream apoptotic cascade (Petronilli et al. 1994; Costantini et al. 1996; Fleury et al. 2002; Ueda et al. 2002; Wei and Lee, 2002; Armstrong and Jones, 2002; Dypbukt et al. 1994; Datta et al. 2002; Armstrong et al. 2002). Also, HIV-infection mediated apoptosis of $CD4^+$ T-cells is preceded by ROS-production and antioxidant depletion. This process has recently been associated with p53, NF-κB and AP-1, pro-apoptotic redox-active factors, that also support expression of viral genes and pro-inflammatory cytokines (Perl et al. 2002). p53, independent of the activating factor, may further stimulate ROS production and induce apoptosis (Sawada et al. 2001; Li et al. 1999).

Moreover, studies evidencing antioxidants' capacity to inhibit apoptosis and recent observations on antiapoptotic molecules with antioxidative properties further argue for a role for ROS in stimulating apoptosis (Melnick, 1996; Kelso et al. 2001; Sato et al. 2002). Additionally, oxidants may direct an apoptotic process into a necrotic one by inactivating caspases or impairing the mitochondrial energy production resulting in subsequent ATP depletion (Samali et al. 1999; Leist et al. 1999). Although possible in the other direction, overwhelming oxidative stress usually always leads to necrosis leaving no possibilities to inhibit or re-regulate the process towards apoptosis.
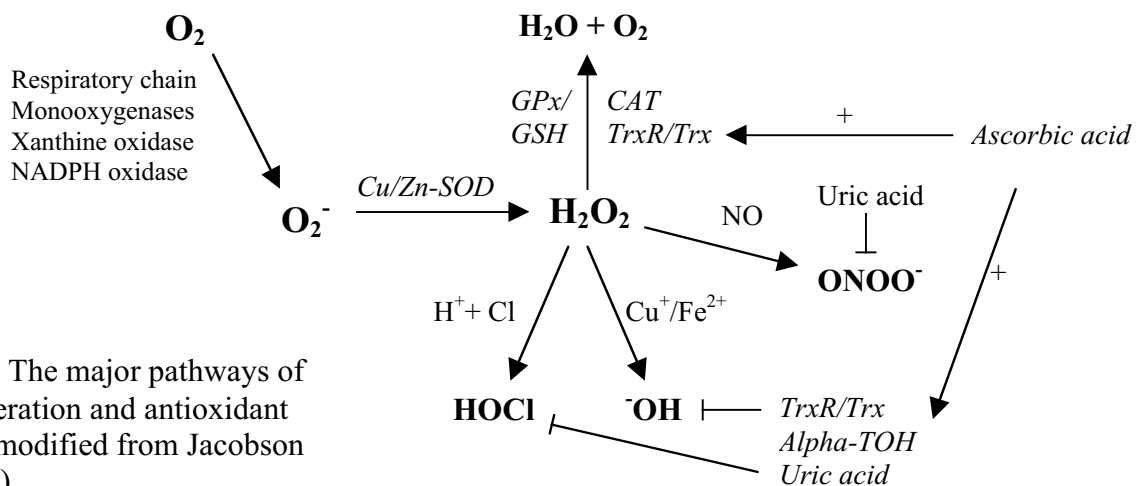

Figure 4. The major pathways of ROS generation and antioxidant defense (modified from Jacobson MD 1996).

4.2. Antioxidative machinery

In an attempt to prevent ROS-mediated damage, cells have developed an antioxidative defense system (Benzie, 2000). The main goal is to maintain the intracellular milieu in a reduced and stable state. This antioxidative machinery consists of several components including heme- or thiol-based enzymatic systems and non-enzymatic scavengers. The most ubiquitous and abundant of them is the glutathione (GSH) system (Deneke and Fanburg, 1989; Anderson, 1998). GSH is formed from the aminoacids glutamate, cysteine and glycine in two ATP-dependent enzymatic reactions by gamma-glutamylcysteine synthetase and glutathione synthetase (Griffith and Mulcahy, 1999). GSH itself functions mainly as a sulfhydryl buffer and helps to detoxify xenobiotics in conjugation reactions catalyzed by glutathione S-transferase. Most importantly glutathione peroxidases, selenocysteine-containing enzymes, use GSH as substrate in the elimination of e.g. hydroxyl radical, peroxynitrite, hydroperoxides and reactive electrophiles. In these reactions two GSH-molecules are oxidized to GSSG, which is converted back to reduced GSH in an NADPH-dependent reaction either by glutathione reductase or the thioredoxin system, described below. Furthermore, as a thiol-containing reductant, GSH maintains so-called thiol-enzymes in their catalytically active forms, and low molecular weight antioxidants, vitamins C and E in their biologically active forms (Fridovich, 1999).

Other major antioxidants include catalase (CAT) and superoxide dismutase (SOD). As referred to by its name, the latter inhibits ROS-mediated damage by scavenging O_2^- (Fridovich, 1995; Fridovich, 1999) Two isoenzymes exist: an essential mitochondrial Mn-SOD and a less essential cytosolic Cu/Zn-SOD. They metabolize two O_2^- molecules to O_2 and H_2O_2; thus another ROS is formed. The general and ubiquitous mechanism to remove H_2O_2 is by peroxisomal heme-containing CAT, which converts two H_2O_2 molecules to O_2 and 2 x H_2O (Kirkman and Gaetani, 1984; Kirkman et al. 1999). Moreover, CAT detoxifies e.g. phenols and alcohols via coupled reactions with H_2O_2.

Another thiol-containing and ubiquitous reducing enzyme system is the thioredoxin (Trx) machinery. Trx functions as a hydrogen donor for other catalytic enzymes (e.g. glutathione peroxidase) and reduces disulfide bonds of diverse proteins (Holmgren, 1984). It protects particularly against peroxide induced stress (Spector et al. 1988; Nakamura et al. 1994) and has also been reported to have anti-apoptotic power

(Saitoh et al. 1998). Like the GSH system, oxidized Trx is converted back to reduced form by a flavoenzyme thioredoxin reductase (TrxR) in an NADPH-dependent reaction (Holmgren, 1985). In addition to Trx, TrxR reduces lipid peroxides, diverse antioxidative selenium containing compounds and converts vitamin C back to its active reduced form.

A distinct, non-enzymatic group of antioxidants of low molecular weight include vitamins E and C, several selenium-containing compounds, lipoic acid, and ubiquinones. Ascorbic acid (AA; vitamin C) and alpha-tocopherol (alpha-TOH; vitamin E) constitute the major water-soluble and lipid-soluble small antioxidants, respectively (Buettner, 1993). As alpha-TOH reduces e.g. peroxyl radicals by its OH-group and converts into tocopheroxyl (chromanoxyl) radical, vitamin C, as its main function, reduces it back to alpha-TOH, the active vitamin E (Sies et al. 1992). Additionally, vitamin C acts as an electron donor for some transmembrane enzymes with oxidoreductase activity (May et al. 1995). As previously mentioned, TrxR reduces oxidized vitamin C (Mendiratta et al. 1998), but its major recycler is GSH (May et al. 1996).

Heme (Fe protoporphyrin IX) is an integral protein to life as delivering oxygen into cells. It circulates incorporated in hemoproteins and occurs unbound (free) only in pathologies sometimes associated with tissue accumulation. Free heme as such is a powerful oxidative molecule and thus a system for its degradation exists. It consists of an oxidative stress–inducible protein HO-1 (heme oxygenase-1, HSP32) and the constitutive isozyme HO-2, which catalyze the oxidation of heme to biologically active molecules: free iron, a gene regulator (Ferris et al. 1999), biliverdin, an antioxidant and carbon monoxide, a heme ligand (Maines, 1997).

Uric acid is produced in liver and represents the end product of the purine metabolism reaction chain. The enzyme xanthine oxidase catalyses the last two reactions from hypoxanthine through xanthine to uric acid as byproducts of which two molecules of H_2O_2 are created. Uric acid is considered a powerful antioxidant (Ames et al. 1981) especially because of its potency in peroxynitrite scavenging (Balavoine and Geletii, 1999; Regoli and Winston, 1999; Whiteman et al. 2002) but also in its ability to chelate transition metal ions and stabilize reactive hypochlorus acid and hydroxyl

radical (Becker, 1993). On the other hand, its increased production indicates increased H_2O_2 production, which may further react with peroxynitrite and form new aggressive oxidative metabolites (Skinner et al. 1998; Vasquez-Vivar et al. 1996; Santos et al. 1999) including alloxan and a nitrite derivative capable of NO release. These both are known for their direct or indirect beta cell toxicity (Lenzen and Panten, 1988). NO may further stimulate peroxynitrate production by reacting with superoxide, thus contributing to beta cell injury (Suarez-Pinzon et al. 1997; Suarez-Pinzon et al. 2001). However, there is evidence that uric acid might further react with and scavenge also these newly formed radicals (Whiteman and Halliwell, 1996; Kooy et al. 1994), which implies to a dual role for this molecule in controlling/affecting the cellular redox status.

4.3. Redox status in beta cells and T1D

Cellular damage in diabetes is most probably associated with various biochemical pathways. There is evidence that oxidative mechanisms participate in the pathogenesis of both beta cell destruction at the prediabetic period and of vascular damage and endothelial dysfunction during the development of further disease complications. According to recent knowledge, the latter are mostly due to increased mitochondrial production of superoxide anion stimulated by prolonged hyperglycemia (Nishikawa et al. 2000). The earlier mentioned NF-κB, an important mediator of cytokine induced pathways of beta cell damage, is also redox-sensitive and assumably also activated by the minor amounts of ROS produced at the initial stage of beta cell destruction. NF-κB is further reported to increase the expression level of iNOS (among other genes) and then through NO-signaling to downregulate the expression levels of beta cell specific genes including Pdx-1, Glut-2, and Isl-1 and also to stimulate the production of cytokines and chemotactic agents amplifying the production of ROS. This cascade then leads to beta cell dysfunction and death through apoptosis or necrosis depending on the overall stress (Cardozo et al. 2001; Ho and Bray, 1999). A chain of events resembling this is also considered to characterize insulitis, during which T-cells and macrophages invade the islets and act as sources of inflammatory mediators and ROS (Rabinovitch et al. 1996b).

Many studies have discovered the general antioxidative capacity to be defective in diabetic patients (Santini et al. 1997; Maxwell et al. 1997; Tsai et al. 1994). Depending on the design of the study, decreased levels of vitamin C, vitamin E, uric acid, GSH and GSH-related enzymes have been detected in diabetics compared to control subjects (Maxwell et al. 1997; Marra et al. 2002; Seghieri et al. 1998; Courderot-Masuyer et al. 2000; Jain and McVie, 1994; Ruiz et al. 1999; Seghrouchni et al. 2002; Sharma et al. 2000; Hoeldtke et al. 2002). Controversial results also exist and imply the need for critical evaluation of the markers measured to assess oxidative stress (VanderJagt et al. 2001; Leinonen et al. 1998; Vessby et al. 2002). Importantly, beta cells themselves have a poorer antioxidative defense system in comparison to other cell types. In specific, the expression levels and activities of antioxidants are low and the adaptive properties to increase antioxidant enzyme production during stress are limited, thus rendering beta cells extremely vulnerable to oxidative damage (Grankvist et al. 1981; Lenzen et al. 1996; Tiedge et al. 1997). Further evidence for these defects have been obtained from substitution studies on insulin producing cells showing effective protection against ROS- or cytokine-mediated cell death by a cocktail of antioxidants CAT+Gpx+SOD (Tiedge et al. 1998; Lortz et al. 2000). Beta cells' own properties are also considered to affect their survival, exemplified by exaggerated ROS-sensitivity during low glucose levels and slow mitochondrial metabolic rate. Thus, normal kinetics of glucose metabolism may protect beta cells against ROS-induced damage (Pipeleers et al. 2001).

The role of Trx has been studied in nonobese diabetic mice overexpressing Trx specifically in beta cells. The incidences of both spontaneous autoimmune and drug- (streptozotocin, a ROS generating agent) induced T1D were reduced (Hotta et al. 1998). Also after major injury (partial pancreatectomy) or transplantation, antioxidative molecules have been shown to increase beta-cell survival through e.g. attenuated apoptotic beta cell death and increased viability (Ribeiro et al. 2003; Laybutt et al. 2002; Bottino et al. 2002; Gunther et al. 2002; Pileggi et al. 2001).

Overall, the existing data indicates oxidative molecules to be important mediators of beta cell damage and thus suggests a role for antioxidant supplementation in diabetes. Due to the complexity and non-specificity of the machinery controlling redox status, this balance is of crucial importance.

5. Virus infections and oxidative stress: Interactions

Malnutrition is known to result in defective and inefficient antioxidative capacity and aggravated exposure to oxidative stress triggered by various pathogens, e.g. viruses (Sofic et al. 2002). According to the traditional view, malnutrition could predispose an individual to infections by weakening the host's immune system and thus allowing the pathogen to multiply and disseminate in the organism. Today it is generally known that many virus infections (e.g. influenzavirus induced pneumonia, HIV, coxsackievirus myocarditis) exert many kinds of oxidative stress in the host (Schwarz, 1996; Xie et al. 2002b; Maeda and Akaike, 1998). Phagocytes become activated and produce ROS (Peterhans et al. 1987) and pro-oxidative cytokines (TNF-α, IL-1β), which further potentiate oxidative and other viral damage through e.g. increased virus multiplication, impaired mitochondrial function and reactive iron accumulation (Polla et al. 1996; Schulze-Osthoff et al. 1992; Schreck et al. 1992; Klempner et al. 1978). Various studies have demonstrated the effect of these cytokines to provoke ROS, especially NO, production within the islets, both in macrophages and beta cells (Rabinovitch and Suarez-Pinzon, 1998; Eizirik et al. 1996; Rabinovitch et al. 1996a; Mandrup-Poulsen et al. 1990). Similarly, the pathogenesis of influenza virus induced pneumonia has been reported to be mostly due to IFN-γ mediated NOS and iNOS activation resulting in NO and further peroxynitrate production (Akaike et al. 1996). The previously mentioned antioxidant-substitution studies confirm ROS production to mediate IL-1β + TNF-α + IFN-γ stimulated beta cell damage (Lortz et al. 2000). These interactions are further strengthened by the knowledge of NF-κB as a mediator of both ROS and cytokine induced apoptosis and the fact that also ROS mediate p53-dependent apoptosis, the pathway commonly used by viruses (Datta et al. 2002; Armstrong et al. 2002).

The exposure to radical stress is further intensified by virus-mediated impairment of host's antioxidative defenses through decreasing concentrations of several ROS scavengers (Xie et al. 2002a; Hennet et al. 1992; Bannister et al. 1986; Staal et al. 1992; Allard et al. 1998). Many *in vitro* studies have characterized this phenomenon and observed reparative effects by supplementing antioxidants. *In vivo* the effect of antioxidant therapy is usually tested as a supplement to some specific antiviral

treatment, because of the antioxidants weak efficiency alone. In some combinations, antioxidative supplement has resulted in improved outcome of the specific therapy for influenzavirus and HIV infections, although the specific mechanisms of protection still remain poorly understood (Allard et al. 1998; Oda et al. 1989). Concerning the immune system, ROS may act as immunomodulators allowing or activating T-cell proliferation, an essential phenomenon in cell-mediated immune response. This idea is based on observations on the ability of several antioxidants to directly inhibit T-cell proliferation or the activation of transcription factors involved in T-cell activation (Hunt N, 1994; Chaudhri et al. 1986). On the other hand, the fatality of HIV infection lies on the exact opposite: increased apoptosis of CD4+ T-cells potentiated or possibly even triggered by the changed redox status (Romero-Alvira and Roche, 1998; Banki et al. 1998).

Only recently it has been shown that the interactions extend further. Malnutrition and deficiency of antioxidants are capable of affecting not only the host, but also the pathogen by increasing its virulence. In a series of experiments Beck et al. first observed that an avirulent strain of CVB3 (3/0), which did not cause any damage in normally fed control mice, established moderate myocardial lesions in both selenium or vitamin E deficient mice. When this avirulent strain was further passaged in a selenium or vitamin E deficient mouse and then reinoculated into a control mouse, severe myocardial damage was provoked. This was shown to be due to six point mutations in the viral genome, which changed an originally avirulent strain into virulent (Beck, 1997). As an example of another virus, ROS are reported to activate the binding of NF-κB to the viral promoter region of HIV resulting in increased viral replication and production of Tax protein. Tax again stimulates NF-κB (Baruchel and Wainberg, 1992) thus creating a vicious cycle for the benefit of the dissemination of the infection.

AIMS OF THE STUDY

The specific aims were

- To investigate the mechanisms of coxsackievirus mediated beta cell death during productive and restricted infective conditions
- To compare the mechanisms of beta cell death during low versus high multiplicity of coxsackievirus infection
- To study the effects of low versus high multiplicity infection on intracellular thiol balance in beta cells
- To elucidate the mechanisms of oxidative beta cell damage and to improve the defense of beta cells against reactive oxygen species by manipulating their thiol supply

GENERAL METHODOLOGY

More detailed protocols of all methods used are provided in the original publications.

1. Cell and tissue culture

1.1. Human islets (I, IV)

Isolated and purified human islets were obtained from Uppsala, Sweden (Nordic Network for Clinical Islet Transplantation, Department of Clinical Immunology and Transfusion Medicine) and Brussels, Belgium (Central Unit of ß-Cell Transplant). All processes followed the protocols approved by local ethics committees (Brandhorst et al. 1998; Ricordi et al. 1988). The cells were sent to Helsinki as free floating islets and further cultured on petri dishes in Ham's F-10 nutrient mixture with supplements. The volume and purity of the islets were determined under light microscope after staining with diphenylthiocarbazone, and only preparations of over 50% purity were selected for experiments.

1.2. Fetal porcine islets (III)

Yorkshire sows, 80 to 95 days pregnant, were obtained from a breeding piggery and killed with high voltage electrical stunning and immediate exsanguination at the slaughterhouse of the Department of Food Technology, University of Helsinki. The fetuses (crown-rump length 21-25 cm) were collected immediately, transported to the laboratory where the pancreases were aseptically removed within two hours after killing and prepared for culture as previously described (Korbutt et al. 1996; Otonkoski et al. 1999). This fetal porcine model was verified suitable for studies considering enterovirus infections in beta cells (Roivainen et al. 2001).

1.3. Insulin producing cell lines (II, IV)

INS-1 is a rat tumor-derived relatively differentiated insulin-producing cell-line, which has been shown to be suitable for the study of beta cell biology (Asfari et al. 1992). RINm5F is a less differentiated rat insulinoma cell line. These lines were kindly provided by Prof. Claes Wollheim (University of Geneva, Switzerland).

MIN6 is a more novel insulinoma cell line of mouse origin. It possesses better functionality defined by closer resemblance to the physiological characteristics of

primary beta cells including better insulin secretory response to glucose and higher intracellular insulin content (Poitout et al. 1996; Ishihara et al. 1993). Thus, it represents a more sophisticated model for studies of beta cell biology *in vitro*.

2. Enterovirus infections

2.1. Viruses (I, III, IV)

Prototype strains of CVB3/Nancy, CVB4/J.V.B., CVB5/Faulkner and CVA9/Griggs (from the American Type Culture Collection, Manassas VA) and the diabetogenic strain CVB4-E2 (from Dr. JW Yoon) were used in the experiments. All virus stocks were passaged in GMK (green monkey kidney) cells and their identities were confirmed by plaque-neutralization assays.

2.2. Viral exposure and infectivity measurements (I, III, IV)

Dilutions of virus preparations corresponding to certain multiplicities were prepared and the cells were exposed to them for 60 minutes at 36°C. The adsorption was followed by removal of the inoculum virus and addition of cell-preparation-specific culture medium. The cultures were incubated in CO_2-athmosphere at 36°C and the culture medium was changed twice a week.

Cell samples were collected after 0 hours, 1, 2 and 3 days of infection. For measurements of total infectivity the samples were frozen and thawed three times to release the virus followed by centrifugation and analysis of the supernatant in a dilution series on GMK cells. To visualize the infected cells, immunocytochemistry for enterovirus antibody was performed for methanol-fixed cells. A sample of non-infected cells served as a negative control. The result was analyzed by confocal microscopy showing infected cells in green.

2.3. Antiviral agents (III)

Restricted infective conditions were created by addition of a selective inhibitor of viral RNA replication into the culture medium immediately after exposure to the virus. In low concentrations (0.1-2 mM) the used inhibitor, guanidine hydrochloride, functions strictly anti-virally against small, unenveloped, RNA containing viruses, like coxsackieviruses (Pfister and Wimmer, 1999; Bovee et al. 1998). We tested its potential in 0.1 mM, 0.5 mM and 1 mM concentrations.

3. Oxidative stress

3.1. Oxidants and antioxidants (II)

We used hydrogen peroxide (H_2O_2) to establish oxidative conditions. H_2O_2 was chosen instead of known beta cell specific toxins (e.g. alloxan and streptozotocin) because of its presence in physiological oxidative processes. We tested 50 µM and 100 µM concentrations, to which INS-1 cells were exposed for 60 min at 37°C followed by a 24-hour incubation in culture medium.

Thiols are efficient reducing molecules that protect cells by interacting with free reactive radicals. Glutathione (GSH) system represents the most important part of the intracellular anti-oxidative machinery. L-cysteine is the precursor of GSH and thus necessary for its synthesis. L-cysteine was chosen instead of the frequently used N-acetylcysteine (NAC) because the deacetylation reaction needed for NAC to enter the intracellular space may not be operative in cell culture models. We tested the ability of 0.1 mM, 1 mM and 5 mM concentrations of L-cysteine to protect the cells from H_2O_2 induced damage. The exposure was similar and simultaneous with the H_2O_2 treatment.

3.2. Thiol analysis (II, IV)

Samples of RINm5F cells were analyzed straight after H_2O_2 and/or L-cysteine treatments for the level of intracellular GSH-content by a spectrofluorometric method. The samples were first exposed to 15 mM monobromobimane in 50 mM N-ethylmorpholine terminated by 100% trichloroacetic acid after 5 min. After quick centrifugation one µl of supernatant was injected onto a Waters Novapak C-18 HPLC column running an isocratic mobile phase (acetonitrile, acetic acid, perchloric acid, pH 3.7). The fluorescent product was detected using Shimadzu RF-10AxL spectrofluorometer with excitation and emission wavelengths at 394 nm and 480 nm (Ahola et al. 1999). Instead, from samples of virus-exposed MIN6 cells the infectivity was first abolished by acidic-treatments after which the same protocol was followed for GSH-measurements.

3.3. Northern blot analysis for gamma-glutamyl cysteine synthetase gene expression (IV)

8 µg of DNase treated total RNA extracted by a commercial kit (RNAeasy, Qiagen) was analyzed by northern blot to detect the level of γ-glutamylcysteine synthetase during a CVB5 infection of low versus high multiplicity at timepoints of one and four days. First the RNA samples were transferred onto nylon membranes and the blots were then hybridized with ^{32}P-labelled (DuPont) complementary RNA probe corresponding to nt 874-1106 of the published sequence (Lu et al. 1992). Finally, the membranes were rinsed, exposed to autoradiography films, stripped and reprobed with ^{32}P-labelled 18S RNA probe to normalize mRNA loading. The result was densitometrically analyzed as a ratio to the loading control.

4.   Detection of the mechanisms of cell death

4.1. Nuclear stainings (I, II, III, IV)

A fluorescent nuclear staining was performed to measure viability and score the mechanistic pattern of cell death. The analysis is based on double staining with Hoechst 33342 (HO) and ethidium homodimer-1 (EthD-1). HO stains all nuclear DNA blue. Instead, EthD-1 only permeates damaged cellular membranes (necrotic cells and late-phase apoptotic cells) and stains DNA red. Viable, pyknotic and necrotic cells are identified by round nuclei with either homogenous blue, bright condensed blue or homogenous red fluorescence, respectively. Apoptotic cells are detected by their fragmented nuclei that exhibit either blue or red fluorescence depending on the stage of the process (Hoorens et al. 1996).

Nuclear DNA fragmentation was further analyzed by TUNEL-staining (terminal dideoxynucleotidetransferase mediated dig-ddUTP nick end labeling). It was combined to cytoplasmic insulin staining to specifically detect apoptotic beta cells. TUNEL is based on tranferase-mediated digoxigenin-labeling of apoptotically cleaved free DNA nick ends. The final exposure to a chemiluminescent dye (CSPD) results in brown nuclei detected under light microscope. Insulin positivity was detected by exposure to guineapig anti-porcine insulin preparation following conjugation with biotinylated IgG and peroxidase-conjugated streptavidin. The cytoplasms were stained red by the reaction product of the used AEC-substrate.

The viability of primary human islets was detected and illustrated by a double staining with calcein and ethidium homodimer-1 performed according to the instructions of the commercial live/dead cell assay kit (L-3224, Molecular Probes). The stained cells were analyzed by fluorescent microscopy showing viable, esterase positive cells in green and dead cells in red.

4.2. Southern blot analysis (II, IV)

DNA was isolated by a similar commercial system (Apoptotic DNA Ladder Kit, Boehringer Mannheim) from all cell/islet preparations followed by RNase-treatment and spectrophotometric quantification. 2 to 3 μg of total DNA per sample was digoksigenin-labeled by transferase, electrophoresed through an agarose gel and detected with antibody-dependent chemiluminiscence. Apoptotic human testis DNA served as a positive control. DNA fragmentation was observed as a ladder pattern on x-ray film.

4.3. Electron microscopy (I, III)

Preparations of human islets and fetal porcine islets were similarly pretreated (2.5 % glutaraldehyde fixation for two hours followed by two rinses with phosphate buffer) to prepare samples for electron microscopic analysis of cell death. The fixed samples were then sent in phosphate buffer to Brussels for further analysis in Prof. L. Bouwens' laboratory. There the samples were embedded in Spurr resin and counterstained with uranyl acetate and lead citrate followed by analysis under electron microscope. Specific signs for apoptotic and necrotic cell deaths and characteristics of beta cells were searched and documented.

5.   Analyses of cellular functions

5.1. Measurement of intracellular insulin and DNA contents (I, II, III)

To confirm the viability and functionality of both primary islets and insulinoma cells their intracellular insulin and DNA contents were measured. The cell samples were first ultrasonically homogenized in redistilled water followed by splitting into separate samples for DNA and insulin measurements. DNA was analyzed in duplicate by a fluorometric method (Hinegardner, 1971) and insulin by a commercial RIA (DPC, Los Angeles).

5.2. Dynamic insulin release (I, III)

The functionality of primary islets was analyzed by dynamic insulin secretory response to stimulation by glucose and theophylline. This was done using a perifusion system described previously (Otonkoski et al., 1999). The flow rate was 0.25 ml / min and fractions were collected every four minutes. The concentrations of insulin in the fractions were measured by a commercial RIA kit (DPC, Los Angeles).

5.3. MTT analysis (II, III)

This analysis was performed to measure the metabolic viability of the H_2O_2 and/or L-cysteine treated INS-1 cells and of PFP cells treated with guanidine-HCl and/or infected with CVB5. Briefly, the cells were first incubated for 120 min with 0.5 mg/ml MTT and 16.7 mM glucose in KRB followed by dissolving the formazan crystals with 0.04 N HCl in isopropanol. The result was recorded as optical densities at 550 nm by a Victor2 multilabel counter (Janjic and Wollheim, 1992).

5.4. Nucleotide measurement (III, IV)

In order to rate the metabolic activity of beta cells, the level of ATP and ADP were measured since their production directly correlates to the level of glucose oxidation. By the side of the MTT analysis, these measurements were performed to test the toxicity of G-HCl and its protective capacity against CVB5 induced functional damage. Briefly, the samples were first abolished of infectivity by acidic treatments after which the nucleotide levels were measured by spectrofluorometry. The level of NADH was simultaneously measured to proportion the ATP level to the cell number. As an indicator of the deteriorating energy metabolism, the levels of purine nucleotide catabolism products (xanthine and hypoxanthine) in MIN6-cell culture media were measured according to the same spectrofluorometric protocol after anti-viral acidic treatment. The NAD level was simultaneously measured to proportion the data to cell number.

6. Nitrite and inducible nitric oxide synthase (iNOS) measurements (I, IV)

To examine the role of nitric oxide in the process of virus induced beta cell death, the level of nitrite was measured from the culture medium samples collected during the first week of infection. After treatments nitrite was spectrophotometrically measured

at 550 nm by a Victor2 (Wallac) multilabel counter using sodium nitrate as standard
(Green et al. 1982).

For the detection of inducible nitric oxide synthase (iNOS) semiquantitative RT-PCR
with specific primers for iNOS and GAPDH was run using 34 and 31 cycles
respectively (Pavlovic et al. 1999). The result was densitometrically quantified as a
ratio of iNOS/GAPDH.

RESULTS AND DISCUSSION

1.   Mechanisms of beta cell death during productive coxsackievirus infection (I, III, IV)

1.1. Human islets (I)

As referred to earlier, enteroviruses often establish subclinical viraemia *in vivo* but also fulminant phenotypes are possible. As an example, the diabetic strain of coxsackievirus B4 has been reported to lead to aggressive, lytic destruction of islet tissue (1979, Yoon et al). To understand the mechanisms of virus-mediated beta cell death in more detail we first infected isolated human islets in productive conditions with prototype strains of CVB3, CVB4, CVB5, CVA9 and the diabetic strain of CVB4 (E2). CVA9 was included because of its genetic similarity to CVBs and previous epidemiologic link to the pathogenesis of T1D (Chang et al, 1992; Roivainen et al, 1998). Since no strategies existed to study the viral effects on beta cells *in vivo*, optimal *in vitro* culture conditions were prepared for this purpose.

In addition to the diabetogenic CVB4-E2 strain, all tested serotypes were able to infect human islets. All CVB-infected cultures were detected with primary morphological changes already after one to two days of infection. At this early time point the morphology was predominantly characterized by nuclear pyknosis (40 % of all cells). Apoptosis was mostly increased by CVB5 (5.9 %). By seven days, all CVB-infected cultures were damaged by secondary necrosis. However, even at this point some islets managed to maintain sound morphology and viability possibly due to the metabolic heterogeneity of beta cells. It is of importance to notice the fact that experimental cell culture systems often lack neighboring phagocytic cells responsible for elimination of the apoptotic cells *in vivo*. Due to this, secondary necrotic cells may emerge with cytolytic characteristics and thus complicate the interpretation of the cell death pattern.

After one week of infection the insulin secretory capacity was most dramatically perturbed by CVB3 and CVB5. All tested CVBs decreased the intracellular insulin content by 40 to 65%. The expression level of inducible nitric oxide synthase (iNOS) was detected unchanged and it was thus concluded that nitric oxide did not mediate beta cell damage.

Interestingly CVA9 infected cells preserved high viability similarly to uninfected control cells. Furthermore, no signs of decreased insulin secretory capacity or intracellular insulin content were observed. It could thus be speculated that CVA9 might cause a persistent infection of islets *in vivo,* which could then result in the initiation or acceleration of beta cell specific autoimmunity.

1.2. Porcine fetal islets (III)

The model of culture-enriched porcine islet cells has been evaluated suitable for studies on the effects of virus infection in respect to the pathogenesis of T1D (Roivainen et al, 2001). Coxsackieviruses replicate well in porcine islets. In comparison to human islets, the porcine endocrine cells are more susceptible to virus induced damage perhaps due to their fetal, immature nature (Roivainen et al, 2001). This phenomenon was also observed in our experiments, in which CVB5 infection killed up to 80% of cells in only two days. The mechanism was predominantly necrotic, though slight apoptosis was observed similarly to the productive CVB-infection in human islets.

1.3 Insulin producing MIN6 cells (IV)

MIN6 cells, a mouse derived insulin producing cell line characterized by good glucose responsiveness, were productively infected with prototype strain of CVB5. The virus replicated well in these cells. However, in comparison to primary cells MIN6 cells were observed with an apparent potential to resist CVB5 infection. Productive infection resulted in gradual increase of necrosis as the dominant form of cell death. After two days approximately 35% of cells were dead, ending up with 60% mortality in one week. Out of the total cell mass only 4% and 2.5% died through apoptosis at the previously mentioned timepoints, respectively. Thus, apoptosis was only slightly and transiently increased. The CVB5-induced cell death pattern followed the one described in human islets and PFP cells.

2. Models of slowly progressing coxsackievirus B5 infection
-Description of the pattern of beta cell death (III, IV)

These studies were undertaken to clarify the mechanisms of coxsackievirus mediated beta cell death in two different settings intended to resemble the conditions associated with a slowly progressing infection *in vivo*. An attack by a foreign microorganism induces a defense response in the immune system in order to maintain the status quo and viability of the host. Due to this fact, no infection invading a normal, functioning organism is ever freely productive. Thus, our primary hypothesis was that a restricted infection created by treatment with an antiviral agent could mimic the trends and effects of a slowly progressing infection *in vivo*. A number of pharmacological agents with specific characteristics to inhibit various individual steps of the cycle of viral multiplication have been developed. One of these, guanidine hydrochloride (G-HCl), specifically inhibits RNA replication and release of small, unenveloped viruses into which category coxsackieviruses are included (Rightsel et al, 1961). On established cell lines, low concentrations of G-HCl (from 0.1 to 2 mM) are reported to function strictly anti-virally without affecting the cellular functionality or structural viability (Pfister and Wimmer, 1999). In a recent study on poliovirus infected Hela-cells, G-HCl-mediated restriction of infection resulted in a switch from pyknotic to apoptotic cell death. Furthermore, the induction of apoptosis was inhibited by a pan-caspase inhibitor (Agol et al, 1998). The protocol of Agol et al. was used as a guideline in our experiments on fetal porcine islets.

In vivo even a small amount of virus particles is enough to establish a local infection. Based on this knowledge we hypothesized, that inoculation of host cells with a low amount of infective viral particles (low multiplicity of infection) could also resemble the situation *in vivo*. Moreover, coxsackievirus infections often remain subclinical *in vivo*, which might be in concert with a small virus load in the target tissue. In order to enable comparisons between different experimental infections it is crucial to somehow standardize the infective conditions. For this purpose, the concept of multiplicity of infection (M.O.I.) has previously been created. By definition M.O.I. displays the number of infective virus particles per cell during an experimental viral exposure in a cell culture model. In general, the higher the M.O.I. the more efficient the progress of infection assuming that the cells exposed are susceptible and support

the multiplication of the specific virus. Together with the strain and serotype of the virus and host-related factors, M.O.I. probably affects both the outcome of infection and the mechanism of host cell death.

2.1. Guanidine-HCl increases the viability of CVB5-infected porcine islet cells but does not preserve their functional integrity

We infected fetal porcine islets with the prototype strain of apparent high multiplicity preparation of CVB5. G-HCl was added into culture media immediately after viral exposure in three different concentrations: 0.1 mM, 0.5 mM and 1 mM. Virus growth was inhibited significantly by the 0.5 mM concentration and blocked totally at 1 mM. Analyzed by nuclear double staining G-HCl dose-dependently protected the infected cells with 1 mM concentration being the lowest effective in preserving viability. It significantly increased the number of living cells at both early (2 to 3 days) and later (5 to 7 days) timepoints of infection. All tested concentrations of G-HCl were nontoxic in control cells revealed by intact MTT and ATP measurements. Regardless of these results and the fact of using exclusively low, strictly anti-viral concentrations of G-HCl, the unfortunate outcome of the dynamic insulin release assay showed perturbance of glucose-responsiveness. These data indicate that G-HCl probably disturbs some step distal to glucose metabolism in the pathway leading from glucose stimulus to insulin secretion in beta cells. Thus, more physiological nontoxic viral inhibitors need to be developed in the future.

2.2. Guanidine-HCl mediates a switch in CVB5-induced cell death pattern

Simultaneously with increasing the viability of infected cells through decreased necrosis, treatment with 1 mM G-HCl increased the amount of apoptotic cells. This was ascertained by means of nuclear double staining and electron microscopy. The present phenomenon is in concert with the earlier mentioned observations on poliovirus infected HeLa-cells (Agol et al, 1998). It has further coherence to previous data on Theiler's murine encephalomyelitis virus (member of the family *Picornaviridae*) and human immunodeficiency virus (HIV), which kill susceptible cells lytically but restricted cells via apoptosis (Jelachich and Lipton, 1996; Martin et al. 1994).

Although it is too early to speculate on the possible implications of this phenomenon for the pathogenesis of virally induced diabetes, we appreciate the likelihood of it being consistent with the hypothesis of beta cell apoptosis resulting from a local enterovirus infection possibly in combination with the following production and release of cytokines within the pancreas (Horwitz et al, 1998; Mandrup-Poulsen, 2001).

2.3. Low vs high multiplicity of CVB5 infection in MIN6 cells and human islets

We infected both MIN6 cells and adult human islets with preparations of either high (> 1000) or low (< 0.5) M.O.I. of prototype CVB5. Both multiplicities reached the same level of viral growth by three days, although the low M.O.I. culture achieved the maximum at a slower pace. As mentioned earlier, MIN6 cells show some resistance against CVB5 infection. Analyzed by nuclear double staining, during the high M.O.I. conditions the viability of MIN6 cells decreased slowly compared to primary cells. By six days 60% of MIN6 cells had died. Expectedly, destruction proceeded even slower in the low M.O.I. culture, where the death rate was only 30% after six days of infection. In human islets the high M.O.I. CVB5 infection lytically ruined the islet morphology in seven days, whereas islets inoculated with low M.O.I. preserved sound integrity.

2.4. Multiplicity of infection -dependent pattern of beta cell death

The morphology-based scoring of cell death after nuclear double staining revealed high M.O.I. CVB5 infection to result in steadily increasing necrosis as the dominant form of MIN6-cell death. Apoptosis was induced transiently, peaking on day two. Instead, within cells inoculated with low M.O.I. both apoptosis and necrosis were induced in moderate levels and the number of apoptotic cells increased linearly with time. Furthermore, the apoptosis/necrosis ratio increased dramatically during low M.O.I. but decreased during high M.O.I. culture clearly illustrating the multiplicity-dependent patterns of cell death.

A similar phenomenon was observed in human islets. TUNEL+Insulin staining showed an increasing amount of apoptotic beta cells at the margins of islets on both days four and seven after low M.O.I. CVB5 infection. Similar results were obtained from two other nuclear double stainings performed. As mentioned earlier, islets

inoculated with high M.O.I. instead showed almost totally ruined morphology after one week of infection. These observations were ascertained by Southern blot analysis of DNA fragmentation from both MIN6 cells and human islets at the timepoint of 4 days. Samples of both cell preparations cultured in low M.O.I. conditions showed a classical ladder-pattern characterizing DNA fragmentation. Similar patterning was evident in a weaker fashion in the sample of high M.O.I. conditions, as expected by the previous staining analyses.

According to these data apoptosis has a pronounced role as a mechanisms of beta cell death after low multiplicity of CVB5 infection. Thus, both of the models created (based on G-HCl restriction and low M.O.I.), result in increased apoptotic beta cell death, consistent with the hypothesis that this also occurs during a pancreatic enterovirus infection *in vivo*.

2.5. Nitric oxide (I, IV)

The recognition of the role of apoptosis during the process of virus mediated beta cell death during low M.O.I. conditions raised interest towards the pathway mediating this response. Nitric oxide (NO), known to hold antiviral properties, is considered important in the immune response against coxsackievirus invasion (Saura et al. 1999; Zaragoza et al. 1998). It has also been presented to act as a mediator of apoptotic cell death (Moncada and Erusalimsky, 2002; Sandau et al. 1997). However, tested on both human islets and MIN6 cells, we found the CVB-mediated cell death not to depend on NO and thus this pathway is also excluded as a mediator of the observed apoptosis. Our results are consistent with the previous observations on NO-independent cytokine- and dsRNA-mediated apoptosis in primary rat islets (Liu et al. 2000a; Liu et al. 2002).

3. Redox balance in stressed beta cells (II, IV)

3.1. Antioxidants protect against oxidative beta cell injury

Beta cells are very vulnerable to oxidative stress because of the weak capacity of their intracellular antioxidative machinery in comparison to other cell types (Lenzen et al. 1996; Tiedge et al. 1997). As previously concluded, oxidative molecules most

probably contribute to the process of beta cell damage and development of T1D. Thus, we analyzed the mechanisms of experimental oxidative injury in insulin producing cells (INS-1 and RINm5F) and tested the capacity of the antioxidant, L-cysteine, to protect from the hypothesized damage.

Exposure to either 50 µM or 100 µM hydrogen peroxide resulted in distinct morphological damage and impaired cellular functions evidenced by decreased intracellular insulin content. Furthermore the intracellular concentration of glutathione (GSH) decreased. Dose-dependent administration of L-cysteine partially protected from both the structural and functional oxidative damage observed by means of nuclear double staining and MTT-analysis. The protective 1 mM concentration of L-cysteine also increased the intracellular GSH-reserve.

Glutathione represents a crucial constituent of the intracellular antioxidative defense machinery and its synthesis is dependent on the availability of L-cysteine. We observed that L-cysteine, administered extracellularly as a substituent antioxidant, is protective against oxidative damage in insulin producing cells. Direct recognition and capture of reactive molecules likely play the most significant role in the protective process, but according to the detected raised intracellular GSH-level also thiol synthesis is utilized to achieve protection. Evidenced by dose-dependent protection and aggravated damage by higher concentrations, the therapeutic dose has to be carefully evaluated to avoid counteractive side effects.

3.2. Mechanisms of ROS-induced beta cell death
Oxidative stress may stimulate various signaling pathways with varying functions, targets and purposes. Apoptotic death is one outcome particularly favored by moderate level of oxidative stress (Lennon et al. 1991; Hampton and Orrenius, 1997).

Out of the tested concentrations, 50 µM hydrogen peroxide represents a strong physiological oxidative burst. In these conditions especially, distinct signs of DNA fragmentation were detected when analyzed by electrophoresis. Furthermore, this apoptotic patterning was abolished by 1 mM L-cysteine, the only concentration also protecting the viability. Supporting our observations, previous studies also acknowledge the anti-apoptotic potential of antioxidants, especially GSH and its

precursor N-acetylcysteine (Melnick, 1996; Heussler et al. 1999; Kelso et al. 2001; Sato et al. 2002; Naderi et al. 2003; Celli et al. 1998; Lee et al. 2001).

3.3. Effects of a coxsackievirus infection on redox balance in insulin producing cells

Attacks by foreign pathogens, like viruses, stimulate the host to produce protective agents. This excites and burdens the cellular metabolism, which inevitably results in the production and release of oxidative radicals in large amounts (Fernandez-Checa et al. 1998). Also direct killing by the attacking microbe may result in leakage of these radicals from the host cell. As mentioned earlier, the antioxidative capacity of the beta cell is low in comparison to other cell types. Thus, even moderate oxidative stress might rapidly use up the antioxidant reserves and even by itself induce detrimental damage. This situation is even more likely when increased free ROS circulate simultaneously with other detrimental molecules such as cytokines, likely to occur due to viral invasion.

In our experimental setting, a high multiplicity CVB5-infection resulted in gradual, permanent decrease of the major intracellular antioxidant, glutathione (GSH). Instead, during low M.O.I. the GSH reserve recovered totally from the initial decrease in the cells that remained viable. This observation is in concert with previous data on CVB3-induced myocarditis *in vivo*, which associated with a similar thiol-profile (Kytö et al, unpublished data). One could consider the recovering thiol-profile to characterize a more physiological stress response, which would logically follow a low-grade infection. Moreover, as mentioned before, such a moderate stress has also previously been linked to the induction of apoptotic cell death (Lennon et al. 1991; Hampton and Orrenius, 1997). Further in line was our Northern blot analysis revealing the expression level of γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione synthesis, to increase dramatically in response to high M.O.I. CVB5-infection and moderately in response to low M.O.I. CVB5-infection. Taken together these data implicate a possible protective role for the intracellular antioxidative system against virus induced beta cell damage.

SUMMARY AND CONCLUSIONS

Enterovirus infections are considered as potential triggers of the autoimmune response towards pancreatic beta cells and the following development of T1D. Same characteristics fit the oxidative molecules, which might aggravate an infective process. The diabetogenic potential is shared between various serotypes in the group of enteroviruses including coxsackieviruses, which represent a widely studied subgroup. These studies were undertaken to uncover the mechanisms of coxsackievirus- and experimental oxidative stress -induced beta cell death and further to reveal their possible interactions in the process of beta cell damage.

Based on the results obtained from the individual studies included in this thesis project the following conclusions can be presented:

1. Coxsackieviruses infect and replicate well in insulinoma cells, fetal porcine pancreatic islet cells and adult human islets. Thus these models are considered suitable for studies concerning virus-mediated effects on beta cells.

2. Productive coxsackie B virus infections of high multiplicity induce primarily nuclear pyknosis in human beta cells, which finally leads to lysis and impaired metabolic functions. In fetal porcine islets the process is more rapid and leads to extensive necrosis in only two days. Based on data from serotype A9 as a representative of coxsackie A viruses, infections due to this subgroup are less aggressive and the viability and functionality of beta cells are maintained.

3. When restricting the replication of coxsackievirus B5 by guanidine hydrochloride in porcine fetal islets, their viability increases dramatically through decreased necrotic cell death. Simultaneously apoptosis is induced. A similar phenomenon was observed in human islets and MIN6 insulinoma cells after inoculation with low multiplicity of coxsackievirus B5. In comparison to the necrotic outcome during high multiplicity infection, low multiplicity conditions favor apoptotic beta cell death. We hypothesize the restrictive and low multiplicity conditions to resemble the situation *in vivo* after a viral attack

and thus propose beta cell apoptosis as a possible outcome of a pancreatic infection.

4. Beta cells are vulnerable to oxidative stress due to their weak antioxidative competence. Our results show a moderate concentration of hydrogen peroxide to induce beta cell apoptosis, while in higher amount necrosis follows as the dominant form of death. 1 mM L-cysteine, the precursor of glutathione, efficiently inhibits oxidative beta cell death, but turns toxic in higher concentrations. By this model of experimental oxidative stress one may conclude that both oxidants and antioxidants act in dose-dependent manner in beta cells. Efficient protection against moderate, *in vivo*-like, oxidative stress may be achieved by carefully evaluated antioxidant supplementation.

5. Virus infections attract the cells of the immune system to produce and secrete cytokines and result further in overproduction of oxidative molecules. We show evidence that a productive coxsackievirus B5 infection of high multiplicity results in deterioration of the beta cell glutathione balance, while during low multiplicity of infection the cells maintain a better viability and are capable of recovering their glutathione reserves. This may be interpreted to indirectly demonstrate a virus-induced stimulation of oxidant production in a dose dependent manner. Furthermore, these data indicate that beta cells use their scarce antioxidative capacity to defend themselves against an infective process. This renders antioxidants important in the battle against virus-induced beta cell injury.

CONCLUDING REMARKS

The pathogenesis of type I diabetes has long been acknowledged to potentially associate with an enterovirus infection and increased oxidative stress. Most of these data derive from epidemiological and clinical patient-based studies. The problem associated with more detailed research on the mechanisms of beta cell damage is the limitation to study this process *in vivo*. Thus, insulin producing cell lines and several *ex vivo* tissue culture models have been created, evaluated and used to serve this purpose.

In the present set of studies we have used *in vitro* models to study in detail the mechanisms of beta-cell death induced by enteroviral infection or oxidative stress. Acknowledging the limitations of these models, we may conclude that apoptotic beta cell death represents a favored outcome of a slowly progressing enterovirus infection and of moderate oxidative stress. Furthermore, a physiological redox balance and antioxidative supplementation may protect from both oxidative and virus induced beta cell damage. Detailed knowledge of the mechanisms of beta cell damage is essential for the development of new anti-viral and anti-oxidative strategies in order to protect pancreatic beta cells.

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