Role of Defective Apoptosis in Type 1 Diabetes and Other Autoimmune Diseases

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ABSTRACT

Lymphocyte development, selection, and education are strictly controlled to prevent autoimmunity, with potentially autoreactive cells being removed by apoptosis. Dysregulation of apoptosis is a central defect in diverse murine autoimmune diseases. In murine models of autoimmune lupus, for example, mutations in the death receptor Fas (CD95) or in its ligand, FasL (CD95L), have been identified and shown to render lymphoid cells resistant to apoptosis. In contrast, select lymphoid subpopulations of mice with autoimmune diabetes manifest an increased susceptibility to apoptosis as a result of impaired activation of the transcription factor nuclear factor-kappa B (NF- κ B), which normally protects cells against tumor necrosis factor-alpha (TNF- α)-induced apoptosis. The genetic basis of this defect in NF- κ B activation is a mutation in the promoter-enhancer region of a gene that encodes an essential subunit (LMP2) of the proteasome. Although no specific genetic defects have been identified in most common forms of human autoimmune disease, functional assays consistently demonstrate heightened apoptosis attributable to multiple death signaling pathways.

I. Introduction

Autoimmunity encompasses a diverse group of diseases that are defined clinically by the target organ or tissue destroyed. Rheumatoid arthritis and type 1 diabetes mellitus (also known as insulin-dependent or juvenile-onset diabetes), for example, result from a presumed T-cell attack on the joints and insulin-secreting beta (β) cells of the pancreas, respectively. Although the clinical manifestations of each autoimmune disease are distinct, the underlying genetics of these conditions are similar, with most showing an association with the human leukocyte antigen (HLA; also known as the human major histocompatibility complex, or MHC) region of the genome or with nearby non-HLA loci (Becker *et al.*, 1998).

Apoptosis may play a role in two different aspects of autoimmune disease. First, controlled apoptotic cell death contributes to normal T-cell selection and education. Thus, interruption of this process might result in the generation of

autoreactive cells. Second, apoptosis might represent a lymphocyte-independent mechanism of organ or tissue destruction. To date, most experimental data as well as identified genetic defects that promote or impair apoptosis have implicated abnormal T-cell selection and development in autoimmunity. Although a target cell apoptotic defect, possibly involving the Fas death receptor, has been proposed to affect the pancreatic islets of individuals with type 1 diabetes (Chervonsky *et al.*, 1997; Itoh *et al.*, 1997; Amrani *et al.*, 1999; Suarez-Pinzon *et al.*, 1999), other studies have suggested that apoptosis is not a major mechanism of β -cell destruction (Kang *et al.*, 1997,1998; Kim *et al.*, 1999; Pakala *et al.*, 1999; Thomas *et al.*, 1999; Kim *et al.*, 2000; Restifo, 2000). This chapter will focus on the role of apoptotic defects that affect education of the lymphoid system in autoimmunity.

A prominent feature of autoimmunity is the failure of autoreactive cells, either during development or subsequently, to undergo negative selection and die. Such apoptotic defects in humans and mice result in autoreactivity and may lead to marked lymphoproliferation. In certain instances, these defects have been attributed to mutations in the genes for proteins that function in apoptotic signaling pathways. One such example is the *lpr/lpr* mouse, a model of human systemic lupus erythematosus (SLE), in which defective apoptosis results in lymphoproliferation and generalized autoimmunity. These animals harbor a spontaneous mutation in the gene for Fas (Watanabe-Fukunaga *et al.*, 1992; Watson *et al.*, 1992; Mountz *et al.*, 1996), a cell-surface molecule also known as CD95 that belongs to the tumor necrosis factor receptor (TNF-R) superfamily. Similarly, the *gld/gld* mouse, which also manifests a lupus-like autoimmune disease, harbors a point mutation in the intracellular domain of the Fas ligand (FasL) (Allen *et al.*, 1990; Lynch *et al.*, 1994; Ramsdell *et al.*, 1994; Takahashi *et al.*, 1994). The identification of these autoimmunity-associated defects in the Fas signaling pathway stimulated a search for similar mutations in humans with lupus. However, only individuals with a rare form of lupus associated with diffuse lymphoproliferation have been shown to possess a mutation in the FasL gene (Wu *et al.*, 1996a). Only patients with the rare Canale-Smith syndrome or autoimmune lymphoproliferative syndrome have been found to harbor a Fas mutation (Rieux-Laucat *et al.*, 1995; Drappa *et al.*, 1996). Not unexpectedly, the lymphoproliferation apparent in these patients resembles that in *lpr/lpr* and *gld/gld* mice and is thought to result from the failure of select lymphocyte populations to undergo apoptosis. Most individuals with lupus do not appear to harbor mutations in the Fas or FasL genes. Indeed, lymphocytes from such individuals manifest an increased susceptibility to apoptosis *in vitro* as well as increased FasL expression (Emlen *et al.*, 1994; Mysler *et al.*, 1994; Desai-Mehta *et al.*, 1996; Koshy *et al.*, 1996; Wu *et al.*, 1996a; Kovacs *et al.*, 1997; Lorenz *et al.*, 1997; Wong *et al.*, 1999).

In most spontaneous forms of human or murine autoimmunity, severe lymphoproliferation is not a prominent feature of the disease. Indeed, we have shown that the pathogenic cells may manifest an increased susceptibility to apoptosis. In the nonobese diabetic (NOD) mouse, for example, a spontaneous model of human type 1 diabetes, lymphocytes are more susceptible to TNF- α -induced apoptosis than are lymphocytes from control animals. This results from a defect in the activation of nuclear factor-kappa B (NF- κ B) (Hayashi and Faustman, 1999), a transcription factor that protects against TNF- α -induced cell death. In addition to the accelerated apoptosis, there is increased FasL expression exhibited by peripheral blood lymphocytes from humans with lupus *in vitro* (Wong *et al.*, 1999). The genetic basis of these human defects remains unknown.

Members of the TNF-R superfamily appear to play an important role in autoimmune disease. These proteins comprise an extracellular domain consisting of cysteine-rich motifs, a transmembrane domain, and a cytoplasmic tail (Liang and Fesik, 1997; Wallach *et al.*, 1999).

Activation of NF- κ B protects cells against TNF- α -induced apoptosis but this transcription factor also contributes to cell death mediated by Fas (Quaaz *et al.*, 1999), another TNF-R family member. In addition, NF- κ B activation in response to TNF- α may contribute to FasL expression (Hsu *et al.*, 1999). The interplay between these various overlapping apoptotic pathways may explain why the apoptotic defects associated with autoimmune disease confer phenotypes of enhanced or diminished T-cell selection.

II. Genetic Risk Factors for Type 1 Diabetes Located in the MHC Region of the Genome

Genetic risk factors for type 1 diabetes map to the MHC region of the genome. In both human type 1 diabetes and two rodent models of this disease (the NOD mouse and BB rat), pancreatic β cells are selectively destroyed as a result of a chronic autoimmune reaction (Figure 1A and B) (Crisa *et al.*, 1992; Rabinovitch and Skyler, 1998). The MHC region of the genome contains immune response genes that are important for T-cell education and for antigen presentation by both MHC class I and class II molecules. Studies of both humans and rodents have suggested that the centrally located MHC class II genes confer the greatest statistical risk for autoimmune disease. However, functional derangement of MHC class II genes has not been demonstrated in humans with autoimmune disease. In contrast, cellular abnormalities in expression of maturation markers or in antigen presentation have been detected in both NOD mice and diabetic humans. These defects include reduced expression of the maturation antigen CD45 and a reduced abundance of conformationally correct complexes of MHC class I molecules and self-peptides on the cell surface (Faustman *et al.*, 1989, 1991; Smerdon *et al.*, 1993; Jansen *et al.*, 1995).

FIG. 1. Insulinitis and diabetes prevalence in the NOD mouse. Sections of a normal pancreas from a 4-month-old BALB/c female mouse (A) and of a pancreas with marked leukocyte infiltration (insulitis) from a 4-month-old NOD female mouse (B). Sections were stained with hematoxylin and eosin (H&E). Arrowheads indicate sites of extensive leukocyte infiltration. (C) Age dependence of diabetes prevalence in male and female NOD mice.

Evidence based on functional assays suggests that human autoimmune diseases are associated with impairment of antigen processing controlled by the MHC. Thus, cytosolic extracts of lymphocytes from either humans with type 1

diabetes or NOD mice exhibit altered patterns of cleavage of test substrates by the proteasome. This results in the generation of peptides that are poorly suited for assembly with MHC class I molecules (Faustman *et al.*, 1989,1991; Smerdon *et al.*, 1993; Jansen *et al.*, 1995). In addition, lymphocytes of individuals with diverse autoimmune diseases — including type 1 diabetes, multiple sclerosis, and rheumatoid arthritis — manifest a reduced expression of peptide-loaded MHC class I molecules on their surface (Faustman *et al.*, 1991; Fu *et al.*, 1993; Li *et al.*, 1995). Moreover, clinical studies have shown that the antigen presentation defect correlates with disease expression in identical twins with type 1 diabetes (Faustman *et al.*, 1991). The genes responsible for antigen processing map to the MHC region of the genome, suggesting that abnormalities in this region might underlie these various conditions.

Candidate genes in the MHC region of the genome in humans and rodents that might be responsible for the antigen presentation defects associated with autoimmune disease include those for the TAP peptide transporters and the LMP proteasome subunits. Thus, for example, both LMP2 and LMP7 are encoded by genes located in the MHC region of the genome (Figure 2). These proteins are expressed constitutively in most cell types but their expression is markedly increased in antigen-presenting cells (APCs) or lymphoid cells in response to exposure to interferon-gamma (γ) (Fruh *et al.*, 1992; Van Kaer *et al.*, 1994; Hisamatsu *et al.*, 1996; Griffin *et al.*, 1998). Knockout (KO) mice that lack specific TAP or LMP genes exhibit abnormal T-cell selection and autoreactivity against transplants of syngeneic normal tissue (Aldrich *et al.*, 1994; Glas *et al.*, 1994; Van Kaer *et al.*, 1994; Wakatsuki *et al.*, 1994).

Ubiquitin-dependent proteolysis mediated by the proteasome, a multisubunit adenosine triphosphate (ATP)-dependent protease, plays important roles in various cellular processes, including cell-cycle progression, gene transcription, and signal transduction (Goldberg, 1995; Coux *et al.*, 1996). In many instances, the target protein is marked for degradation or processing by both phosphorylation and ubiquitination. Cleavage of endogenous proteins by the proteasome also generates small peptide fragments that contribute to T-cell education as a result of their presentation by MHC class I molecules. Although, in general, the proteasome exhibits minimal variability in substrate selectivity and subunit composition, incorporation of the LMP2 and LMP7 subunits during assembly of the proteasome changes its specificity for self-proteins in such a manner that the suitability of the generated peptides for presentation in the peptide-binding groove of MHC class I molecules is increased (Belich *et al.*, 1994; Gaczynska *et al.*, 1996). The abundance of LMP2 mRNA in lymphocytes derived from NOD mice is reduced, compared with that in lymphocytes from control animals (Figure 2) (Yan *et al.*, 1997), which likely explains, at least in part, the altered T-cell education toward self apparent in these mice.

FIG. 2. Identification of a point mutation in the shared promoter-enhancer region of the LMP2 and TAP1 genes in the NOD mouse. The mutation creates a CAAT box in the shared promoter-enhancer region. CAAT box-binding proteins likely act as negative regulators of gene transcription. Northern blot analysis reveals that the abundance of both LMP2 and TAP1 mRNAs is reduced markedly in splenocytes derived from adult NOD female and male mice with type 1 diabetes, compared with those in splenocytes from control BALB/c mice.

III. The NOD Mouse: A Spontaneous Model of Type 1 Diabetes

Type 1 diabetes usually is caused by T-cell-mediated autoimmunity, with a prediabetic state characterized by the production of autoantibodies specific for proteins expressed by pancreatic β cells, including insulin. In general, the autoantibodies recognize intracellular proteins and likely are generated in response to islet death. The NOD mouse frequently is studied as a rodent model of human type 1 diabetes. The etiology of diabetes in the NOD mouse is complex and multifactorial (Delovitch and Singh, 1997; Rabinovitch, 1998; Atkinson and Leiter, 1999). Both $CD4^+$ and $CD8^+$ T cells mediate the autoimmune response, with underlying functional defects being present in bone marrow-derived APCs. Many $CD4^+$ and $CD8^+$ T-cell lines and clones with diabetogenic potential that are targeted to a variety of identified and unidentified antigens have been established from both the islets and spleen of NOD mice. Destruction of

pancreatic β cells appears to be mediated by both necrotic and apoptotic death triggered by invasion of islets by leukocytes, a process referred to as insulinitis (Rabinovitch, 1998). Although insulinitis is not apparent in NOD mice up to 3 weeks of age, its prevalence increases in both female and male animals after 5 weeks of age. A clear sex difference is observed with respect to the onset of diabetes, however (Figure 1C). In NOD females, the onset of diabetes occurs as early as 10 weeks, with the number of affected animals increasing with age (Makino *et al.*, 1980). The cumulative prevalence of diabetes in NOD females by 50 weeks of age is $\approx 70\text{--}80\%$. In contrast, only about 20% of NOD males are affected by diabetes at this age. The large numbers of leukocytes apparent in the islet infiltrates of NOD mice are suggestive of lymph node formation around islets (Figure 1A and B). A strain-specific characteristic of NOD mice is the accumulation of many T lymphocytes in peripheral lymphoid organs, the pancreas, and submandibular salivary glands. This T-cell accumulation may reflect low interleukin (IL)-2 concentrations and the resistance of thymocytes and peripheral T cells to the induction of apoptosis. Such apoptotic resistance may be an early phenotype of lymphoid lineages prior to disease initiation (Lamhamedi-Cherradi *et al.*, 1998).

Type 1 diabetes in the NOD mouse, like that in humans, exhibits a marked genetic component that maps to the MHC region of the genome. We have identified a specific proteasome defect in NOD mouse lymphocytes that results from downregulation of expression of the LMP2 proteasome subunit (Figures 2 and 3) (Hayashi and Faustman, 1999), which is encoded by a gene located in the MHC genomic region. This defect both prevents the proteolytic processing required for the production and activation of NF- κ B, which plays an important role in immune and inflammatory responses, and increases the susceptibility of the affected cells to apoptosis induced by TNF- α (Figure 4). The proteasome dysfunction in NOD mice is both tissue and developmental stage specific; it is not apparent in islet cells.

IV. Defects in Proteasome-mediated NF- κ B Activation and T-cell Education in NOD Mice

The proteasome mediates the processing and activation of the transcription factor NF- κ B (Figure 5). NF- κ B is activated in response to various extracellular stimuli, including IL-1, lipopolysaccharide, and TNF- α (Thanos and Maniatis, 1995; Verma *et al.*, 1995; Baeuerle and Baltimore, 1996; Baldwin, 1996). It contributes to regulation of the gene expression for cytokine production, cell adhesion, lymphocyte maturation, and protection from TNF- α -induced apoptosis, as well as antigen processing and presentation by MHC class I molecules (Bohline *et al.*, 1988; Cross *et al.*, 1989; Tan *et al.*, 1992; Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996). Insights into the various biological functions of

FIG. 3. Schematic representation of the central role of the proteasome in NF- κ B activation and antigen presentation in an antigen-presenting cell (APC). Proteasomes containing the LMP2 subunit generate self-peptides for presentation on the cell surface by MHC class I molecules, a process that is required for T-cell education by self-antigens. Such LMP2-containing proteasomes also are required for processing NF- κ B subunit precursors and degradation of I κ B α , which underlies NF- κ B activation. The activation of NF- κ B is essential for lymphocyte maturation, protection against apoptosis, and cytokine balance. Lymphoid cells of adult NOD mice fail to produce LMP2 and are thus defective in antigen presentation and susceptible to apoptosis.

NF- κ B have been provided by the generation and characterization of KO mice lacking either subunits of this protein or associated regulatory factors (Burkly *et al.*, 1995; Kontgen *et al.*, 1995; Weih *et al.*, 1995; Franzoso *et al.*, 1997; Bushdid *et al.*, 1998; Caamano *et al.*, 1998; Kanegae *et al.*, 1998; Hu *et al.*, 1999; Li *et al.*, 1999a,b; Takeda *et al.*, 1999).

Active NF- κ B exists predominantly as a heterodimer composed of p65 (RelA) and either p50 or p52 subunits. The p50 and p52 subunits are generated constitutively but their abundance is increased markedly by various extracellular stimuli, including IL-1 and TNF- α . These proteins are generated as a result of the proteasome-mediated removal of the carboxyl termini of p105 and p100 precursors, respectively (Fan and Maniatis, 1991; Schmid *et al.*, 1991; Palombella *et al.*, 1994; Coux and Goldberg, 1998; Lin *et al.*, 1998; Sears *et al.*, 1998). In resting cells, NF- κ B is sequestered in the cytoplasm as a result of its association with I κ B α or other members of the I κ B family of inhibitory proteins (Ghosh and Baltimore, 1990; Hayashi *et al.*, 1993a,b). Cell stimulation results in the phosphorylation of I κ B α by the I κ B kinase (IKK) complex and its degradation by the ubiquitin-proteasome pathway, thereby allowing the p50-p65 or p52-p65 heterodimer to translocate to the nucleus and initiate transcription of target genes (Figure 3) (Ghosh and Baltimore 1990; Oeri *et al.*, 1991; Palombella *et al.*, 1994;

FIG. 4. Impaired granulocyte-macrophage (GM) colony formation and increased sensitivity to TNF α -induced apoptosis in NOD mouse spleen cells. Spleen cells derived from 6-week-old male or female BALB/c or NOD mice were mixed with 1.3% methylcellulose dissolved in culture medium and layered onto a bed of 0.53% agarose in culture medium. Cells were cultured for 3 weeks in the presence of GM-colony stimulating factor (CSF) (20 ng/ml) and in the absence or presence of TNF- α (20 ng/ml), as indicated. [Reprinted with permission from Hayashi T, Faustman D 1999 NOD mice are defective in proteasome production and activation of NF- κ B. *Mol Cell Biol* 19:8646–8659. Copyright American Society for Microbiology.]

MacKichan *et al.*, 1996; Belich *et al.*, 1999). Complexes of p65 and p105 also have been detected but these do not appear to translocate rapidly to the nucleus in response to cell stimulation (Sun *et al.*, 1994; Lin *et al.*, 1998).

Our laboratory has sought to understand why, in type 1 diabetes, T cells treat pancreatic β cells as foreign. We therefore have attempted to understand the process of T-cell education to self-antigens and how this process is altered in individuals with type 1 diabetes. T-cell education requires the presentation of self-antigens, a task that is undertaken by “professional” APCs such as macrophages, dendritic cells, and B cells. Until recently, it was thought that autoimmunity results from the inappropriate activation of T cells by foreign antigens (e.g., viral proteins) that generate cross-reactivity with self-antigens, which was considered an MHC class II defect. However, we proposed, and presented evidence for the notion, in both NOD mice and humans with type 1 diabetes, that interruption of the presentation of self-antigens by MHC class I molecules underlies the development of autoimmune disease (Faustman *et al.*, 1991). This proposal was based on the contention that such MHC class I-mediated presentation of self-peptides is essential for the development of normal tolerance. Previously, MHC class I proteins were thought to function primarily in the

T-cell Education to Self Peptides

FIG. 5. Impaired expression of LMP2 in NOD mouse splenocytes. (A) Schematic representations of 26S and 20S proteasomes. (B) Lysates of spleen cells from adult male (M) or female (F) BALB/c or NOD mice were subjected to immunoblot analysis with antibodies specific for the indicated 20S proteasome subunits or, as controls, with antibodies to various cyclin-dependent kinases (CDKs) or to the transcriptional factor TAF_{II}250.

presentation of peptides derived from foreign intracellular proteins, especially viral proteins, for the generation of cytotoxic T cells. Subsequent studies in transgenic mice deficient in chaperone proteins required for the intracellular assembly of MHC class I complexes confirmed the importance of self-peptide presentation by MHC class I molecules in T-cell education to self (Aldrich *et al.*, 1994; Glas *et al.*, 1994; Van Kaer *et al.*, 1994).

In our attempt to discover the basis for the impairment in presentation of self-peptides by MHC class I molecules in the NOD mouse, we found that the abundance of LMP2 mRNA in lymphoid cells from these animals was markedly reduced, compared with that in control animals. This defect in LMP2 expression

in the NOD mouse was shown to be attributable, at least in part, to a specific mutation in the shared bidirectional promoter-enhancer region of the LMP2 and TAP1 genes in the MHC class II region of the genome (Figure 2). The reduced abundance of LMP2 interrupts the proteasome-mediated generation of self-peptides for presentation by MHC class I molecules and the consequent development of T-cell tolerance to self-antigens (Yan *et al.*, 1997). It also prevents the processing of NF- κ B precursor proteins and the degradation of I κ B α required for activation of NF- κ B (Hayashi and Faustman, 1999), events important for T-cell maturation and normal immune and inflammatory responses. The LMP2 expression defect in NOD mice is specific for lymphoid lineage cells and becomes apparent after 10 weeks of age (Hayashi and Faustman, 1999).

The interruption by the LMP2 defect in NOD mice of both self-peptide presentation by APCs as well as normal T-cell development — two phenotypes we had established as important in both murine and human autoimmune diabetes — suggests that the onset of LMP2 downregulation is an essential trigger for disease initiation. The expression of MHC class I molecules in islets is upregulated early during islet invasion by T cells in both humans and NOD mice with type 1 diabetes. This phenomenon probably defines target selection by augmenting self-antigen presentation, thereby promoting cytotoxic T-cell attack mediated by poorly educated, LMP2-deficient T cells.

V. Increased Sensitivity of NOD Mouse Lymphocytes to TNF- α -induced Apoptosis

Recent reports indicate that NF- κ B is an important protector of cells from TNF- α -induced apoptosis (Beg *et al.*, 1995). Embryos of mice lacking the NF- κ B p65 subunit, IKK β or IKK γ , manifest marked hepatic apoptosis that appears to result from the associated defects in NF- κ B activation (Beg and Baltimore, 1996; Li *et al.*, 1999b, Rudolph *et al.*, 2000). The activation of NF κ B by the ubiquitin-proteasome pathway also is thought to protect cells from TNF- α -induced cell death (Figure 3) (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996; Wu *et al.*, 1996b). The antiapoptotic effect of NF- κ B is likely mediated by the activation of genes that encode cell survival-promoting factors.

We investigated the effect of TNF- α on the viability of adult NOD mouse lymphocytes, in which TNF- α -induced activation of NF- κ B is impaired. Whereas incubation of BALB/c mouse splenocytes with various concentrations (2–20 ng/ml) of TNF- α for 24 hours had virtually no effect on cell survival, TNF- α induced a dose- and time-dependent decrease in the survival of splenocytes derived from male or female NOD mice (Hayashi and Faustman, 1999; Hayashi *et al.*, 2000). Similarly, whereas incubation of BALB/c mouse splenocytes with TNF- α (10 ng/ml) for up to 48 hours had no effect on cell viability,

the survival of NOD splenocytes already was reduced markedly after incubation with the same concentration of TNF- α for only 12 hours (Hayashi and Faustman, 1999; Hayashi *et al.*, 2000). The toxic effect of TNF- α on NOD mouse lymphocytes appeared more pronounced for female than for male animals. Exposure of lymphocytes from LMP2 KO mice to TNF- α also resulted in marked cell death (Hayashi and Faustman, 1999; Hayashi *et al.*, 2000). Agarose gel electrophoresis confirmed that TNF- α induced a pattern of internucleosomal DNA fragmentation characteristic of apoptosis in lymphocytes from NOD mice and LMP2 KO, whereas it did not induce DNA fragmentation in those from BALB/c mice (Hayashi and Faustman, 1999). It is thus likely that the toxicity of TNF- α for NOD mouse lymphocytes is attributable to the NF- κ B inactivation due to defective proteasome function.

TNF- α also reduced the viability of spleen cells derived from 7-day-old NOD mice but to a lesser extent than it did in cells derived from adult animals. It had no effect on the viability of spleen cells derived from 7-day-old BALB/c mice. Whereas TNF- α had no effect on the viability of cultured macrophages derived from 13.5-day BALB/c or NOD mouse fetal liver, it induced a dose- and time-dependent decrease in the viability of such cells derived from LMP2 KO mouse fetal liver at the same stage of development (Hayashi and Faustman, 1999). Similarly, TNF- α had no effect on the viability of cultured BALB/c or NOD mouse embryonic fibroblasts, whereas TNF- α treatment of such cells derived from LMP2 KO mice resulted in prominent cell death (Hayashi and Faustman, 1999,2000). Although disruption of the NF- κ B p65, IKK β , or IKK γ genes is associated with marked abnormalities in liver development (Beg *et al.*, 1995; Beg and Baltimore, 1996; Li *et al.*, 1999b; Rudolph *et al.*, 2000), hematoxylin-eosin staining of liver sections from 6-week-old NOD mice did not reveal any apparent defects (Hayashi and Faustman, 1999).

VI. Impaired Granulocyte-Macrophage Colony Formation by NOD Mouse Spleen Cells

NF- κ B also plays an important role in the maturation of lymphocytes and monocytes. We therefore examined the development of the granulocyte-macrophage (GM) cell lineage with splenocytes isolated from 6-week-old NOD and BALB/c mice. Colony-formation assays revealed that, whereas GM-colony-stimulating factor (CSF) induced the formation of clusters of mature GMs in BALB/c mouse splenocytes, the formation of such clusters was impaired in splenocytes from NOD mice (Figure 4, A-D). Furthermore, whereas exposure of GM-CSF-treated spleen cell cultures from BALB/c mice to TNF- α had no effect on cell viability or colony development, TNF- α induced the death of all cells in NOD mouse cultures (Figure 4, E-H).

The specificity of the developmental defect and cytotoxic effect of TNF- α in the GM lineage of NOD mice was investigated by examining colony-forming units (CFUs) of erythrocytes in cultures of spleen cells derived from 6-week-old BALB/c and NOD animals. Erythrocyte colony formation appeared normal in erythropoietin-supplemented cultures of NOD mouse spleen cells, compared to that observed in spleen cells from BALB/c mice (Hayashi and Faustman, 1999). Moreover, TNF- α had no effect on erythrocyte colony formation, which is known to require NF- κ B, in spleen cells from either BALB/c or NOD mice. These results suggest that a lack of NF- κ B activation in GM precursors derived from NOD mice at 6 weeks of age impairs the maturation of these cells and renders them susceptible to the cytotoxic effect of TNF- α . In contrast, NF- κ B appears to be functional in the erythrocyte lineage of these mice, which seem to develop normally and be resistant to TNF- α -induced apoptosis. Given that TNF- α had no effect on the viability of cultured macrophages derived from 13.5-day BALB/c or NOD mouse fetal liver, the proteasome defect in NOD mice appears to be specific for both cell type and developmental stage.

VII. Gender, Age, and Tissue Specificity of Proteasome Dysfunction and Disease Expression in NOD Mice

The prevalence of diabetes is markedly greater in NOD females than in NOD males. Most human autoimmune diseases also are expressed preferentially in females. Consistent with a role for defective proteasome activity and consequent impaired NF- κ B function in NOD mouse diabetes, cytosolic extracts of splenocytes from male NOD mice were able to convert a small proportion of recombinant NF- κ B p105 to p50. However, the product of this reaction appeared to differ in size slightly from that of the p50 subunit produced by extracts of BALB/c mice (Hayashi and Faustman, 1999). Splenocyte extracts from NOD females did not generate any detectable p50 protein in this assay. Furthermore, as mentioned previously, both the time course and dose-response relation for the effect of TNF- α on cell viability revealed that the sensitivity of splenocytes from NOD females to this cytokine was greater than that of cells from NOD males (Hayashi and Faustman, 1999; Hayashi *et al.*, 2000).

The characteristics of KO mice that lack NF- κ B subunits or LMP2 overlap partially with those of NOD mice (Van Kaer *et al.*, 1994; Burkly *et al.*, 1995; Kontgen *et al.*, 1995; Weih *et al.*, 1995; Horwitz *et al.*, 1997). However, LMP2-deficient mice do not develop diabetes by 32 weeks of age (D.L. Faustman, unpublished observation), consistent with the contribution of multiple chromosomal regions to disease penetrance in both NOD mice and humans. The homogeneous nature of the gene defect in all tissues of LMP2 KO mice differs from the apparent developmental stage and tissue specificity of the proteasome defect in NOD mice, which might underlie target selection in disease expression.

LMP2-deficient and other KO mice with defects in the assembly of MHC class I molecules with self-peptides destroy transplanted syngeneic tissues from control animals (Li and Faustman, 1993; Vidal-Puig and Faustman, 1994; Frelund *et al.*, 1998). Target cell loss thus might result from preferential direct attack by cytotoxic T lymphocytes in the early stages of autoimmune disease.

The marked proapoptotic effect of TNF- α in NOD mouse lymphocytes also suggested a possible role for this cytokine in early β -cell destruction in these animals. Such a mechanism of β -cell death would require that β cells exhibit the same proteasome defect as that apparent in NOD mouse lymphocytes. This defect is characterized by loss of LMP2 expression, aberrant NF- κ B activation, increased sensitivity to the cytotoxic effect of TNF- α , and reduced expression of peptide-filled MHC class I molecules on the cell surface. However, one of the early pathological features of autoimmune diabetes in both humans and rodent models is hyperexpression of correctly assembled MHC class I molecules on the surface of β cells (Foulis, 1987; Ono *et al.*, 1988; Weringer and Like, 1988; Hanafusa *et al.*, 1990; Kay *et al.*, 1991; Vivés-Pi *et al.*, 1996; Stephens *et al.*, 1997), a phenomenon that requires intact proteasome function. Studies of both humans and animals with diabetes or other autoimmune diseases suggest that discordance in the regulation of MHC-linked genes between tissues might confer target specificity for attack by cytotoxic T lymphocytes (Hayashi and Faustman, 1999).

Macrophages and fibroblasts derived from 13.5-day NOD mouse embryos exhibited normal cell growth and resistance to TNF- α cytotoxicity. In contrast, TNF- α exhibited a marked proapoptotic effect in the corresponding cell types derived from LMP2 KO mice (Hayashi and Faustman, 1999,2000; Hayashi *et al.*, 2000). TNF- α also induced a relatively small decrease in the viability of spleen cells derived from 7-day-old NOD mice but had no such effect on the corresponding cells from BALB/c mice. In contrast, lymphoid cells of splenic origin, lung macrophages (Kupffer cells), and GMs from 6- to 8-week-old NOD mice exhibit reduced LMP2 expression, impaired NF- κ B activation, and increased sensitivity to the cytotoxic effect of TNF- α (Hayashi and Faustman, 1999). Furthermore, consistent with a role for the proteasome and NF- κ B in normal cell growth, culture of spleen cells from 6-week-old NOD mice with GM-CSF failed to induce normal expansion of the GM cell lineage. The islets of Langerhans, liver, and erythrocytes of 6- to 8-week-old NOD mice appear normal. The ability of NOD mouse macrophages to activate regulatory T cells in an autologous mixed lymphocyte reaction also has been shown to be impaired (Atkinson and Leiter, 1999).

The age-dependent proteasome defect in the macrophages of NOD mice likely explains some of the important features of disease development in these animals. Thus, female NOD mice show no signs of autoimmunity up to 3 weeks of age. At 5 weeks and older, insulinitis begins to appear. By 8 weeks of age,

autoantibodies are detectable. The insulinitis gradually increases in intensity, with complete destruction of islets usually apparent by 30 weeks of age (Makino *et al.*, 1980). Furthermore, the outcomes of various interventions and treatments in NOD mice are age dependent. For instance, the administration of TNF- α to animals older than 6 weeks sometimes prevents the development of diabetes, whereas the same treatment in animals younger than 4 weeks has no effect or a detrimental effect (Yang *et al.*, 1994). Therefore, both the time course of the histopathology of autoreactivity and the paradoxical responses to TNF- α treatment parallel the altered developmental regulation of LMP2 expression and NF- κ B activity in these animals.

VIII. Defective Proteasome Function and Autoimmunity

The ubiquitin-proteasome pathway plays an essential role in many important biological processes (Maniatis, 1999). Protein degradation by this pathway thus generates peptides for presentation by MHC class I molecules and either activates or inactivates transcription factors. In general, proteasome subunit composition varies minimally among eukaryotic cells. However, the interferon- γ -induced expression of the MHC-encoded proteasome subunits LMP2 and LMP7 is thought to promote the generation of endogenous peptides compatible with the peptide-binding cleft of MHC class I molecules (Akiyama *et al.*, 1994; Belich *et al.*, 1994). The MHC-encoded proteasome subunits also play a role in general proteasome function, including the processing and activation of NF- κ B.

The defect in proteasome function in NOD mouse splenocytes is attributable to a loss of expression of the LMP2 subunit and was evident from the impaired proteolytic processing of the p105 precursor of the NF- κ B subunit p50 *in vitro* as well as from the lack of degradation of phosphorylated I κ B α in these cells in response to TNF- α . This defect confers sensitivity on the affected cells to the apoptotic action of TNF- α (Figure 6). The role of LMP2 in NF- κ B activation was confirmed by observations that 1) cytosolic extracts of lymphocytes from LMP2 KO mice also failed to convert p105 to p50 and 2) only NOD mouse tissues that lack LMP2 subunit showed impaired activation of NF- κ B and sensitivity to TNF- α -induced apoptosis (Hayashi and Faustman, 1999,2000). The defect in LMP2 protein production in NOD mice is both developmental stage (age) and tissue specific. Dysfunction of a gene in the MHC region of the genome thus virtually abolishes the activity of a transcription factor that plays important roles in both immune and nonimmune cellular functions. The NOD mouse therefore represents a newly defined mosaic model of discordant MHC gene expression that exhibits marked proteasome dysfunction in an age- and tissue-specific manner.

The delayed maturation of lymphocytes and cytokine abnormalities apparent in NOD mice that spontaneously develop type 1 diabetes are mirrored, in part, by

FIG. 6. Model for TNF- α -induced apoptosis in NOD mouse lymphocytes. The TNF- α signaling pathway generates an unknown proapoptotic signal. The defect in the activation of NF- κ B prevents induction of the expression of a gene (or genes) that encodes an antiapoptotic factor (or factors), resulting in an increased susceptibility to apoptosis.

the phenotypes of KO mice lacking NF- κ B subunits or LMP2 (Van Kaer *et al.*, 1994; Sha *et al.*, 1995; Beg and Baltimore, 1996; Snapper *et al.*, 1996; Franzoso *et al.*, 1997; Horwitz *et al.*, 1997; Iotsova *et al.*, 1997; Caamano *et al.*, 1998; Tanaka *et al.*, 1999). The clinical relevance of the phenotypes of the NOD mouse and of these various KO animals to human disease is supported by the existence of nearly identical cytokine and lymphocyte maturation defects in humans with type 1 diabetes.

In conclusion, we have demonstrated the existence of a marked defect in proteasome function in lymphocytes from autoimmune diabetes-prone NOD

mice. This defect results from a deficiency of the LMP2 subunit, which is encoded by a gene located in the MHC region of the genome. It results in both impaired processing of self-peptides for presentation by MHC class I molecules as well as the inability to activate NF- κ B. A similar age-related defect in GMs is proposed to confer target specificity in autoimmunity toward tissues with intact LMP2 expression. Abnormal processing of intracellular proteins thus may contribute to the pathogenesis of type 1 diabetes.

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