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Recent progress in research on beta-cell apoptosis by cytokines

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1. ABSTRACT

Pancreatic beta-cell apoptosis plays a critical role in the pathogenesis of type 1 diabetes mellitus. As death effector molecules, perforin, Fas ligand, tumor necrosis factor (TNF)-alpha, Interleukin (IL)-1, interferon (IFN)-gamma, and nitric oxide have been claimed. Recently, combinations or synergisms between IFN-gamma and TNF-alpha or IL-1β are being revisited as the death effectors, and signal transduction of such synergisms has been explored to find molecular mechanism of beta -cell death. Among the regulators of apoptosis, nuclear factor-kappaB (NF-kappaB) has emerged as a master switch of cytokine-induced beta -cell dysfunction and death. By employing TNF-alpha / IFN-gamma synergism model which causes beta -cell apoptosis, we found that the antiapoptotic X-linked inhibitor of apoptosis (XIAP) molecule is upregulated by NF-kappaB in response to TNF-alpha and XIAP induction was inhibited by IFN-gamma-induced signal transducer and activator of transcription-1 (STAT1) activation, which explains the death of beta -cells by TNF-alpha /IFN-gamma synergism.

2. INTRODUCTION

Type 1 diabetes mellitus (T1D) occurs as a result of selective immune-mediated destruction of insulin producing pancreatic islet β-cells with consequent insulin deficiency (1). In genetically susceptible individuals, body immune system mistakenly launches an attack on β-cells. Thus, antigen-presenting cells (APC) present specific β-cell antigens (autoantigens) to autoreactive T cells, and T cells recognizing β-cell autoantigens subsequently impose death on β-cells. In nonobese diabetic (NOD) mice, a prototypic animal model of T1D, pancreatic islets are infiltrated by diverse inflammatory/immune cells that comprise T or B cells, macrophages and dendritic cells. Such diverse inflammatory cells themselves and inflammatory mediators such as cytokines and free oxygen radicals released from them all contribute to β-cell destruction. To explain the above observation, an inflammatory model for the pathogenesis of T1D has been suggested (2). Initially, liberation of β-cell antigen is caused by neonatal β-cell mass remodeling or by environmental factors, which induces presentation of β-cell antigens to $CD8^+$ T cells that

Table 1. Death mediators involved in β-cell apoptosis

Contact-dependent apoptosis	Contact-independent apoptosis
Perforin/granzyme ($CD8^+$ T cells)	Soluble cytokines (IFN-γ, TNF-α, IL-1β, etc)
FasL (some diabetogenic $CD4^+$ T cells)	NO, ROS (from $CD4^+$ T cells and/or macrophage)
Other membrane-bound cytokines	

causes β-cell damage via perforin pathway. Liberated β-cell components are taken up by dendritic cells in the islets and transported to regional pancreatic lymph nodes (PLN), where the antigens are presented to autoreactive $CD4^+$ T cells. After activation, $CD4^+$ T cells home to the islets and activated T cells together with released cytokines finally cause β-cell death.

Recent evidence points to apoptosis as the main form of β-cell death in animal models of T1D (2-5). The initiation phase of the β-cell apoptosis is mediated by contact-dependent mechanisms or by contact-independent cytokine action via induction of proapoptotic signaling in β-cells (Table 1). Autoreactive T lymphocytes contribute significantly to β-cell death in T1D (6, 7). Previous adoptive transfer experiments indicated that both $CD4^+$ and $CD8^+$ T lymphocytes are important final effectors in β-cell destruction (7, 8). T lymphocytes exert contact-dependent cytotoxicity upon target cells using mainly two separate arms- the perforin-granzyme ($CD8^+$ T cells) and the Fas-mediated pathways ($CD4^+$ T cells) (9, 10). However, Fas-Fas ligand (FasL) system cannot fully explain β-cell death by $CD4^+$ T cells (7), the most important death effector cells in T1D (3, 11, 12) . Besides contact-dependent cytotoxicity, $CD4^+$ T cells induce contact-independent target cell death via soluble mediators such as tumor necrosis factor- α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ). Macrophages and dendritic cells may also play a major role as a source of oxygen radicals or other soluble cytotoxic mediators (13). Among cytotoxic effector molecules, previous reports showed that combinations of proinflammatory cytokines exert much stronger or synergistic effects on β-cell viability *in vitro,* since most single cytokines have negligible effect on islet cell viability. IFN-γ/IL-1β combination (14) has been considered as a strong candidate for death effectors in T1D, while recent *in vivo* results do not fully support its role (15, 16). We and other investigators (17, 18) have suggested that IFN-γ/TNF-α synergism is a critical death mediator in the final effector phase of β-cell destruction in T1D (18-20) Based on the results we obtained before, we proposed that $CD8^+$ T cells lyse β-cells via perforin-mediated cytotoxicity, whereas $CD4^+$ T lymphocytes (as a major source of IFN-γ and/or TNF-α) act in collaboration with macrophages (as a major source of TNF-α) (19) to induce β-cell death through delayed-type hypersensitivity-like reaction in which mononuclear cells and cytokines from them work together and lead to pronounced inflammatory reaction. However, the exact signal transduction mechanisms in β-cell death by such cytokine synergisms have been unclear. Many studies have implicated nuclear factor kappa B (NF-κB), whose activity is stimulated by both TNF-α and IL-1 (21), as an important player in β-cell apoptosis (22-24).

It is well established that NF-κB activation by TNF family members plays an antiapoptotic role and that NF-κB activity is essential for prevention of TNF-α-induced death (25-28). However, NF-κB activation was suggested to have proapoptotic effects after activation by IL-1, ultraviolet light, or chemotherapeutic agents (29), where it mediates the synthesis of FasL (30) and, possibly other death mediators (31). The purpose of this review is to focus on apoptotic machinery in pancreatic β-cells, especially the protective role of NF-κB against TNF-α induced death signal, where interesting advances have been achieved employing *in vitro* models and transgenic or knock-out mouse systems.

3. ROLE OF NF-κB IN β-CELL DEATH OF T1DM

Understanding of the gene regulation and final effector mechanisms has become an area of intense research due to the importance of apoptotic β-cell death in T1D. In an attempt to elucidate the death signals for β-cell apoptosis, we studied which cytokine (combination) could lead to pancreatic β-cell death using MIN6N8 insulinoma cells (18). A combination of IFN-γ and TNF-α, but not either cytokine alone, induced a classical caspase-dependent apoptosis in murine insulinoma and pancreatic islet cells. IL-1β had a minor effect on insulinoma cell death. IFN-γ treatment seems to sensitize otherwise resistant insulinoma or primary pancreatic β-cells to TNF-α-mediated apoptosis by activation of signal transducer and activator of transcription-1 (STAT1). Transfection of phosphorylation-defective STAT1 abrogated islet cell apoptosis by IFN-γ/TNF-α combination, suggesting that STAT1 phosphorylation plays a critical role in the IFN-γ-induced induction of TNF-α susceptibility and illustrating a novel signal transduction in IFN-γ/TNF-α synergism (18). In the case of TNF-α that could be from T cells and/or macrophages (32), coexistence of IFN-γ (or other cytokines inducing TNF-α susceptibility) might be critical because β-cells are resistant to TNF-α alone as stated above which appears to be associated with the concomitant activation of the antiapoptotic process such as NF-κB activation (25, 28, 33).

Previous papers have implicated NF-κB as an important player in the protection of target cells against TNF-α induced apoptosis (22, 23, 34). However, other papers reported increased cell death by NF-κB activation after hypoxic injury or reactive oxygen species (ROS) treatment of neuronal cells or lymphocytes (35, 36). In the case of pancreatic islet cells, we have reported antiapoptotic role of NF-κB in pancreatic β-cell death by TNF-α. In brief, specific inhibition of NF-κB activation by transfection or adenoviral transduction of inhibitor of κB (IκBα) 'superrepressor' (IκBα-SR) sensitized insulinoma cells to TNF-α-induced apoptosis (37). In contrast, other reports have described a proapoptotic role for NF-κB in pancreatic β-cell death after IL-1β or IFN-γ/IL-1β treatment (38-42). The difference may reflect the involvement of different death effectors and NF-κB activators. Furthermore, *in vivo* circumstances would be

quite different from simple *in vitro* ones in that multiple stimuli including membranous/soluble forms of cytokines and other death effector molecules coexist at different concentrations depending on the stage of development of T1D. If IL-1β is a dominant death effector of pancreatic β-cell death in the development of autoimmune diabetes *in vivo* as claimed in previous papers (43-45), then NF-κB activation by IL-1 could be detrimental to pancreatic islet cells (39-42), contrary to TNF-α induced NF-κB activation.

To address these issues, we examined the *in vivo* function of β-cell NF-κB in a mouse model for autoimmune T1D. We produced transgenic NOD and C57BL/6 mice expressing a degradation-resistant form of IκBα (mIκBα), which functions as an NF-κB inhibitor (46) in pancreatic β-cells (RIP-mIκBα/NOD and RIP-mIκBα/C57BL/6 mice) in collaboration with Dr. Sherwin at Yale University (47). When islet cells of RIP- mIκBα/C57BL/6 mice were treated with TNF-α/IFN-γ, mIκBα-islet cells were more susceptible to apoptotic death relative to normal counterparts. In contrast, IL-1β plus IFN-γ treatment resulted in fewer mIκBα-islet cells death compared to normal counterparts which could be explained by compromised induction of inducible NO synthase (iNOS). To further confirm these *ex vivo* findings, we produced mice with β-cell-specific deletion of IκB kinase β (Ikkβ), a central mediator of NF-κB activation ($Ikk\beta^{\Delta\beta\text{-}cell}$ mice) in collaboration with Dr. Karin at University of California San Diego (21, 48, 49). Islet cells from $Ikk\beta^{\Delta\beta\ cell}$ mice exhibited a significantly elevated death response relative to normal counterparts when incubated with TNF-α/IFN-γ. Similar to mIκBα-islet cells, less cell death was seen in islets of $Ikk\beta^{\Delta\beta\ cell}$ mice after IL-1β plus IFN-γ treatment compared with normal counterpart islets. Hence, the effect of NF-κB on β-cell viability *in vitro* was dependent on the cytokine combination. When we directly examined the role of β-cell NF-κB in development of autoimmune diabetes *in vivo*, development of diabetes was significantly accelerated in RIP- mIκBα/NOD mice compared with nontransgenic littermates, suggesting that the net effect of NF-κB in β-cells is a protective one in the course of T1D and TNF-α plays a dominant role in β-cell destruction *in vivo* compared to IL-1β. When we adoptively transferred purified diabetogenic $CD4^+$ T cells from BDC2.5-SCID mice carrying the rearranged TCR-α and -β genes of a diabetogenic $CD4^+$ T cell clone (50) to irradiated RIP-mIκBα/NOD recipients, injection of anti-TNF-α almost completely abrogated the development of diabetes, suggesting that $CD4^+$ T cells use TNF-α as a critical death effector in RIP-mIκBα/NOD mice. We then compared incidence of diabetes after transfer of $CD4^+$ T cells between RIP-mIκBα/NOD mice and nontransgenic littermates. The development of diabetes after BDC2.5 $CD4^+$ T cell transfer was also accelerated in RIP-mIκBα/NOD mice compared with nontransgenic littermates. Hence, NF-κB activation within β-cells plays an important protective role against $CD4^+$ T cell-mediated killing through TNF-α-dependent mechanism. These results are consistent with a recent paper reporting that diabetogenic $CD4^+$ T cells produce TNF-α and IFN-γ, which are highly representative of cytokines in natural T1D (51).

4. ROLE OF NF-κB-MEDIATED XIAP UPREGULATION AGAINST β CELL APOPTOSIS

As was shown in the previous section, NF-κB is an important mediator of antiapoptotic activity against TNF-α/IFN-γ-induced pancreatic β-cell death. Then, what molecules are downstream players of NF-κB activation? Among the NF-κB target genes, the inhibitor of apoptosis proteins (IAPs) are potent natural suppressors of apoptosis and function by directly inhibiting the activity of caspases, the principal effectors of apoptotic cell death (52). Among the IAPs, X-linked inhibitor of apoptosis (XIAP) is the most potent inhibitor against caspase-3, -7, and -9 (53). When we examined the XIAP expression in MIN6N8 insulinoma cells, we noticed that XIAP level was inversely correlated with the progression of MIN6N8 cell death and caspase-3-like activation induced by IFN-γ/TNF-α or IκBα-SR/TNF-α. Further, the inhibition of XIAP expression by antisense oligonucleotide rendered MIN6N8 cells susceptible to apoptosis by TNF-α alone. Adenoviral XIAP expression also significantly inhibited the apoptosis of MIN6N8 cells by IFN-γ/TNF-α and IκBα-SR/TNF-α, which suggests that the decrease in XIAP level was not simply a result of apoptosis but contributed to apoptosis. To further investigate the role of endogenous XIAP in MIN6N8 cell apoptosis, we synthesized cell-permeable second mitochondria-derived activator of caspases (Smac) peptide that binds XIAP and blocks their caspase-inhibitory activity (54-56). Transduction of cell-permeable Smac peptide rendered MIN6N8 cells susceptible to apoptosis by TNF-α alone, again supporting that XIAP is an important antiapoptotic molecule in insulinoma cells (57).

We also studied the possible regulation of XIAP expression by IFN-γ in an attempt to understand the mechanism underlying the cytotoxic synergism between IFN-γ/TNF-α. IFN-γ suppressed TNF-α-induced upregulation of XIAP at protein level in MIN6N8 cells. Since IFN-γ did not affect TNF-α induced NF-κB activation in MIN6N8 cells (37), these results suggest that IFN-γ suppresses the expression of XIAP at the level of translation independent of NF-κB, which lowers the threshold for caspase activation and apoptosis induction by TNF-α (57).

In an attempt to elucidate the XIAP regulation in primary β-cells instead of insulinoma cells, we examined the effect of NF-κB inhibition on expression of antiapoptotic genes in β-cells of RIP-mIκBα mice. Importantly, induction of XIAP and cellular FLICE-inhibitory protein (c-FLIP) by TNF-α was abrogated in mIκBα-islet cells, suggesting a requirement for NF-κB in the induction of antiapoptotic molecules. Under the same condition, expression of two other apoptotic inhibitors cIAP1 and TNF-receptor associated factors 2 (TRAF2) was also abolished in mIκBα-islets. Because the proapoptotic effect of mIκBα was most significant when cells were

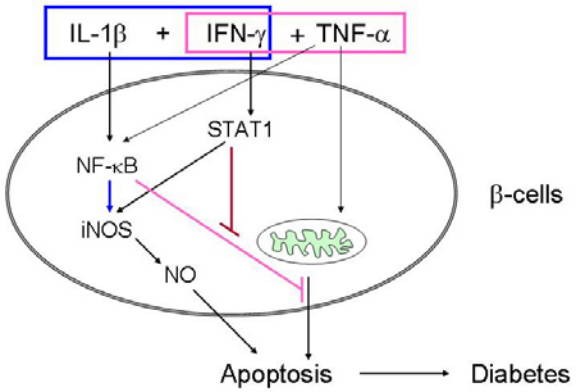

Figure 1. Diagram of the signaling pathways involved in β-cell death by IFN-γ/TNF-α synergism. $CD4^+$ T lymphocytes (as a major source of IFN-γ and TNF-α) act in collaboration with macrophages (as a major source of TNF-α) to induce β-cell death through delayed-type hypersensitivity-like reaction in which mononuclear cells and cytokines from them work together, leading to pronounced inflammatory reaction. NF-κB is activated by IL-1β or TNF-α. When activated by IL-1β, NF-κB plays a proapoptotic role by inducing iNOS and producing nitric oxide (NO). In contrast, NF-κB activated by TNF-α plays an antiapoaptotic role by inducing transcription of antiapoptotic molecules such as XIAP that inhibits caspases downstream of mitochondrial events. STAT1 activated by IFN-γ inhibits translation of antiapoptotic proteins

subjected to TNF-α plus IFN-γ treatment, we examined the effect of IFN-γ on XIAP and c-FLIP expression. IFN-γ did not affect mRNA level of XIAP but decreased XIAP and c-FLIP protein levels in both nontransgenic islets and mIκBα-islet cells. mIκBα transgene and IFN-γ had additive inhibitory effects on XIAP expression (47). These findings all suggest that XIAP plays an important regulatory role in TNF-α-induced β-cell apoptosis, and NF-κB activation by TNF-α could be an antiapoptotic signal through upregulation of XIAP. However, we do not rule out the involvement of other NF-κB-dependent antiapoptotic molecule (s) such as other IAPs (22) and/or antiapoptotic Bcl-2 family members (58). Protective role for XIAP in β-cell injury has also been demonstrated in other types of β-cell death such as an allograft model (59). While NF-κB in nonlymphoid cells such as β-cells plays an antiapoptotic role in TNF-α-induced cell death, NF-κB in lymphoid cells such as macrophages or dendritic cells is critical in the inflammatory and immune responses. We recently reported that NF-κB is activated in macrophages after contact with β-cells undergoing secondary necrosis through Toll-like receptor 2 (TLR2) and that such inflammatory response of macrophages or dendritic cells contributes to the initiation of T1D (60)

5. ROLE OF STAT1 IN β-CELL DEATH OF T1DM

We have suggested an important role for STAT1 in β-cell death by IFN-γ/TNF-α synergism, since STAT1 was phosphorylated by IFN-γ and transfection of phosphorylation-defective STAT1 rendered MIN6N8 insulinoma cells resistant to cell death by IFN-γ/TNF-α (18). However, other investigators reported that experimental allergic encephalomyelitis (EAE), an autoimmune disease involving central nervous system (CNS), was aggravated in *STAT1*$^{-/-}$ mice (61, 62), which is in contrast to our suggestions that STAT1 renders target cells susceptible to cytokine-induced death. Thus, we studied *in vivo* role of STAT1 in a mouse model for T1D using *STAT1*$^{-/-}$ mice. Primary islet cells from *STAT1* $^{-/-}$ mice were resistant to death by IFN-γ/TNF-α or IFN-γ/IL-1β (63). When we produced *STAT1*$^{-/-}$ NOD mice by backcrossing *STAT1*$^{-/-}$ mice onto the NOD background and checked the incidence of diabetes, no diabetes or insulitis was observed up to 50 weeks after birth. When we transferred highly diabetogenic BDC2.5 $CD4^+$ T-cells from NOD/BDC2.5-SCID mice (4) into irradiated NOD/*STAT1*$^{+/-}$ mice, the incidence of diabetes was decreased to less than 20% in NOD/*STAT1*$^{-/-}$ mice, indicating that STAT1 deficiency in islet cells render them resistant to cell death by diabetogenic $CD4^+$ T cells *in vivo* (63). However, when we conducted adoptive transfer of lymphocytes from diabetic NOD mice into irradiated NOD/*STAT1*$^{-/-}$ mice, the final incidence of diabetes after adoptive transfer was not significantly decreased in NOD/*STAT1*$^{-/-}$ mice compared with control mice, while the development of diabetes was delayed. This inconsistency could be due to $CD8^+$ T cells equipped with STAT1-independent death effectors such as perforin. Because β-cells in NOD/*STAT1*$^{-/-}$ mice appear to be susceptible to effector T cells other than $CD4^+$ T cells, the complete absence of diabetes or insulitis in NOD/*STAT1*$^{-/-}$ mice may not be explained by the resistance of *STAT1*-null β-cells against cytokine-induced apoptosis alone. Indeed, we observed skewed Th1/Th2 phenotypes in NOD/*STAT1*$^{-/-}$ mice (63). Taken together, these results suggest potential therapeutic potential of STAT1 blockade or inhibitors of its upstream Jak kinases in the prevention or treatment of T1D.

6. CONCLUSIONS AND PERSPECTIVES

There have been remarkable advances in the research on the molecular and immunological pathogenesis of T1D. In this review, we summarized recent progress in the signal transduction in β-cell death by cytokines. This review is based mostly on the data from our laboratory, and we regret that we could not accommodate all of the valuable data from other laboratories. We showed the protective role of NF-κB in β-cell death by TNF-α- or TNF-α/IFN-γ synergism and identified XIAP as one of the target molecules of NF-κB. We also showed a critical role of STAT1 in pancreatic β-cell death by cytokines and in the development of T1D (Figure 1). Our results provide a new insight into the signal transduction of β-cell apoptosis by cytokines and suggest a therapeutic potential of XIAP (or other antiapoptotic molecules) or regulation of STAT1 (or its upstream Jak kinases) in the prevention/treatment of T1D. Although NF-κB activation is often assumed to upregulate proapoptotic genes, studies cited above clearly demonstrated that the

timing of NF-κB activation significantly influences apoptotic outcomes following various combinations of stimuli. For example, inhibition of NF-κB activation during TNF-α/IFN-γ leads to increased apoptosis, while inhibition of NF-κB activation following IL-β/IFN-γ prevents apoptosis. The processes that influence β-cell survival in T1D are diverse and complex. By exploring signal transduction in β-cell apoptosis, we hope new targets for intervention of T1D are available in the future.

NF-κB activation may serve as a target in type 2 diabetes (T2D) also. Recent studies have shown that inhibition of NF-κB or its upstream kinase Ikkβ in inflammatory cells or hepatocytes attenuates the development of obesity-induced insulin resistance and T2D (64, 65). However, because β-cell destruction may also contribute to the pathogenesis of advanced T2D, it is important to elucidate the long-term effect of NF-κB inhibition on β-cell survival before development of antidiabetic agents targeting this pathway.

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Abbreviations: APC: antigen presenting cell; c-FLIP: cellular FLICE-inhibitory protein; FasL: Fas ligand ; IAPs: inhibitor of apoptosis proteins; IFN: interferon; IκB: inhibitor of κB; IκBα-SR: IκBα superrepressor; Ikkβ: IκB kinase β; IL: interleukin; iNOS: inducible NO synthase; NF-κB: nuclear factor kappa B; NO: nitric oxide; NOD:nonobese diabetic; PLN: pancreatic lymph nodes ; RIP: rat insulin promoter ; ROS: reactive oxygen species; Smac: second mitochondria-derived activator of caspases; STAT1: signal transducer and activator of transcription-1; T1D: type 1 diabetes mellitus; T2D: type 2 diabetes mellitus; TNF: tumor necrosis factor; XIAP: X-linked inhibitor of apoptosis.

Key Words : Apoptosis, Diabetes, XIAP, NF-kappaB, TNF-alpha, IFN-gamma, STAT1, Signal transduction, Review

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