β-Cell death during progression to diabetes

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The hallmark of type 1 diabetes is specific destruction of pancreatic islet β-cells. Apoptosis of β-cells may be crucial at several points during disease progression, initiating leukocyte invasion of the islets and terminating the production of insulin in islet cells. β-Cell apoptosis may also be involved in the occasional evolution of type 2 into type 1 diabetes.

Type 1 diabetes is an autoimmune disease resulting from specific destruction of the insulin-producing β-cells of the islets of Langerhans of the pancreas[1]. It has two distinct phases: insulitis, when a mixed population of leukocytes invades the islets; and diabetes, when most β-cells have been killed off, and there is no longer sufficient insulin production to regulate blood glucose levels, resulting in hyperglycaemia. Individuals can have covert insulitis for a long time (years in humans, months in rodent models) before it finally progresses to overt diabetes, and sometimes it never does.

Type 1 diabetes is an old disorder — descriptions of it appear in ancient Egyptian and Greek writings. It is also a common disease, currently affecting about 0.5% of the population in developed countries and increasing in incidence. In addition, there is mounting evidence that a fraction (variously estimated at 5–15%) of people originally diagnosed as type 2 diabetic may actually have a slowly progressing and less severe form of type 1 termed 'latent autoimmune diabetes of adults'. It is surprising, then, that we remain so ignorant about the aetiology and pathogenesis of autoimmune diabetes. We do not know what triggers it, have little understanding of the genetic and environmental factors regulating its progression, and have a confused view of the final effector mechanism(s). Consequently, we still do not know how to prevent or reverse type 1 diabetes in a sufficiently innocuous manner to be therapeutically useful. Faced with the difficulties of addressing these issues in humans, many investigators have turned to small-animal models of diabetes, in particular the non-obese diabetic (NOD) mouse and transgenic derivatives of it (Box 1).

Here we focus on the crux of autoimmune diabetes — β-cell death. We explore the possibility that physiological destruction of β-cells is a crucial event at disease outset, initiating autoimmunity. We also evaluate what is known about the mechanism(s) of β-cell death in the terminal phases of the autoimmune attack, and weigh the contention that destruction of β-cells can convert type 2 into type 1 diabetes.

Death as the initiator

Type 1 diabetes is primarily a T-lymphocyte-mediated disease[1,2]. Immunohistological analyses show that most

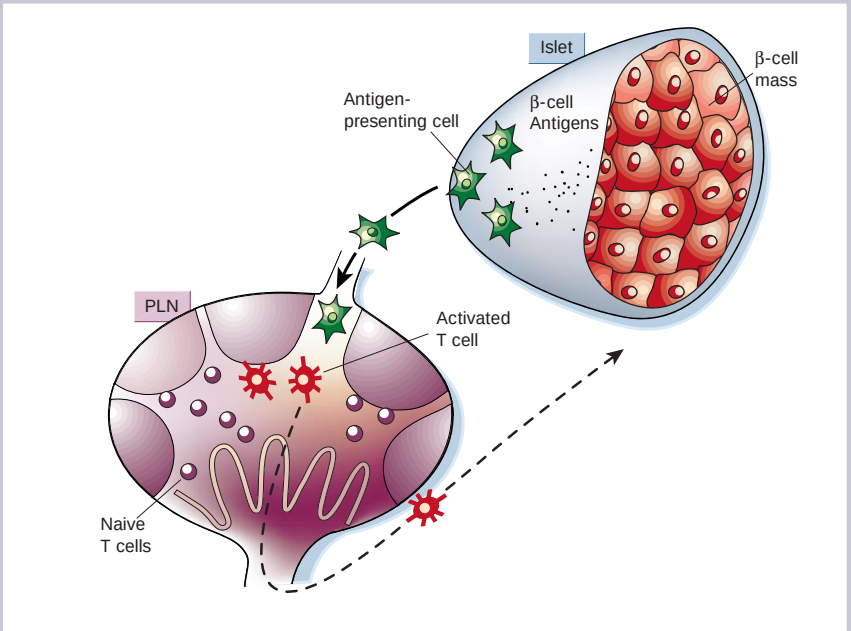

Figure 1 Proposed scheme for the initiation of type 1 diabetes. Naive T cells (purple spheres) circulate through the blood and lymphoid organs, including the pancreatic lymph nodes (PLNs). In the nodes, they encounter APCs (probably mature DCs) displaying on their surface MHC molecules carrying antigens in the form of peptide fragments. In this case, the antigens derive from proteins synthesized by pancreatic islet β-cells, picked up (in soluble form, as cell bits or as apoptotic cells) when the APCs (probably DCs in immature state) resided in the islets. A minute fraction of the naive T cells can recognize MHC molecule/β-cell antigen complexes, become activated (red asterisks), and then access tissues including the pancreas, where they re-encounter cognate antigen, and are reactivated and retained.

leukocytes in the islet infiltrate are T cells. Disease does not develop in NOD mice that are genetically athymic or T lymphopenic, or were thymectomized at birth; likewise, it is dampened or even abrogated by reagents that interfere with T-cell function. Finally, diabetes can be transferred by injecting T cells from diseased donors into healthy NOD recipients — the purest demonstration being inoculation of a single T-cell clone into a lymphocyte-deficient NOD mouse. Although most of these data derive from rodent diabetes models, there is ample indication that the human disease is also mediated by T lymphocytes, and has a similar islet histology and positive response to treatment with T-cell inhibitors.

T cells with diabetogenic properties fall into both the $CD4^+$ helper and the $CD8^+$ killer classes, although both may be required for optimum disease activity[1,2]. They respond to several antigens that are specifically, but perhaps not uniquely, synthesized by pancreatic islet β-cells, including peptides derived from insulin, glutamic acid decarboxylase and the tyrosine phosphatases 1A-2 and 1A-2β (phogrin). For $CD4^+$ T cells, the antigens are peptides presented by major histocompatibility complex (MHC) class II molecules found primarily on specialized antigen-presenting cells (APCs); for $CD8^+$ T cells, they are peptides displayed by MHC class I molecules present on most cell types.

Where and when the autoimmune response is initiated

Where do naive, potentially diabetogenic T cells first encounter their β-cell-derived antigens? The most likely possibilities are in the islets themselves; in the lymph nodes draining the islets (the pancreatic lymph nodes; PLNs); or at some distant site (although in this case the original stimulus would not be the β-cell antigen itself, but rather a molecular mimic of it introduced by infection or diet). Data from two transgenic mouse models of diabetes[3,4] support the second explanation, suggesting a scheme (Fig. 1) that is highly analogous to that established for T-cell responses to antigens introduced through the skin.

Like all naive T lymphocytes, naive β-cell-reactive, potentially diabetogenic T cells do not usually access the tissues (including the pancreas), but circulate freely through the blood and lymphoid organs. At some point, β-cell-derived antigens are taken up by APCs (probably dendritic cells; DCs) in the islets, inducing maturation of the APCs and their migration to the immediately draining PLNs. Here, they display the β-cell-derived antigens to naive β-cell-reactive T cells in circulation and activate them. As part of the activation process, the T cells acquire the ability to migrate through the tissues; in the islets, they re-encounter cognate antigen, become reactivated and are retained, thereby initiating insulitis. An unknown fraction of the activated and/or re-activated T cells may re-enter the circulation. Much evidence suggests that a similar sequence of events unfolds to initiate insulitis in standard NOD mice[3].

This scheme raises the issue of when β-cell antigens are first made available for uptake by islet-resident APCs. An answer has come from a T-cell receptor (TCR) transgenic mouse model of diabetes[3]. Insulitis begins abruptly and highly synchronously in these animals at 15–18 days of age. At and after this time, it is possible to detect the relevant β-cell-derived antigen in the PLNs (and in no other lymph nodes) by monitoring activation and proliferation of transferred transgenic T cells; before this time, the PLNs seem to be free of the relevant antigen even though it is synthesized by β-cells from birth[5]. There seems to be no difference in the timing or extent of β-cell antigen release to the PLNs in NOD versus non-diabetes-prone C57Bl/6 mice[6], implying that this event, albeit crucial to the process, does not explain the propensity of NOD mice to develop diabetes.

How autoimmunity is initiated

In regard to how β-cell antigens are first made available, an intriguing possibility is that they are taken up by APCs in the islets because of the death of β-cells. Many groups have shown that a wave of physiological β-cell death takes place in the islets of juvenile rodents, peaking at 14–17 days after birth[7–10]. Significant death at this age was first postulated on the basis of mathematical modelling studies, taking into account the kinetics of β-cell division, changes in cell volume, and the evolution of cell mass[7]. Confirmation came from quantifying dying cells on pancreas sections[8,9]: the frequency of dying cells was low even at peak times (4–13%), but, given that dead cells can be very difficult to detect owing to rapid engulfment and disposal, it is possible that a significant fraction (estimated at one-third) of the β-cells in juvenile rodents turns over. A wave of β-cell death was observed in both normal and diabetes-prone strains, although perhaps augmented in some of the latter[7]. Interestingly, an analogous perinatal wave, peaking at birth, has been demonstrated in humans, leading to the suggestion that extension of this wave might be the cause of hyperinsulinaemia in infancy[11].

The significance of such a wave of β-cell death in the context of initiation of autoimmunity is that the engulfment of apoptotic and necrotic cells can be a potent activation signal for APCs, in particular DCs[12]. Most recently, emphasis has been placed on necrotic cells as an immunogenic (as opposed to a tolerogenic) stimulus, but apoptotic

Box 1
The NOD mouse and simpler murine models

Discovered in the late 1970s, the non-obese diabetic (NOD) mouse has become the most commonly used animal model of autoimmune diabetes[2]. Disease develops spontaneously in this strain, sharing several crucial features with the human disorder — for example, a protracted course of pathology, disease that is primarily mediated by T lymphocytes, and disease that is under complex polygenic control, by far the most important contributor being the MHC. The NOD mouse has permitted many of the outstanding issues in the diabetes field to be addressed by direct experimentation, and results from this animal have heavily coloured our view of the pathogenesis of diabetes. But, because of the asynchrony and complexity of the disease in this model, vital issues have remained unresolved. Thus, diverse strategies have been taken to simplify the NOD disease in order to highlight particular features by ablation or exaggeration.

One strategy has been the generation of T-cell receptor (TCR) transgenic mice to focus on the role of T lymphocytes. Because of allelic exclusion, mice carrying already rearranged TCR transgenes have a T-cell repertoire highly skewed for the transgene-encoded specificity — even monoclonal when mutations blocking rearrangement of endogenous TCR genes (SCID, $RAG^{0/0}$) are introduced. Such a skewed repertoire has proved useful for synchronizing disease unfolding and for monitoring the behaviour of pathogenic T cells. There are now several examples of TCR transgenic mice with repertoires enriched for a diabetogenic specificity[98,99] — either MHC class-I-restricted $CD8^+$ or class-II-restricted $CD4^+$ T cells, and T cells recognizing either a natural antigen or a genetically engineered neo-antigen synthesized specifically by β-cells.

Another strategy has been to direct expression of molecules of interest specifically to islet β-cells under the dictates of the insulin promoter, permitting assessment of the effects of expression at enhanced levels, ectopically, locally or in isolation. Examples include mice expressing MHC molecules, cytokines, co-stimulatory molecules and neo-antigens[99,100].

Thus, there now exists a set of powerful murine models of diabetes, each one with its inherent interests and disadvantages. We do not know, however, which model most faithfully mimics which variant of human diabetes. Given the heterogeneous symptoms, courses and genetics exhibited by affected individuals, it may well be that human diabetes is really a multiplicity of diseases. It seems that an open-minded view is called for at this point.

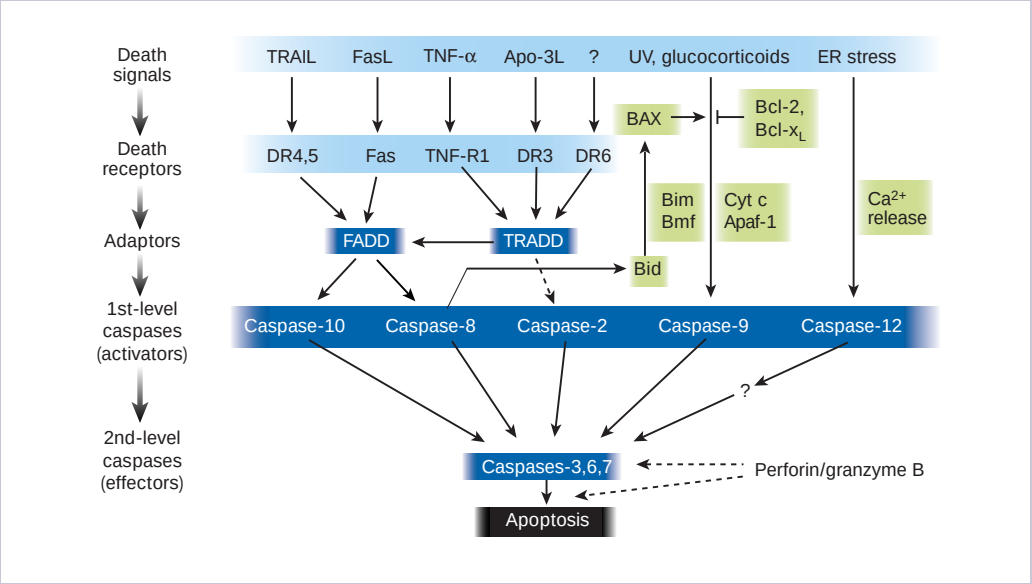

Figure 2 Intracellular death signalling molecules. Apoptosis is the end result of a cascade of interactions and activities[97]. Mammals have two main networks of apoptosis signalling molecules, which are largely distinct. One is initiated by death receptors at the cell surface and is not controlled by Bcl-2 family members; the other is initiated by pro-apoptotic BH3-only members of the Bcl-2 family (such as Bid, Bim or Bmf), and is inhibited by anti-apoptotic Bcl-2 homologues (such as Bcl-2, Bcl-x_L). When cell-surface receptors such as Fas, TNF-RI and death receptors (DR) 3–6 bind their ligands, the receptors cluster and signalling complexes are assembled, including adaptors (FADD, TRADD) and first-level pro-caspases (such as caspase-8 and -2; and caspase-10 in humans). These pro-caspases are cleaved to generate active caspase forms, which activate downstream caspases, which in turn cleave several cell substrates to effect cell death. Other pathways initiated by ultraviolet light, glucocorticoids and stress, for example, do not involve cell-surface receptors, but rather mitochondrial perturbations and/or Ca^{2+} release. Other first-level caspases (caspase-9 and -12) are mobilized, followed by poorly characterized downstream events. Central to mitochondrial involvement are pro- and anti-apoptotic members of the Bcl family, which regulate exit of cytochrome *c*, culminating in the assembly of an 'apoptosome'. The perforin/granzyme B pathway is also poorly defined, although at some point caspase-3 is mobilized. Perforin/granzyme B may also directly cleave cellular substrates. Dashed arrows indicate uncertainty, a controversial link, or one with limited experimental underpinning. No arrows emanate from Bmf and Bim because the pathways are currently ill defined.

cells can also provoke an immune response under the appropriate conditions, for example, when present in high enough numbers, physiologically stressed, or exposed to inflammatory cytokines such as tumour-necrosis factor (TNF)-α and interleukin (IL)-1β (ref. 13). In addition, several studies have linked the presentation of autoantigens and production of autoantibodies with apoptotic cells, notably in systemic lupus erythaematosus[14]. Thus, it seems plausible that this wave of β-cell death prompts uptake of β-cell antigens in the islets followed by their ferrying to the PLNs, where they become available to naive circulating β-cell-reactive T cells. In support of this, an earlier wave of apoptosis in the kidney (peak at 10 days) is correlated with an earlier arrival of kidney-derived antigens in the renal lymph nodes (at 10 days)[3,10,15].

The wave of β-cell death in juvenile animals might stem from one or both of two processes. First, at various times during development, certain tissues undergo a remodelling process involving apoptosis[16] and/or necrosis[17]. A classic example is the loss of interdigital tissue in mammalian limbs during fetal development; examples in the neonatal window include death in the kidney, nervous system, heart and adrenal cortex. The precise mechanisms involved are poorly understood, but both cell-autonomous and extracellular controls have been implicated in different contexts, and the latter can involve either the imposition of a positive signal or the removal of a negative one[18]. Second, the wave of β-cell death peaks around the time of weaning — a known stage of massive metabolic and synthetic perturbations such as changes in glycogenolysis, gluconeogenesis, lipogenesis and hormone production, including insulin and glucagon[19]. Such changes might be an extracellular signal for triggering tissue remodelling, and/or they might induce tissue stress. The latter is likely to be relevant to the pancreatic islets, as β-cells are unusually sensitive to overproduction of proteins and to exposure to several cytokines[20].

The signalling networks involved in cell-stress-induced apoptosis are beginning to be identified. One major pathway, distinct from that implicated in death mediated by members of the TNF receptor family, mobilizes cytochrome *c*, Apaf1, caspase-9, and in some cases caspase-3 (refs 21–25; and Fig. 2). Yuan and co-workers[26] reported that stress in the endoplasmic reticulum (ER), usually provoked by an excess of misfolded proteins, specifically activates caspase-12, which eventually leads to apoptosis. Cells from caspase-12-deficient mice are resistant to ER-stress-induced death, but do undergo apoptosis in response to other death stimuli[26]. Also involved in this pathway are IRE-1, TRAF-2, calpain, caspase-7 and perhaps caspase-9 (refs 27–29; and Fig. 2). It is imperative to assess these two pathways in the context of insulitis onset.

Thus, we and others[7] propose that a wave of physiological β-cell death, which takes place in all individuals, is crucial in the initiation of type 1 diabetes. It has pathological consequences in diabetes-prone individuals, who have an autoreactive T-cell repertoire that can be mobilized by the exposed β-cell antigens and may also be subject to additional genetic or environmental susceptibility factors. This resulting indiscriminate release of β-cell antigens might explain why it has been so difficult to identify convincingly 'the antigen'[30] that initiates autoimmune diabetes. It also eliminates the need for invoking molecular mimicry by microbial T-cell epitopes to initiate the autoimmune process — a notion that has attracted much interest but has little direct experimental support[31] — although microbes might still precipitate insulitis prematurely or cause it to convert more rapidly to diabetes.

Proof will require juvenile β-cell death to be linked experimentally to onset of insulitis; that is, by blocking the death wave or inducing it prematurely, we should detect a reduced or earlier leukocytic infiltrate, respectively. It may also prove possible to track apoptotic β-cell fragments as they are engulfed by APCs in the pancreas and transported to the PLNs, as has been done so elegantly in an intestinal system[32]. Finally, it will be important to define the death signalling pathways that are mobilized during the wave of β-cell death in juvenile animals.

Death as a terminator

Massive, specific destruction of pancreatic islet β-cells is the hallmark of type 1 diabetes. Thus, it is surprising that we still have so little definitive information — either from rodent models or from affected

humans — on how this comes about or why it is so specific. For humans, this most probably reflects the fact that once diabetes is diagnosed, most of the β-cell destruction has already taken place.

Cellular mechanisms of β-cell death

Two principal cellular mechanisms have been proposed (refs 1,2; and Fig. 3). The 'recognition-linked' mechanism attributes the death of β-cells to cytotoxic T-cell recognition of autoantigens presented by MHC molecules at their surface. Thus, killing is provoked by direct recognition of a target on the β-cell and requires T-cell/β-cell contact. In mice, this would imply recognition of MHC class-I-restricted antigens by $CD8^+$ T cells because their β-cells do not express MHC class II molecules. Several observations are consistent with this scheme: (1) $CD8^+$ T cells follow $CD4^+$ T cells into the islets when splenocytes from diabetic donors are transferred into healthy NOD recipients; (2) both $CD4^+$ and $CD8^+$ T cells are required for transfer of diabetes even though the former can invade the host's islets alone; (3) some $CD8^+$ T-cell clones derived from diabetic NOD mice can provoke diabetes on transfer into lymphocyte-deficient recipients, or are diabetogenic in monoclonal TCR transgenic mice[33,34]; (4) diabetes but not insulitis is reduced when insulitic, pre-diabetic NOD mice are injected with anti-MHC class I or anti-CD8 monoclonal antibodies; (5) $CD8^+$ T cells isolated from diabetic animals can attach to and lyse β-cells *in vitro*. Also perhaps relevant is that $CD8^+$ T cells frequently dominate islet infiltrates in humans with diabetes (for example, see ref. 35). However, other evidence argues against this scheme: (1) some $CD4^+$ T-cell clones can provoke diabetes in the absence of $CD8^+$ T cells in transfer or TCR transgenic systems[36]; (2) splenocytes from diabetic donors can transfer disease into recipients lacking MHC class I molecules on their islets[37]; and (3) diabetes can develop in a TCR transgenic model in the absence of antigen-specific contact between T and β-cells[38].

The second, 'activation-linked', mechanism is an indirect one, by which the mere proximity of β-cells to angry T cells provokes their death. This scheme is more consistent with two perplexing observations: $CD4^+$ T cells can cause diabetes in mice in the absence of $CD8^+$ T cells; but MHC class II molecules are not detected on murine β-cells. According to this scheme, β-cell autoantigens are picked up by APCs (macrophages or DCs) resident in or recruited to the islets, and are presented to primed $CD4^+$ T cells provoked to circulate through the tissues. As a result of such T-cell/APC interactions, the activated T cells may directly kill the bystander β-cells, for example through perforin or Fas/Fas ligand (FasL) interactions (pathway i in Fig. 3b); produce soluble mediators that induce β-cell death (ii in Fig. 3b); or activate the cytocidal functions of macrophages in the vicinity (iii in Fig. 3b). The β-cells themselves may enter the melee, induced to synthesize soluble mediators that hasten their own demise (iv in Fig. 3b). The nature of the soluble mediators involved is controversial: interferon (IFN)-γ, IL-1, TNF-α, IL-6 and nitric oxide (NO) have all been implicated (see below).

Molecular mechanisms: cell–cell contact

Recent efforts have been directed at dissecting the molecular mechanisms responsible for the β-cell death that preludes type 1 diabetes[39]. According to several mouse models[36,40], apoptosis is probably the main mode of destruction. For humans, finer mechanistic studies are essentially restricted to experiments on β-cells cultured *in vitro* or expression profiling of *ex vivo* material; for mouse models, the range of genetically engineered knockout strains has added an important perspective. Nonetheless, the results acquired so far have often been contradictory and unsatisfying.

In recognition-linked killing (and also the surface-receptor-mediated variant of activation-linked killing; Fig. 3b, pathway i), most attention has been devoted to perforin and Fas/FasL. The perforin/granzyme pathway (Fig. 2), which leads to insertion of tubular perforin complexes into the cell membrane and consequent osmotic lysis, is the main mode of death induced by cytotoxic T cells. Both NOD mice[41,42] and a class-I-restricted TCR transgenic model[43] showed normal insulitis, but a reduced incidence and delayed onset of diabetes when rendered perforin-deficient, suggesting that perforin has a role in terminal β-cell destruction. In direct contradiction to these results, however, two class-I-restricted TCR transgenic models developed severe diabetes on a perforin-deficient background[42,44], and a third model also did neonatally, although disease was attenuated when perforin-deficient transgenic T cells were transferred into a normal recipient[45]. Perhaps the explanation for these inconsistent data lies in other effects of the perforin mutation; for example, perforin also seems to be involved in the homeostasis of memory $CD8^+$ cells[46]. Alternatively, different $CD8^+$ T-cell clones, recognizing various β-cell antigens, might intervene at diverse stages in the disease process, relying on different effector mechanisms.

Results on the role of Fas/FasL in β-cell death have been equally as confusing. Fas, which is expressed on activated lymphocytes and some cells in tissues, is probably the main cell-surface receptor transmitting death signals (Fig. 2), and FasL is expressed primarily on activated T lymphocytes[47]. The first evidence that this receptor/ligand pair might be involved in terminal β-cell destruction came from the observation that NOD mice homozygous for the *lpr* mutation (a mutation of the *fas* gene) did not develop diabetes; moreover, transfer of splenocytes from diabetic standard NOD mice into NOD *lpr*/*lpr* recipients did not provoke diabetes, although it did in wild-type hosts[48]. The homozygous *lpr* mutation also had an effect in one TCR transgenic model[42], but not in another[45].

These findings prompted several studies demonstrating Fas on β-cells isolated from rodents and humans, and its upregulation during progression to diabetes (for example, see ref. 48), although the

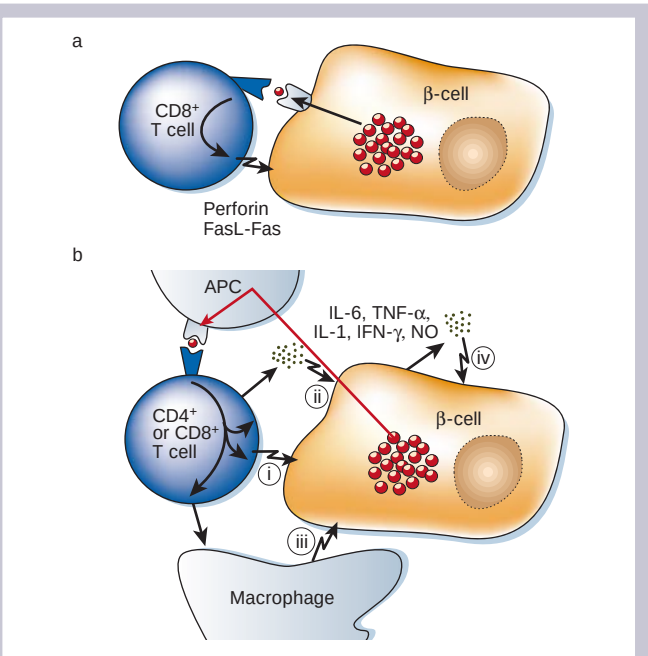

Figure 3 Proposed cellular mechanisms of β-cell death. **a**, According to 'recognition-linked' mechanisms, the T cell (in this case $CD8^+$ T cell) is activated by direct recognition of islet β-cell antigens (red dots) presented by MHC molecules (in this case class I molecules) on β-cells. Activation provokes killing of the β-cell through cell/cell contact using, for example, the Fas/FasL or perforin pathways. **b**, According to 'activation-linked' mechanisms, the T cell (either $CD4^+$ or $CD8^+$) recognizes β-cell antigens presented indirectly by APCs (macrophages, DCs) in the vicinity. The resulting activation provokes β-cell death mediated by surface receptors (such as FasL/Fas, TNF-α/TNF-R) (i); cytokines and other soluble death mediators produced by the T cell (ii); activation of macrophages and stimulation of their cytocoidal activities (iii); and activation of β-cells and stimulation of their production of cell death mediators (iv).

complex cellular composition of inflamed islets renders histological interpretation difficult, and Fas upregulation and its function have been disputed[49]. In addition, *in vitro* studies have documented cytokine-induced Fas upregulation on cultured murine and human islets and its ability to transmit apoptosis signals[49,50]. But it has also been reported that insulitis was abrogated in NOD *lpr/lpr* mice[51], meaning that the lack of diabetes in these animals might have nothing to do with terminal β-cell destruction, but rather to another pleiotropic effect of this mutation on the lymphoid system or to a need for some β-cell death to initiate the autoimmune response (see above).

Indeed, islets from NOD *lpr/lpr* mice were not protected from autoimmune attack when transferred into diabetic wild-type recipients[52,53], and even wild-type islets were protected when introduced into NOD *lpr/lpr* hosts[52]. The explanation for these contradictory transfer results seems to be that lymphocytes residing in *lpr/lpr* mice strongly upregulate FasL[54], rendering them potent killers of any introduced cells expressing Fas[55]. This neat explanation is not completely satisfying, however, as it has been reported that a homozygous *gld* (or FasL) mutation, which lacks the pleiotropic effects of the *lpr* mutation, also inhibited diabetes in NOD mice, and that transfer of diabetogenic T cells into lymphocyte-deficient NOD severe combined immunodeficient (SCID) *lpr/lpr* mice also resulted in attenuation of diabetes[56].

A role for membrane TNF/TNF receptor II (TNF-RII) interactions in recognition-linked killing of β-cells in a $CD8^+$ T-cell-dependent diabetes model has been suggested[57], although, again, these data are difficult to interpret because of the pleiotropic mutations in the mouse strains from which they derive. Interestingly, however, TNF-RII expression was upregulated during disease unfolding in a $CD4^+$ T-cell-dependent TCR transgenic model[58].

Molecular mechanisms: soluble mediators

An implication of perforin, Fas/FasL or membrane TNF-α/TNF-R in terminal β-cell destruction cannot be taken as evidence for a recognition-linked mode of death, because T-cell/β-cell contact is also a feature of a variant of the activation-linked mechanism (pathway i in Fig. 3b). Nonetheless, the other variants all entail soluble mediators (pathways ii–iv in Fig. 3b) and, as discussed above, because there is evidence to support this latter class of mechanisms, much effort has been devoted to identifying the factors that might be involved.

Untangling the relative roles of the different pro-inflammatory cytokines in β-cell death is problematic because these cytokines are produced by more than one cell type and have effects on many targets. In addition, the same cytokine might act as a membrane or soluble form (with a different range of activities) or might engage several receptors (again with different effects). Moreover, the pro-inflammatory cytokines form a network of molecules, which influence each other's synthesis and activities. The difficulties encountered are illustrated by TNF-α. This cytokine exists in a soluble or membrane-bound form, interacts with TNF-RI and -RII, and promotes activities, such as inflammation, differentiation, survival and death, in many cell types[59]. Elevated levels of TNF-α expression have been correlated with β-cell destruction *in vivo*[60], and TNF-α can contribute directly or indirectly to β-cell destruction *in vitro*[61,62], but its effects on the unfolding of diabetes *in vivo* are confusing. Systemic injection of TNF-α gave different results according to the age of the recipient: enhancing disease before 3 weeks, and blocking afterwards[63]. This differential effect has been mimicked in transgenic mice expressing TNF-α specifically in the β-cells of NOD mice[4]. These results probably reflect changing populations of TNF-α producers and users throughout the development of the animal and disease progression.

Thus, interpreting experiments using mice with a genetic deficiency in TNF-α may not be straightforward. NOD mice deficient in TNF-RI developed normal insulitis but reduced diabetes; they also showed less than the usual diabetes when transferred with T cells from wild-type NOD donors[64]. To circumvent some of the problems, a transplant model was developed with the transfer of both islets from various knockout mice and diabetogenic lymphocytes from TCR transgenic mice into NOD-SCID recipients. Transplants carrying mutations in TNF-RI, but not Fas, IFN-γ receptor or inducible NO synthetase (iNOS) resisted rejection, although this turned out to reflect an indirect effect on lymphocyte residence in the islets rather than a direct effect on β-cell killing[65].

Another difficult area is the role of free radicals and reactive oxygen intermediates in terminal β-cell destruction[39]. Inflammation leads to the induction of iNOS and consequent production of NO in macrophages and other cell types. IL-1, TNF-α and IFN-γ have all been implicated in this increased production, but IL-1β may be primordial[66]. Elevated concentrations of NO lead to DNA damage and apoptosis by a poorly defined mechanism. More specifically related to diabetes, *in vitro* both macrophages[67] and islet β-cells[68] can be induced to synthesize NO, and both exogenously and endogenously produced NO provokes β-cell destruction[61]. *In vivo*, iNOS concentrations increase in parallel with diabetes onset in a model of T-cell transfer[69] and after cyclophosphamide treatment of insulitic animals[70]; moreover, an inhibitor of iNOS activity can block diabetes transfer[69]. Thus, it was surprising to find that iNOS-deficient NOD mice developed diabetes as usual (J. Fenyk-Melody and J. Mudger, personal communication) and that islets from such animals were attacked efficiently by diabetogenic T cells in the transplant model mentioned above[65].

Currently, debate is focused on just how critical NO is in 'natural' diabetes, and whether the main culprit is NO produced exogenously by macrophages or NO produced endogenously by β-cells (or both). Resolving the latter issue is important because it might suggest a mechanism for the specificity of β-cell death: at least *in vitro*, both islet α- and β-cells are sensitive to NO-induced destruction[66], but only the latter show elevated iNOS concentrations after cytokine treatment[66]. Notably, cytokine-induced death of human β-cells seems not to depend critically on iNOS *in vitro*[71], but it has been suggested that NO serves to induce the expression of Fas on human islets, rendering them susceptible to T-cell attack[72].

Other oxygen-reactive species might also be involved in β-cell destruction during diabetes development. Peroxynitrite[73] and hydrogen peroxide[74] are involved in β-cell killing *in vitro*, whereas overexpression of catalase[75], manganese superoxide dismutase[75,76], glutathione peroxidase[75] and thioredoxin[77] have such an influence on cytokine-mediated killing *in vitro* or on diabetes development in NOD mice. In addition, the ALR mouse strain constitutively expresses, both systemically and in β-cells, high levels of molecules associated with dissipating free-radical stress, including manganese superoxide dismutase and glutathione peroxidase, and islets from these animals resist cytokine-induced β-cell death through cytokines *in vitro* and through diabetogenic T cells *in vitro* and *in vivo*[78].

Why is it so complex?

So the picture that we currently have is blurred: data from different diabetes models implicate distinct death effector systems with no clear indication of which, if any, are involved in the development of human diabetes. It should be remembered that different intracellular death signalling pathways may be implicated in the β-cell apoptosis induced by diverse agents[79], and that murine and human β-cells may show variable susceptibilities to apoptosis mediated by the different pathways[80]. In particular, one has to question the relevance of *in vitro* studies in light of increasing evidence that the removal of cells from their normal tissue environment can launch a very specific cell death program, 'anoikis', mediated by *bmf* — a member of the pro-apoptotic Bcl-2 family[81].

It is also important to remember that affected individuals can present with heterogeneous clinical parameters[82], and thus diabetes in man could be a set of related disorders with possible differences in inciting antigen, primary effector cell type and, most relevant here, mechanism of β-cell destruction. It is not known which of the mouse

models will prove best for studying each of the human variants. Neither is it known whether islet grafts are subject to the same or different mechanisms of destruction — an important issue given the increasing interest in using grafts to attenuate disease.

Death as a connector

By contrast, type 2 diabetes is non-autoimmune in nature[83]. Its hallmarks are insulin resistance issuing from any of several causes and inadequate insulin secretion. In advanced stages of disease, β-cell function may degenerate to such a degree that insulin therapy becomes necessary. As mentioned above, a significant fraction of individuals originally diagnosed with type 2 diabetes are cryptic type 1 diabetics or evolve with time to a type 1 state, which is defined as exhibiting anti-β-cell autoimmunity. What connects the two types?

The nature of the β-cell abnormality underlying reduced insulin secretion in humans with late type 2 diabetes may be multifactorial. Hyperglycaemia can lead to alterations in glucose sensing by the β-cell, and evidence has been presented for a genetic component that results in defects in β-cell function, a reduction in β-cell mass, or a combination of the two. A modest reduction in the β-cell mass of type 2 individuals at autopsy has been reported[84], but conflicting results abound[85,86]. When β-cell mass is reduced, amyloid deposits are often seen[84,86], but the extent to which they contribute to β-cell failure has been disputed[85]. It may be worth keeping in mind that type 2 diabetics would be expected to have an elevated β-cell mass owing to hyperplasia, so the comparable masses sometimes found in diabetic compared with normal individuals might still reflect induction of β-cell death — only longitudinal studies can give a true indication. *In vitro* experiments have shown that elevated glucose concentrations, analogous to those seen in type 2 diabetics, can induce apoptosis of cultured human islets[87].

Better evidence for β-cell death in late stages of type 2 diabetes comes from animal models. Perhaps the most convincing are data from the gerbil, *Psammomys obesus*, which does not naturally develop diabetes, but can in captivity when fed a high-energy diet. The disease begins with a state of insulin resistance/hyperinsulinaemia/normoglycaemia and evolves to one of hypoinsulinaemia/hyperglycaemia. During the development of hyperglycaemia, an increase in apoptosis occurs, culminating in a severe disruption of the islet architecture[88,89]. This increased β-cell death could result from hyperglycaemia, as suggested by *in vitro* studies showing that islets from diabetes-prone gerbils exhibit a progressive apoptosis on culture at high glucose concentrations[89,90]. *In vitro* studies on other rodent models[91,92], have also shown apoptosis of β-cells after incubation in elevated concentrations of glucose or free fatty acids.

Almost nothing is known about the mechanism(s) of this β-cell death. One possible clue has come from studies on islets from human type 2 diabetics at autopsy showing a decrease in the ratio of anti-apoptotic to pro-apoptotic members of the Bcl-2 family: decreased Bcl-x_L; increased Bad, Bid and Bik[87]. Interestingly, increased levels of apoptosis and islet cell Bad were also characteristic of mice that are diabetic because of a genetic deficiency in insulin receptor substrates (IRS-2)[93]. These findings support the notion that apoptosis signals channel through the mitochondria and argue against surface-receptor-mediated signals (Fig. 2).

Another clue may come from Perk knockout mice, which develop diabetes at an early age owing to massive disappearance of β-cells[94]. The protein kinase Perk couples protein folding in the ER to polypeptide biosynthesis by phosphorylating one of the subunits of eukaryotic translation initiation factor 2 (eIF2α), which attenuates translation in response to ER stress such as insulin biosynthesis in states of hyperglycaemia or hyperlipidaemia[94,95]. In the absence of Perk, translation could not be muted by this mechanism, the ER became distended, the ER stress transducer IRE1α was activated, and massive loss of β-cells and diabetes occurred. IRE1α lies upstream of caspase-12 (ref. 28), which is specifically activated during the ER stress response (ref. 26; and Fig. 2), so it is likely that this is the death effector pathway mobilized in such contexts. Notably, individuals affected with Wolcott–Rallison syndrome develop diabetes as infants and have a mutation in the human equivalent of Perk[96]. Thus, abnormalities in Perk or associated molecules might underlie any reduced β-cell mass that occurs in the more usual cases of type 2 diabetes.

It seems, then, that β-cell death may be what connects type 2 and type 1 diabetes in those occasional cases where the former evolves into the latter. Depending on genetic and/or environmental influences on a particular individual's course of pathogenesis, a fraction of type 2 diabetics may exhibit abnormal β-cell death; and, again reflecting a combination of genetic and environmental effects, a subfraction of these may show mobilization of T cells reactive to β-cell autoantigens, eventually culminating in autoimmune, insulin-dependent diabetes. If so, the scheme hypothesized to unfold is very reminiscent of the one that we proposed above for initiating type 1 diabetes in more standard cases, as are some of the players speculated to take part (metabolic fluxes, ER stress, caspase-12). Animal models permitting direct comparison of early- and late-onset autoimmune diabetes need to be engineered to evaluate this notion. □

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