CHOP deletion does not impact the development of diabetes but suppresses the early production of insulin autoantibody in the NOD mouse

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Abstract

C/EBP homologous protein (CHOP) has been proposed as a key transcription factor for endoplasmic reticulum (ER) stress-mediated β-cell death induced by inflammatory cytokines *in vitro*. However, the contribution of CHOP induction to the pathogenesis of type 1 diabetes is not yet clear. To evaluate the relevance of CHOP in the pathogenesis of type 1 diabetes *in vivo*, we generated CHOP-deficient non-obese diabetic (*NOD.Chop*$^{-/-}$) mice. CHOP deficiency did not affect the development of insulitis and diabetes and apoptosis in β-cells. Interestingly, *NOD.Chop*$^{-/-}$ mice exhibited a delayed appearance of insulin autoantibodies compared to wild-type (wt) mice. Adoptive transfer with the diabetogenic, whole or CD8^{+}-depleted splenocytes induced β-cell apoptosis and the rapid onset of diabetes in the irradiated *NOD.Chop*$^{-/-}$ recipients with similar kinetics as in wt mice. Expression of ER stress-associated genes was not significantly up-regulated in the islets from *NOD.Chop*$^{-/-}$ compared to those from wt mice or NOD-*scid* mice. These findings suggest that CHOP expression is independent of the development of insulitis and diabetes but might affect the early production of insulin autoantibodies in the NOD mouse.

Keywords: CHOP; Type 1 diabetes; Non-obese diabetic mouse; ER stress; autoantibody; apoptosis.

Introduction

Type 1 diabetes is a chronic autoimmune disease resulting from specific destruction of the insulin-producing β-cells by an inflammatory reaction in and around the pancreatic islets[1]. Two major molecular mechanisms of β-cell destruction via direct cell-mediated cytotoxicity, such as Fas/ Fas Ligand (FasL)[2] or perforine/granzyme[3] and indirect cytokine-mediated cytotoxicity, have been proposed to be involved in type 1 diabetes[4-7]. Inflammatory cytokines including interleukin-1β(IL-1β), tumor necrosis factor α(TNF-α) and interferon γ(IFN-γ) are detected within the islets and the exposure to soluble mediators including cytokines, nitric oxide (NO), and oxygen free radicals probably causes β-cell death in the course of insulitis and type 1 diabetes [8-12].

Possible mechanisms of cytokine mediated β-cell death include i) the activation of the stress-activated protein kinases such as cJUN NH_2-terminal kinase(JNK), p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase, ii) the release of death signals from mitochondria and iii) triggering of endoplasmic reticulum (ER) stress[12, 13]. The ER stress response is triggered by the accumulation of unfolded proteins in the lumen of the ER. Upon the initiation of ER stress, the cells activate intracellular signaling pathways that transmit information from the ER to the cytoplasm and nucleus; this is known as the unfolded protein response (UPR)[14, 15]. The UPR promotes cell survival by adjusting the ER protein-folding capacity through translational attenuation and degradation of unfolded proteins, and up-regulation of the ER chaperones. But if homeostasis cannot be re-established, apoptosis is induced via transcriptional induction of C/EBP homologous protein (CHOP)[16], the activation of JNK [17, 18], and/or the activation of caspase-12[19].

The β-cells of the pancreas have a highly developed ER to secrete insulin, and are susceptible to ER stress[20]. The relevance of ER stress-mediated β-cell apoptosis to the pathogenesis of diabetes has been proven in several genetic syndromes[21-23] and in type 2 diabetes[24, 25]. NO-induced apoptosis in β-cells is shown to be mediated by the ER stress pathway. NO donors trigger an ER stress response induced by the depletion of ER Ca^{2+} content in β-cells leading to CHOP expression and apoptosis. CHOP-deficient islets have been shown to be much more resistant to NO-induced, ER stress-mediated β-cell apoptosis than wild-type islets [26, 27]. These observations suggest that the ER stress pathway via CHOP expression might be relevant for β-cell destruction in the pathogenesis of type 1 diabetes[16,

28]. A recent study, however, demonstrated that the attenuation of ER stress with siRNA-mediated CHOP reduction does not have an impact on cytokine-mediated β-cell death[29]. The contribution of CHOP induction in cytokine-mediated β-cell apoptosis or in the pathogenesis of type 1 diabetes is still controversial.

This study was therefore conducted to investigate the role of CHOP in the pathogenesis of a spontaneous type 1 diabetes model studying the non obese diabetic (NOD) mouse whose disease pathogenesis, specifically in relation to autoimmune-mediated β-cell destruction, is most likely similar to that in human type 1 diabetes.

Materials and methods

Mice

Female NOD mice and NOD-*scid* mice were purchased from Clea Japan (Tokyo Japan) and CHOP knockout mice on C57Bl/6 background (*B6.Chop*$^{-/-}$) were obtained by the method described previously[26, 30]. We generated NOD mice with the CHOP gene deleted by backcrossing with *B6.Chop*$^{-/-}$ mice to NOD mice. The targeted allele was introgressed into the NOD background using a marker-assisted "speed congenic" breeding approach wherein backcross segregants were fixed for homozygosity for NOD alleles at NOD/B6 polymorphic markers at known NOD diabetes susceptibility (idd) loci. Idd loci were fixed by backcross 4. Homozygous CHOP-deficient NOD background mice (*NOD.Chop*$^{-/-}$) were produced by the intercrossing of heterozygotes at N10, and a permanent line of *NOD.Chop*$^{-/-}$, wild-type mice (*NOD.Chop*$^{+/+}$) and heterozygous mice (*NOD.Chop*$^{+/-}$) were established at N10.

The mice were maintained in the Laboratory Animal Center for Biomedical Research at Nagasaki University under specific pathogen-free conditions. All animal experiments described in this study were approved by the institutional animal experimentation committee, and were conducted in accordance with the Guidelines for Animal Experimentation.

Monitoring blood glucose levels

Blood glucose levels were monitored weekly with a One-Touch Ultra (Johnson & Johnson, Tokyo, Japan). Mice with blood glucose levels above 250 mg/dl for two consecutive measurements were considered diabetic.

Histology

Pancreatic sections were histologically analyzed by fixing tissues in 10% formalin and staining the paraffin-embedded samples with hematoxylin and eosin. A minimum of 30 islets from each mouse were microscopically observed by two different observers for the presence of insulitis, and the levels of insulitis were scored according to the following criteria: 0, no lymphocyte infiltration; 1, islets with lymphocyte infiltration in less than 25% of the area; 2, 25-50% of the islet infiltrated; 3, 50-75 % of the islet infiltrated; 4, more than 75% infiltrated or small retracted islets.

Measurement of insulin autoantibodies and serum IgG level

The mouse serum were collected and kept –20℃. The levels of insulin autoantibody (IAA) in serum were evaluated by using a 96-well filtration plate micro IAA assay as previously described[31]. The index value of 0.01 was chosen as the cut-off limit of normal serum level of IAA in non-diabetic mouse strains. The level of serum IgG was quantified with Mouse IgG ELISA Quantitation Kit (Bethyl laboratories, INC, TX, USA). The 96-well plates were precoated with 100ul of 1:100 carbonate-bicarbonate buffer dilutied primary antibody in kit at room temparature (RT) for 1 hour, and then washed 3 times, followed by addition of 100ul of 1:2 serially dilutied 500ng/ml standard IgG into seven wells and 1:100 dilutied samples into other assigned wells respectively. The plates were incubated at RT for 1 hour, and the wells were washed 5 times. Then 100ul of 1:50000 diluted horseradish peroxidase conjugated antibody was added into well, after 1 hour incubation at RT, the samples were washed as previous described, color was developed with TMB and terminated by 2M H_2SO_4. IgG concentration (μg/ml) was calculated according to the standard cure measured under 450nm.

Adoptive transfer of diabetes

The $3x10^7$ whole splenocytes or $2x10^7$ splenocytes depleted $CD8^+$ splenocytes with Auto-MACS (Milteny Biotech, Bergisch Gladbach, Germany) from newly diabetic female NOD mice were injected intraperitoneally into 8-12 wk-old male recipient mice which were irradiated at 750 rad 4 hours before the transfer[32].

Immunohistochemistry

The kidneys from mice treated with 1.0 mg/kg of tunicamycin (i.p.) and the pancreata were fixed in 10% formalin and embedded in paraffin. Tissue sections (5-μm thick) were sectioned and deparaffinized. The sections were then stained using the labeled streptavidin-biotin method (Histofine Staining Kit, Nichirei Co., Tokyo, Japan) with 10% rabbit serum, followed by incubation with mouse anti-insulin monoclonal antibody (Nichirei Co., Tokyo, Japan) and rabbit anti-Chop polyclonal antibody (Santa Cruz, CA, USA)(1:200 dilution).

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining

For the confirmation of apoptosis by immunofluorescence analysis, TUNEL staining was employed to detect double-stranded DNA breaks. After the pancreatic tissues were fixed in 4% PFA 4°C for 15 minutes followed by immersion in PBS with 0.5 % Tween 20 and 0.2 % bovine serum albumin, we used Mebstain Apoptosis kit direct (MBL, Nagoya, Japan). After the pancreatic tissues were rinsed with distilled water, they were incubated with a 50ul terminal deoxynucleotidyl transeferase solution at 37°C for one hour. The stained pancreatic tissues were analyzed by confocal microscopy (LSM5, PASCAL; Carl Zeiss, Jena, Germany). After washing in PBS, the cells were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), and scanned by confocal microscopy. The number of apoptotic cells was counted in the areas of islets in four-nonconsecutive slices of pancreatic tissues derived from each mouse.

Real-time quantitative PCR

Islets were isolated from 20-week-old female mice by pancreatic digestion with collagenase (Wako Pure Chemical Industries, Ltd., Japan). Islets were purified by Histopaque (Sigma-Aldrich) density

gradient centrifugation. Isolated islets were stored at -80 °C until use. Total RNA was extracted from the isolated islets and kidneys. cDNA synthesis was done using primers with SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). cDNAs were used as templates in SYBER green real-time PCR assays on a LightCycler (Roche Diagnostics). The primers used in the PCR reaction for inositol requiring 1 (IRE1), PKR-like ER kinase (PERK), JNK1, JNK2, and Caspase-12 were obtained from SA Biosciences. The primers for CHOP and Binding immunoglobulin protein (Bip) were synthesized as described in the previous study[29]. Sample data were analyzed according to the comparative cycle threshold method and were normalized by stable reference genes of GAPDH and 18S selected by geNorm VBA applet among three house keeping genes including β-actin, GAPDH and 18S. All results were expressed as a percent of the value in control extracts.

Statistical analysis

Group differences were analyzed by the Mann–Whitney U test, χ^2 test or Student's t test and differences between Kaplan-Meier survival curves were estimated by the log-rank test with the use of Dr SPSS II for Windows software (SPSS Inc., Chicago, IL, USA). *P* values less than 0.05 was considered statistically significant. Insulitis levels were analyzed by Ridit analysis, and levels of *t* higher than 1.96 or lower than -1.96 were considered statistically significant.

Results

CHOP deletion did not affect the spontaneous development of diabetes and insulitis

The spontaneous incidence of disease was compared in both female and male *NOD.Chop*$^{-/-}$ mice, *NOD.Chop*$^{+/-}$ mice and *NOD.Chop*$^{+/+}$ mice. In female mice, the onset of diabetes began at 15 weeks of age in *NOD.Chop*$^{-/-}$ and *NOD.Chop*$^{+/+}$ mice and began at 19 weeks of age in *NOD.Chop*$^{+/-}$ mice. The incidence of diabetes was essentially identical among these three groups until 50 weeks of age (P=0.86) **(Figure 1, left).** In male mice, the incidence of spontaneous diabetes was also essentially identical among these groups (P=0.21) **(Figure 1, right).** The levels of insulitis did not significantly differ between female *NOD.Chop*$^{-/-}$ and *NOD.Chop*$^{+/+}$ mice at 12 and 20 weeks of age (Ridit score, T = 0.37 at 12 weeks, T = 0.8 at 20 weeks) **(Figure 2).**

CHOP deletion suppressed the expression of insulin autoantibody at 8 weeks of age

The mean levels and frequencies of IAA were compared in female *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice at 4, 8, 12 and 16 weeks of age. Interestingly, the expression of IAA at 8 weeks of age in *NOD.Chop*$^{-/-}$ mice occurred at a significantly lower level and frequency than in *NOD.Chop*$^{+/+}$ mice (*P<0.005, Mann–Whitney U test , **P<0.05, χ^2 test). In contrast, no significant differences in the levels and frequencies of expression of IAA were found at 4, 12 and 16 weeks of age. We also examined the levels of total serum IgG and no significant differences were found between both groups at any age (**Table 1**). Thus, CHOP deficiency suppressed early phase of insulin autoantibody expression independent of total serum IgG production.

CHOP deficiency did not affect diabetes in an adoptive transfer study with whole or CD8$^+$-depleted splenocytes

Autoimmune diabetes can be adoptively transferred to irradiated NOD recipients by the injection of whole or CD8$^+$-depleted splenocytes (SPCs) from a diabetic NOD donor[33]. In this study, we performed an adoptive transfer study involving the transfer of diabetogenic SPCs into irradiated *NOD.Chop*$^{+/+}$ or *NOD.Chop*$^{-/-}$ recipients to test whether CHOP contributes to islet β-cell death in the effector phase. Since it is possible that direct cell-mediated cytotoxicity of β-cell death by CD8$^+$ T cells may compensate for the lack of CHOP, CD8$^+$ depleted cells were also transferred. We found no

significant differences in the rates of diabetes development between *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice with whole splenocytes or CD8^{+}-depeleted SPCs **(Figures 3A, B).**

Apoptosis in the islets of *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice in spontaneous and an adoptively transferred diabetes models

To verify that ER stress via CHOP expression is not critical in β-cell damage in NOD mice, we evaluated apoptotic cells in the islets between 20-wk-old female *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice by TUNEL staining **(Figure 4A).** Apoptotic islet cells were frequently observed in both *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice. We also found sufficient number of apoptotic cells in the islets of irradiated *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice adoptively transferred with diabetogenic SPCs **(Figure 4B)**. In each study, there were no significant differences of the frequency of apoptotic cells in the islets between *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice **(Figure 4C)**. This suggests that spontaneous or T cell-mediated direct β-cell damage is independent of ER stress via CHOP expression.

Expression of ER stress genes was not enhanced in the islets from pre-diabetic wild-type NOD mice

We evaluated the expression of CHOP and other ER stress-associated molecules in the islets of pre-diabetic *NOD.Chop*$^{+/+}$ or *NOD.Chop*$^{-/-}$ mice (~20 weeks of age), since CHOP expression is often observed in the pre-apoptotic stage of target cells [30, 34-37]. As expected, an immunohistochemistry study showed that CHOP was expressed in kidneys from tunicamycin-treated mice as a positive control and in the islets from pre-diabetic *NOD.Chop*$^{+/+}$ mice and NOD-*scid* mice, but not in the islets from *NOD.Chop*$^{-/-}$ mice **(Figure 5A)**. Quantitative analysis revealed that the levels of CHOP mRNA in the islets in pre-diabetic *NOD.Chop*$^{+/+}$ mice did not differ from those in the islets in NOD-*scid* mice of the same age. We also investigated the mRNA expression of other molecules of the ER stress-mediated apoptosis pathway including Bip, IRE1, PERK, JNK1, JNK2 and Caspase-12 normalized by the reference genes of GAPDH and 18S in the pre-diabetic *NOD.Chop*$^{+/+}$, *NOD.Chop*$^{-/-}$ and NOD-*scid* mice. No significant differences in mRNA expression in any molecule were observed among the three groups **(Figure 5B)**.

Discussion

Both type 1 diabetes and type 2 diabetes are characterized by deficits in β-cell mass and increased β-cell apoptosis[38-43]. It has been suggested that ER stress induced β–cell apoptosis may be a common cause in type 1 and type 2 diabetes [26, 27, 44, 45]. In type 1 diabetes, exposure to cytokines and NO during insulitis inhibits expression of the sarcoendoplasmic reticulum pump Ca^{2+} ATPase (SERCA) 2 in β-cells and deplete endoplasmic reticulum calcium stores, leading to ER stress and apoptosis together with increased levels of ER stress-associated molecules such as IRE-1α, CHOP and ATF-4[27]. The pathogenesis of type 2 diabetes is characterized by both insulin resistance and impaired insulin secretion due to decreased β-cell function and β-cell mass [46, 47]. High levels of free fatty acids (FFAs) palmitate triggers ER stress and induces β–cell apoptosis together with increased levels of ER stress-associated molecules [44, 45]. Thus, cytokines and palmitate trigger ER stress in β-cells evidenced by increased expression of ER stress-associated molecules including CHOP. A recent study, however, demonstrated that siRNA-mediated CHOP reduction as well as PBA which acts as a chemical chaperone attenuate palmitate-mediated ER stress and β-cell death but not cytokine-mediated ones[29]. Furthermore, CHOP deletion in several mouse models of type 2 diabetes also demonstrated improved glycemic control and the maintenance of β-cell mass via the UPR and oxidative stress response[48, 49].

In the present study, we investigated the impact of CHOP deletion in the NOD mouse, a model of type 1 diabetes, in order to determine whether ER stress-mediated β-cell death via the CHOP is involved in the pathogenesis of type 1 diabetes. We found that CHOP deletion does not affect the development of insulitis and diabetes. We also demonstrated that CHOP expression is not increased in islets from pre-diabetic NOD mice compared to those from NOD-*scid* mice. A previous study has shown that CHOP expression is frequently observed in islets from obese type 2 diabetes patients but not from type 1 diabetes patients[50]. These findings suggest that CHOP is fundamental factor that links ER stress and apoptosis in β-cells in type 2 diabetes but not type 1 diabetes.

It is possible that other mechanisms of β-cell death such as direct cell-mediated cytotoxicity induced by activated $CD8^+$ T cells (CTLs) may compensate for the lack of CHOP-mediated cell death in the *NOD.Chop*$^{-/-}$ mice, resulting in the similarity of the kinetics of diabetes development with the kinetics in wild-type mice. However, our adoptive transfer study with $CD8^+$-depleted splenocytes confirmed that

CHOP deletion does not have an influence on the development of diabetes, even without CTL-mediated cytotoxicity.

We also investigated the mRNA expressions of JNK[17, 18] and caspase-12[19, 51] in order to rule out the possibility of the involvement of other apoptosis pathways mediated by ER stress. We did not find any evidence of enhancement of the expression of ER stress response genes or ER stress-associated apoptosis genes in the *NOD.Chop*$^{-/-}$ mice or the wild-type mice in comparison to control mice. Our results indicate that ER stress response in the islets is not a major mechanism of cytokine induced β-cell death in the pathogenesis of type 1 diabetes. It has been proposed that possible other pathways contributing to the cytokine induced β-cell death include an activation of stress-activated kinases and the release of mitochondrial death signals[12]. JNK group of MAPK is activated by IL-1β in β-cells and inhibitors of JNK prevent cytokine-induced apoptosis in insulin-producing cells[52, 53]. The JNK1 is encoded by the *Mapk8* gene and *Mapk8*$^{-/-}$ mice exhibit profound defects in insulin resistance and obesity caused by eating high fat diet, suggesting that JNK1 is implicated in type 2 diabetes[54, 55]. In contrast, JNK2 encoded by the *Mapk9* gene play an important role for type 1 diabetes since *Mapk9* deletion suppress the development of insulitis and diabetes in the NOD background mice. However the β-cells derived from *Mapk9*$^{-/-}$ NOD mice and wild type NOD mice are found to be equally sensitive to cytokine induced apoptosis *in vitro*[56], suggesting that JNK2 is independent of cytokine induced β-cell death. We investigated the mRNA expression of both JNK1, JNK2 in the islets of *NOD.Chop*$^{+/+}$, *NOD.Chop*$^{-/-}$ and NOD-*scid* mice but no significant differences were observed among the three groups.

Mitochondria are key organelles for β-cell function but also play an important role in triggering apoptosis. Members of the Bcl-2 protein family regulate the mitochondrial response to pro-apoptotic signals[57] and prevent release of cytochrome c which execute dell death. It has been reported that overexpression of Bcl-2 partially protects cytokine-induced β-cell death in mouse [58]and human[59]. However, overexpression of Bcl-2 *in vivo* does not prevent spontaneous diabetes in NOD mice[60].

Thus, proposed pathways contributing to cytokine-induced β-cell death could not fully explain the pathogenesis of type 1 diabetes *in vivo*. Alternative approaches with engineered NOD background mouse are need to be investigate to clarify the relevance of cytokine-induced β-cell death in type 1 diabetes.

We found that the expression of IAA was suppressed in *NOD.Chop*$^{-/-}$ mice at 8 weeks of age. NOD

mice usually express IAA as early as 4 weeks of age, and the early appearance of IAA at 4 to 8 weeks is strongly associated with the subsequent early development of diabetes[31]. Since the development of diabetes was not suppressed in *NOD.Chop*$^{-/-}$ mice, CHOP might be relevant for the production of autoantibody independent of the development of autoimmune diabetes. A previous study demonstrated that the ER stress response via the transcription factor X-box binding protein 1 (XBP-1) is required for the terminal differentiation of B cells to plasma cells[61]. The expression of CHOP as well as XBP-1 has been shown to be rapidly induced in the B cells after stimulation with IL-4[62]. CHOP deficient mice exhibit lower levels of serum IgM secretion after lipopolysaccharide (LPS) stimulation independent of the differentiation of plasma cells[63]. Although the spontaneous IAA in NOD mice is predominantly IgG, CHOP deficiency did not affect on the total serum IgG levels in this study. CHOP might play a crucial role in the early production of antigen-specific autoantibody such as IAA in the NOD mouse.

In this study, we demonstrated here that a lack of CHOP does not affect the natural course of diabetes development in NOD mice. Our results, taken together with these previous findings, suggest that the mechanisms of β-cell death could be fundamentally different between type 1 diabetes and type 2 diabetes, and different therapeutic approaches will be required to prevent β-cell death and reverse diabetes in each types of diabetes[12].

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Figure legends

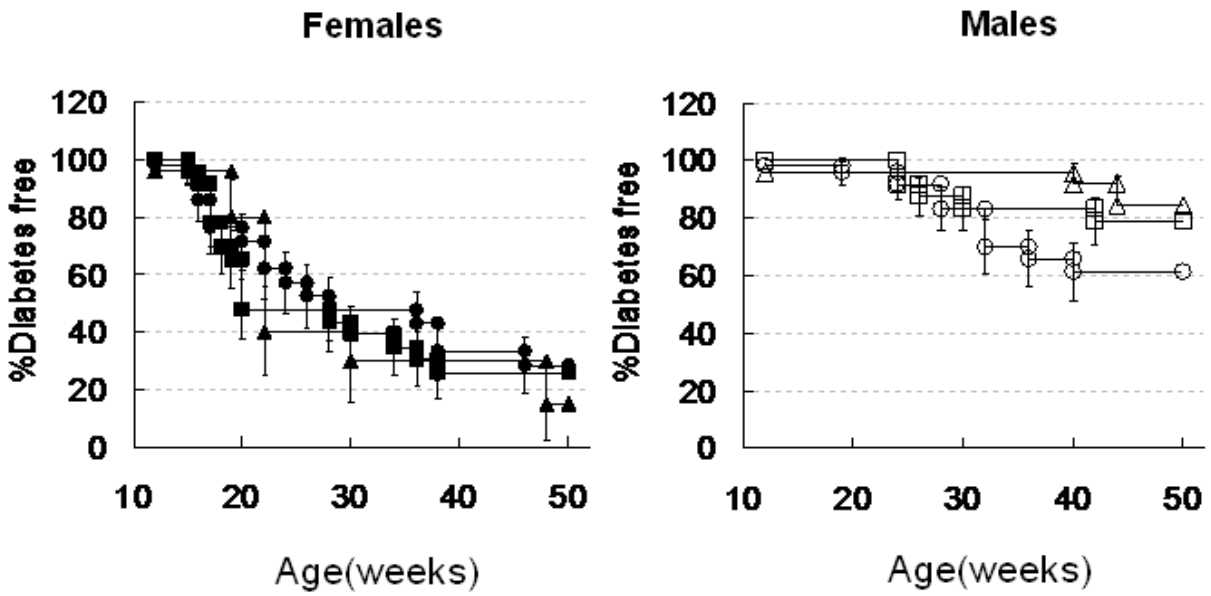

Figure 1. Incidence of spontaneous diabetes in *NOD.Chop*$^{-/-}$ mice (filled squares, n=23 females and open squares, n=24 males), *NOD.Chop*$^{+/-}$ mice (filled triangles, n=10 females and open triangles, n=13 males) and *NOD.Chop*$^{+/+}$ mice (filled circles, n=21 females and open circles, n=24 males). All three groups in each gender developed diabetes at the same rate (log-rank test, P = 0.86 for females and 0.21 for males).

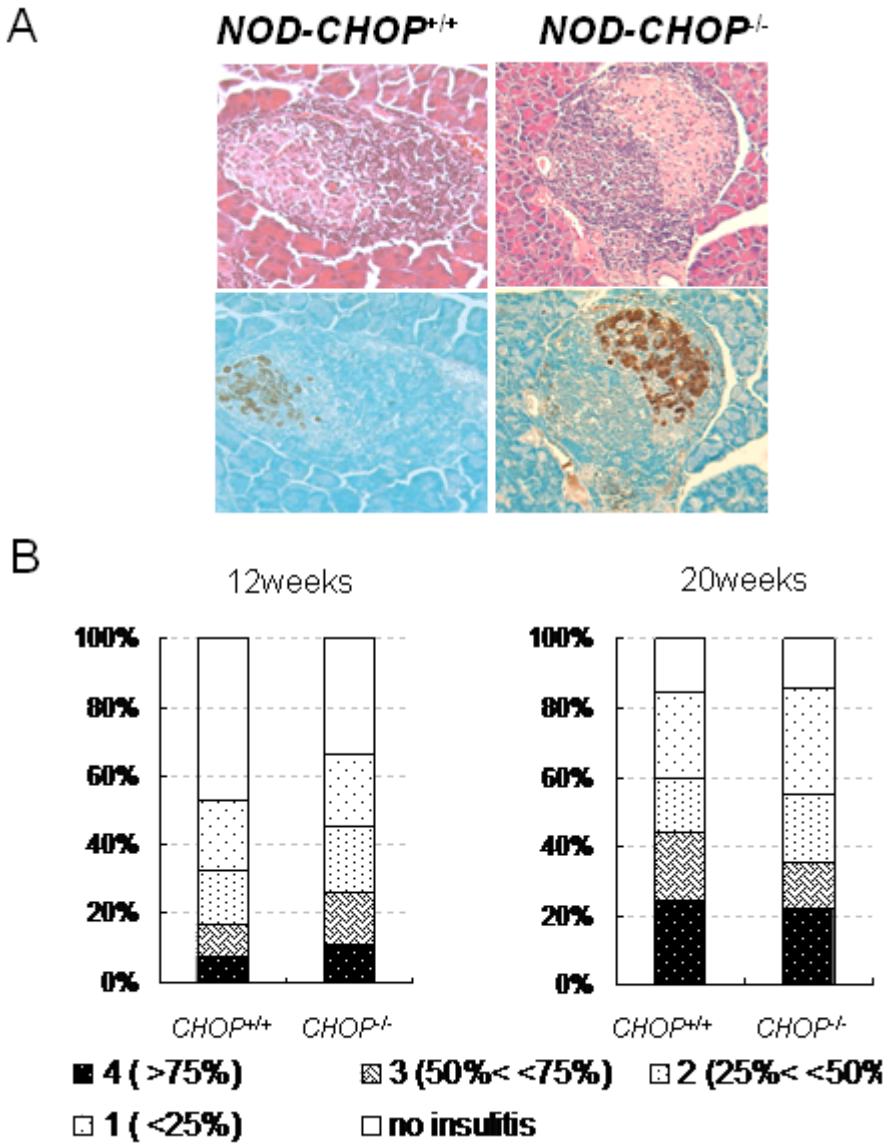

Figure 2. (A) Hematoxylin eosin staining and immunoperoxidase staining with anti-insulin monoclonal antibody derived from 20-wk-old female *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice. **(B)** Levels of insulitis in *NOD.Chop*$^{+/+}$ mice (n=5) and *NOD.Chop*$^{-/-}$ mice (n=5) at 12 and 20 wks of age. A level of T > 1.96 determined by Ridit analysis was regarded as significant. No significant difference was found between the two groups at 12 wks of age (T=0.37) and at 20 weeks of age (T=0.8).

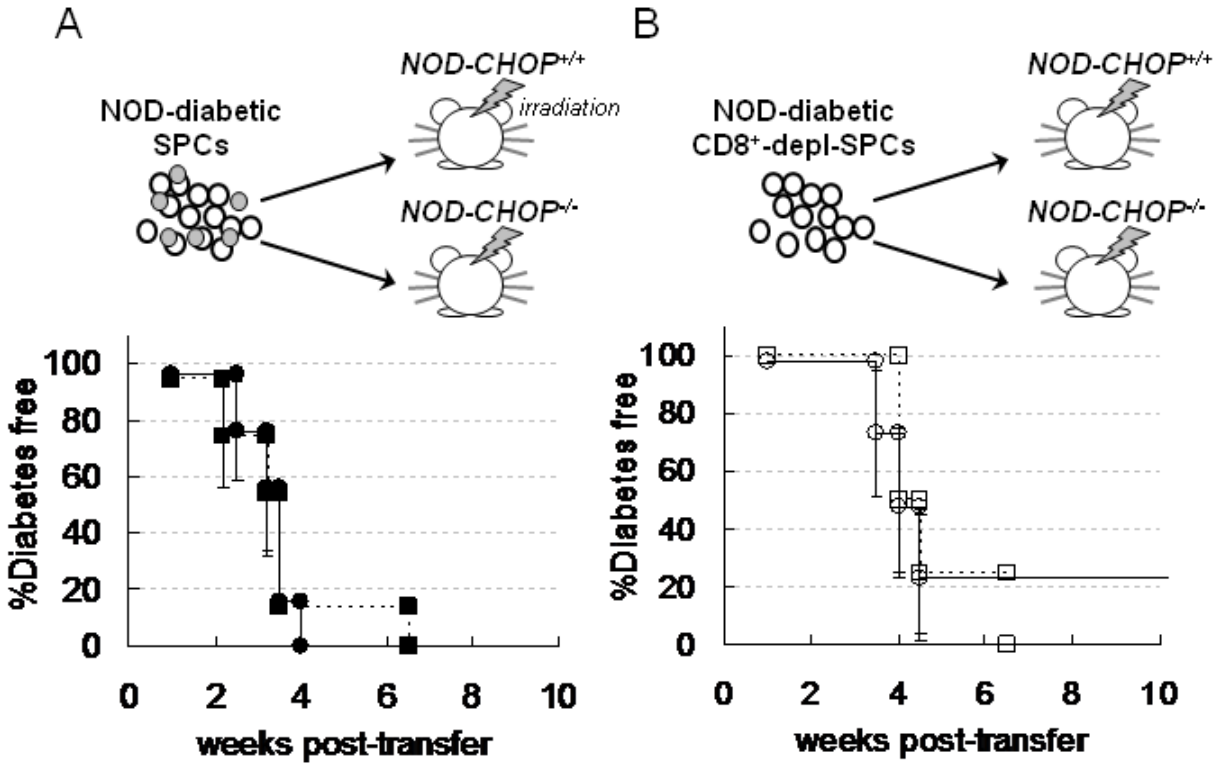

Figure 3. Adoptive transfer study. **(A)** Whole SPCs ($3x10^7$) isolated from newly diabetic NOD mice were transferred into irradiated *NOD.Chop*$^{-/-}$ mice (n=5 filled squares) and *NOD.Chop*$^{+/+}$ mice (n=5 filled circles). **(B)** $CD8^+$-depleted SPCs ($2x10^7$) were transferred into irradiated *NOD.Chop*$^{+/+}$mice (n=4 open circles)or *NOD.Chop*$^{-/-}$ mice (n=4 open squares).

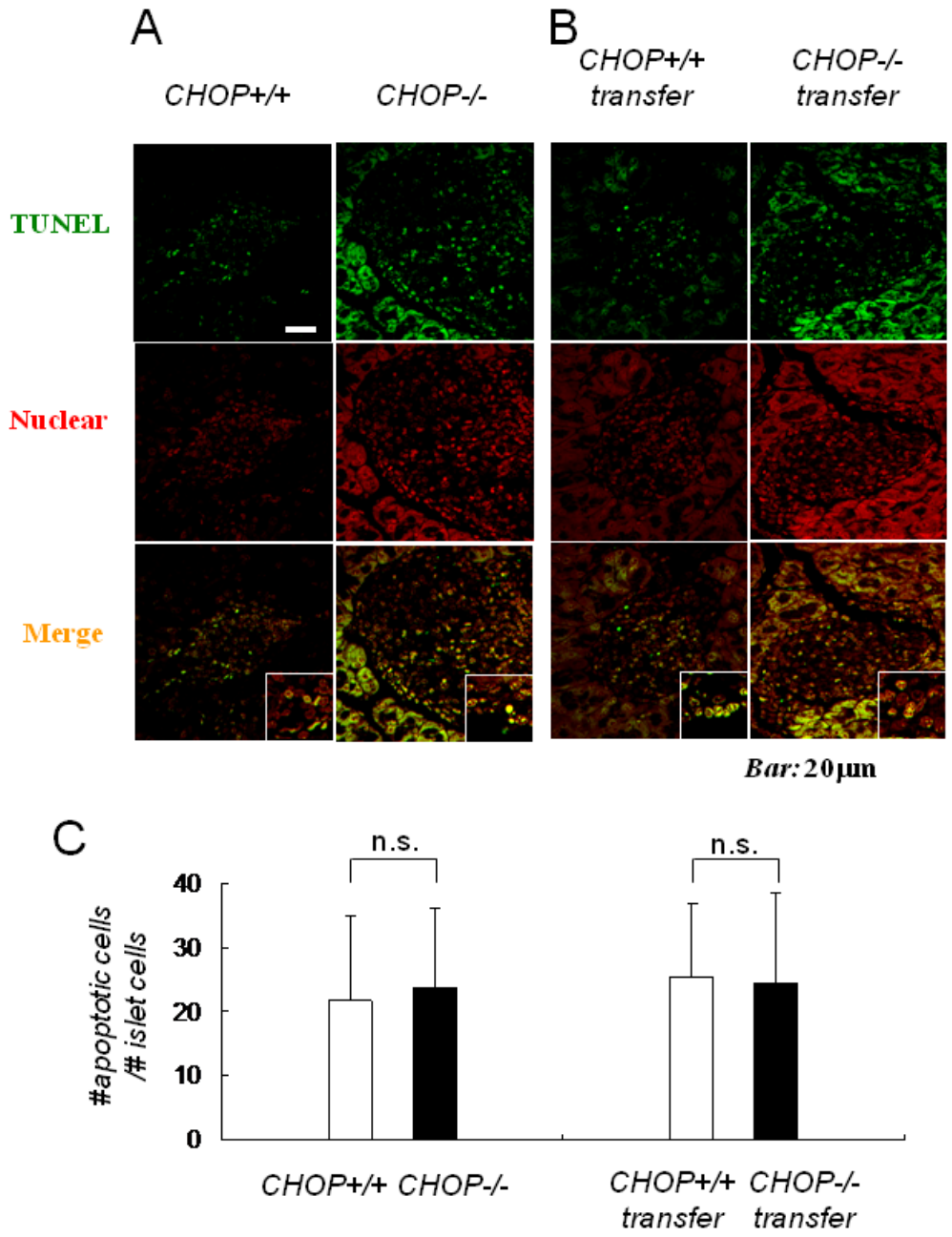

Figure 4. TUNEL staining of islets from 20-wk-old female *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ mice **(A)** and those of irradiated *NOD.Chop*$^{+/+}$ and *NOD.Chop*$^{-/-}$ recipient mice adoptive transferred whole SPCs **(B)**. TUNEL staining (green) was employed and the TUNEL image was merged with nuclear image (red) by the merged view.(Bar 20μM). **(C)** Percentages of apoptotic cells among islet cells in *NOD.Chop*$^{+/+}$ mice (n=5) and *NOD.Chop*$^{-/-}$ mice (n=5) at 20 wks of age and freshly diabetic *NOD.Chop*$^{+/+}$ (n=4) and *NOD.Chop*$^{-/-}$ recipient mice (n=4). The number of apoptotic cells was counted in the areas of islets in four-nonconsecutive slices of pancreatic tissues derived from each mouse. No significant difference was found between both mice in each study.

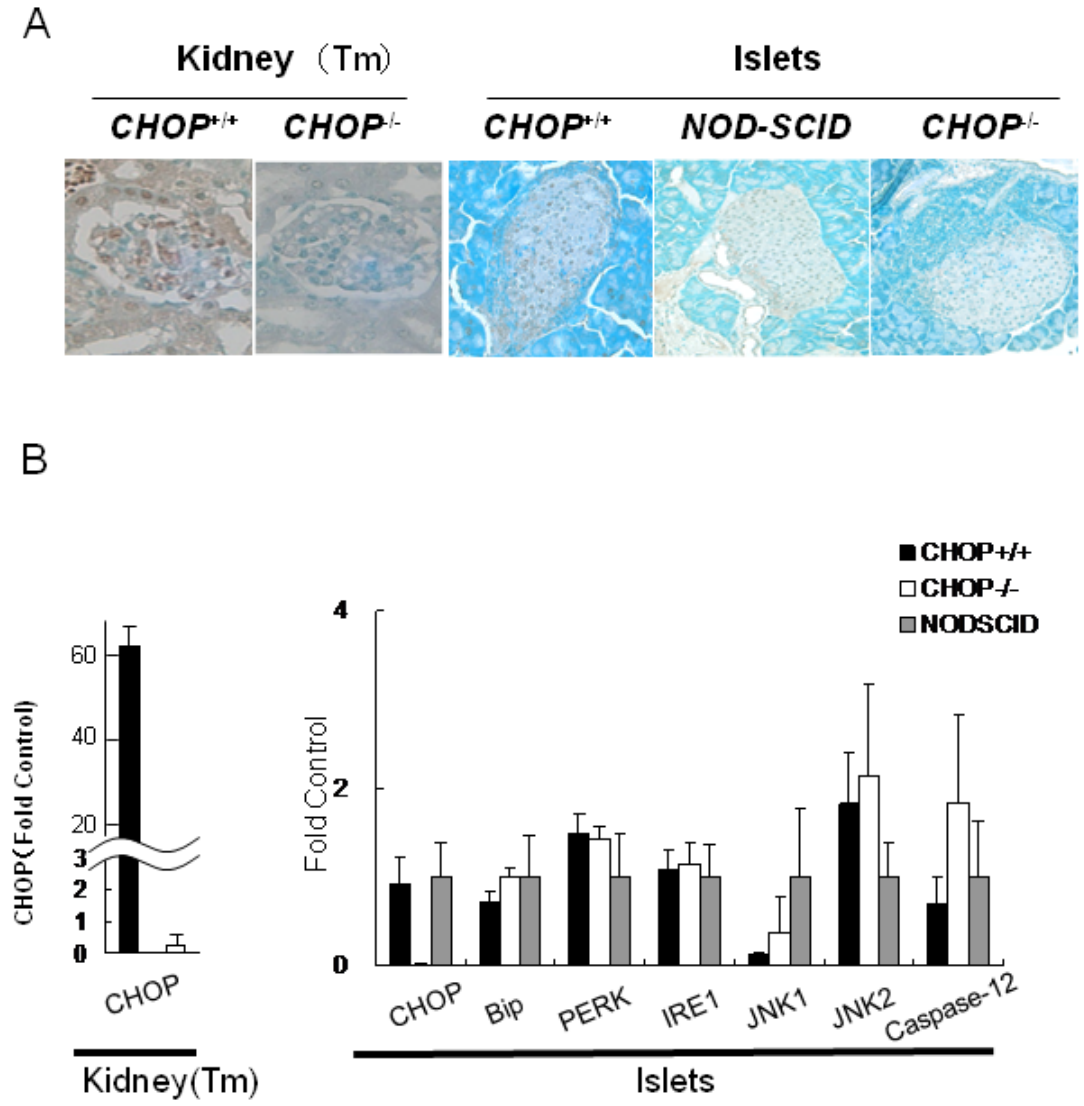

Figure 5. (A) Immunoperoxidase staining and real-time RT PCR of mouse kidneys and islets in *NOD.Chop*$^{+/+}$ mice, *NOD.Chop*$^{-/-}$ mice and *NOD-scid* mice. The kidneys were injected (i.p.) with tunicamycin (Tm) in *NOD.Chop*$^{+/+}$mice and *NOD.Chop*$^{-/-}$ mice. The kidneys were removed 24 hr after injection and stained with anti-CHOP polyclonal antibody. The islets of *NOD.Chop*$^{+/+}$ and *NOD-scid* mice expressed CHOP in similar proportions. **(B)** Changes to the mRNA levels of genes involved in ER stress and apoptosis of ER in islets from *NOD.Chop*$^{+/+}$ (n=6), *NOD.Chop*$^{-/-}$ (n=6) and *NOD-scid* mice (n=3). Sample data were normalized by stable reference genes of GAPDH and 18S. Values are means+SE and the data are expressed as a percentage of the mRNA levels in islets isolated from control *NOD-scid* mice at the same time.

Table 1.

	Age in weeks	4	8	12	16
Mean levels of IAA	*NOD-Chop*$^{+/+}$	1.7±0.3	41.4±17.7	142.8±70.4	146.7±81.9
index (x10^{-3})	*NOD-Chop*$^{-/-}$	1.8±0.2	4.0±1.1*	70.4±40.3	146.7±81.9
Total levels of IgG	*NOD-Chop*$^{+/+}$	13.2±0.6	14.9±0.5	13.2±0.5	11.8±0.3
(μg/ml)	*NOD-Chop*$^{-/-}$	11.8±0.4	13.4±0.7	12.0±0.6	11.7±0.5
Frequencies of IAA	*NOD-Chop*$^{+/+}$	0/15 (0.0)	13/42 (31.0)	16/41 (39.0)	14/26 (53.8)
positive/ total (%)	*NOD-Chop*$^{-/-}$	0/15 (0.0)	4/39** (10.3)	13/39 (33.3)	13/24 (54.2)

The results are shown as means ± S.E. and a level of ≥ 1.00 x10^{-3} was regarded as positive for IAA (*P<0.005, Mann–Whitney U test, **P<0.05, χ^2 test). IgG level was analyzed by student-t test. This table includes the data from two different experiments at 4-12week and 8-16 week of age.