Modulation of Glucocorticoid-Induced GAD Expression in Pancreatic β-Cells by Transcriptional Activation of the GAD67 Promoter and Its Possible Effect on the Development of Diabetes

Kyung Soo Kim,1 Yup Kang,1 Sung E. Choi,1 Ju Hee Kim,1 Hyeon Man Kim,2 Beichen Sun,3 Hee-Sook Jun,3 and Ji-Won Yoon3

GAD is a pancreatic β-cell autoantigen in humans and nonobese diabetic (NOD) mice. Modulation of GAD expression in pancreatic β-cells has been suggested to be associated with the development of autoimmune diabetes. Hormonal changes through environmental stimuli are considered to influence the expression of the disease. We determined whether steroid hormones would modulate the expression of GAD in pancreatic β-cells. We treated NOD mouse β-cells (MIN6N8a cells) with various steroids, including testosterone, estradiol, progesterone, and cortisol, and examined the expression of GAD67 mRNA. We found that only cortisol enhanced the expression of GAD67, whereas the other steroid hormones had no effect. When we treated MIN6N8a cells with a synthetic glucocorticoid, dexamethasone, we found that GAD67 mRNA expression was stimulated in a dose- and time-dependent manner. Cells treated with 100 nmol/l dexamethasone for 6 h showed a 10-fold increase in the expression of GAD67 mRNA and an increase in GAD67 protein. The upregulation of GAD67 expression in β-cells by dexamethasone was found to be due to the transcriptional activation of the GAD67 promoter. We then examined whether dexamethasone would influence the development of diabetes in NOD mice. Injection of dexamethasone into neonatal NOD mice resulted in a significant increase in the expression of GAD67 mRNA in pancreatic β-cells and the development of insulin and diabetes. We conclude that glucocorticoid hormones can modulate GAD expression by the transcriptional activation of the GAD promoter and may influence the development of autoimmune diabetes in NOD mice. Diabetes 51:2764–2772, 2002
that one line showed a low incidence of type 1 diabetes, whereas the other line showed no difference in the incidence of the disease as compared with NOD controls (17). Conversely, a transgenic NOD mouse line that expresses GAD65 in all tissues showed an increased incidence of diabetes compared with wild-type NOD mice (18). In addition, the suppression of GAD expression in β-cells of NOD mice prevents the development of type 1 diabetes (19). These results suggest that the expression of GAD in β-cells may be involved in the modulation of β-cell–specific autoimmunity.

It has been suggested that changes in the functional and metabolic state of the β-cells may affect the development of type 1 diabetes, and the expression of GAD seems to be affected by the functional and metabolic state of the β-cells (20–21). For example, there is an increase in the expression of GAD in islets cultured under hyperglycemic conditions (22), and the accumulation of glutamate through the perturbation of the GABA network by oxidative damage or nutrient stress could induce GAD expression (21). Environmental stresses through infectious agents, psychological stress, and hormonal changes were suspected to influence the expression of autoimmunity type 1 diabetes (23–27). Castration increases the incidence of diabetes (26–28), and psychological stress accelerates the disease (29,30). This investigation was initiated to determine whether steroid hormones affect the expression of GAD on pancreatic β-cells and the development of diabetes. We found that glucocorticoids significantly increased GAD67 expression in NOD mouse β-cells, and this increase in GAD expression was mediated by the transcriptional activation of the GAD67 promoter and is associated with the development of diabetes.

**RESEARCH DESIGN AND METHODS**

**Cells and animals.** MIN6N8a cells, a mouse insulinoma cell line with a NOD background (11,32), were provided by Dr. Michiyasu Yamasaki (Tokyo, Tokyo, Japan). The cells were cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD), 1 g/l glucose, 100 μg/ml streptomycin (Gibco BRL), and 100 units/ml penicillin (Gibco BRL) and maintained at 37°C under 5% CO₂. NIH3T3 cells were obtained from ATCC (Manassas, VA). NOD mouse embryonic fibroblast (MEF) cells were prepared as described elsewhere (33). NOD mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and were bred and maintained at the animal facilities at Aju University and the University of Calgary under specific pathogen-free conditions. The animal studies were conducted in accordance with the Principles of Laboratory Animal Care (National Institutes of Health publication no. 85–23).

**Hormone treatment.** To determine the effect of steroid hormones on GAD67 transcription, we incubated MIN6N8a cells with 1 μmol/l cortisol (Korea Upjohn, Seoul, Korea), testosterone, β-estradiol, or progesterone (Sigma, St. Louis, MO) or with 0.1, 10, 100, or 1,000 nmol/l dexamethasone (Sigma) in DMEM supplemented with 10% fetal bovine serum, 1 g/l glucose, and 100 μg/ml streptomycin. Cells incubated without hormone were used as a control. At various times (0–8 h) after treatment, cells were harvested for RT-PCR analysis. To determine whether dexamethasone-induced GAD67 expression is inhibited by blocking the glucocorticoid receptor (GR), we incubated MIN6N8a cells with a GR blocker, RU486 (0–10 μmol/l), for 2 h before incubation with 100 nmol/l dexamethasone for 6 h, and cells were harvested for RT-PCR analysis. To investigate the effect of glucocorticoid on GAD67 expression in vivo, we injected dexamethasone (0.1 mg/kg body wt) subcutaneously into neonatal (2–3 days of age) and intraperitoneally into adult (5–6 weeks of age) NOD mice. Animals that received an injection of the same volume of PBS were used as a control. After 6 h, the tail portion of the pancreas was removed for RT-PCR analysis.

**RT-PCR.** Total RNA was extracted by RNAzol B solution (TEL-TEST, Friendwood, TX) according to the supplier’s protocol, and GAD67 mRNA expression was determined by semiquantitative RT-PCR using an RNA PCR kit (Takara Shuzo, Otu, Shiga, Japan). One microgram of total RNA was used to synthesize the first strand of the cDNA using AMV reverse transcriptase and a random 9-mer oligonucleotide. PCR was performed with oligonucleotide primer sets for mouse GAD67 (forward 5′-ACAGTGACCAAGGTTGGCGCTC-3′, reverse 5′-GAAACCCTGACAGGTTGCTTC-3′) for 30–32 cycles at 95°C for 30 s, 64°C for 30 s, and 72°C for 60 s. As an internal standard, β-actin mRNA was amplified using primer sets for mouse β-actin (forward 5′-CATGTTTGAGCCTTCACACCCCC-3′, reverse 5′-GCCATCTTCTGCTGTTGACGTC-3′) for 34 cycles under the same PCR conditions. The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. The expression of GAD67 was determined quantitatively by densitometric analysis of the stained bands and normalized with respect to the β-actin signal. For RT-PCR analysis of GAD65, insulin, and HSP60, the following primer sets were used: mouse GAD65: forward 5′-CCTTCCCTCTCGGCGCTGAC-3′, reverse 5′-GGATGATACTCACCCACAGC-3′, and mouse HSP60: forward 5′-ATGAGCTTACGAGGTTGGGAGAGTC-3′, reverse 5′-GCTCATCATTACGGGGTTTCT-3′.

**Immunoblotting.** MIN6N8a cells (1 × 10⁶) were incubated with 100 nmol/l dexamethasone for 12 h and lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris–Cl [pH 7.5]). The cell lysate was mixed with 1 μl of antibody (AB20, Abcam, Cambridge, CA), and the immune complex was collected with 100 μl of protein A Agarose beads (Calbiochem, San Diego, CA). The precipitated proteins were fractionated by 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electrottransfer (60 volts for 4 h). The membrane was blocked in 5% skim milk–PBS solution for 1 h and incubated with anti-GAD67 antibody. After washing, the membrane was incubated with horseradish peroxidase–conjugated anti-rabbit IgG. The reactive band was detected by an enhanced chemiluminescence kit (Amer sham Pharmacia Biotech, Piscataway, NJ).

**Flow cytometric analysis.** To determine the expression of GAD protein after dexamethasone treatment, we treated MIN6N8a cells with 100 nmol/l dexamethasone for 24 h and fixed them with 4% paraformaldehyde in PBS. The cells were stained with anti-GAD67 antibody (Chemicon) and then with FITC-labeled secondary antibody; labeling intensity was analyzed by FACScan (Beckton-Dickinson, San Jose, CA).

**Luciferase assay.** To investigate the transcriptional regulation of the GAD67 gene by dexamethasone, we determined the activity of the GAD67 promoter using a luciferase reporter assay. A total of 1,150 bp of the 5′-flanking region of the mouse GAD67 gene (34) was amplified with GAD67 promoter-specific primer set (5′-AGGTTACCTAAAGGATGGAGACGGGAGGAG 5′-GAGAGACGCTGCGATCCATCTAG) and subsequently digested with KpnI and BgII site of pGL2-Basic vector (Promega, Madison, WI) to construct pGL2-GAD67P. MIN6N8a cells grown subconfluently in six-well plates were transfected with 1 μg of pGL2-GAD67P and 1 μg of control pSV-β-galactosidase plasmid (Promega) using Lipofectamine (Gibco BRL). The transfected cells were treated with 100 nmol/l dexamethasone for 9 h. The cells were washed in PBS and scraped into a lysis buffer (Luciferase assay kit, Promega). An aliquot of the cell lysate was added to the luciferase assay reagent, and then the activity was measured in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

**MTT assay.** To measure the cell growth and viability, we conducted an MTT assay. MIN6N8a cells treated with or without 0.1 or 1 μmol/l dexamethasone were washed with Krebs-HEPES buffer and preincubated for 30 min at 37°C under 5% CO₂ in the same buffer. As a positive control, MIN6N8a cells were also treated with 0.1–0.5 mmol/l sodium nitroprusside and incubated under the same conditions. The cells were then treated with 0.5 mg/ml MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) in the same buffer for 1 h. After aspiration of the supernatant, formazan crystals were dissolved by the addition of isopropanol. The absorbance was read at 540 nm on a spectrophotometer (Bio-Rad, Hercules, CA).

**In vivo treatment with dexamethasone.** To determine the effect of dexamethasone on the development of insulitis, we injected dexamethasone intraperitoneally into neonatal NOD mice on day 2 (0.5 mg/kg body wt); day 3 (0.3 mg/kg body wt); and days 4, 5, and 6 (0.1 mg/kg body wt). The pancreas was removed at 8 weeks of age for the examination of insulitis. The onset of diabetes was monitored as described previously (35).

**Histology.** Pancreata were removed, fixed with formalin, embedded in paraffin, serially sectioned at 4.5 μm, and stained with hematoxylin and eosin. A total of 20–30 islets per animal were examined and classified as follows: 0, intact islet; 1, early insulitis, accumulation of mononuclear cells at the periphery of the islet; 2, intermediate insulitis, infiltration of mononuclear cells at the periphery and ~50% of the islet center; 3, late insulitis, massive
infiltration of mononuclear cells throughout the islet; 4, end-stage insulitis, small, retracted islets with or without residual infiltration.

Statistics. GAD67 expression in the pancreas was analyzed by one-way ANOVA using the SPSS program; these results are expressed as means ± SD. The degree of insulitis was analyzed by the Mann-Whitney U test. Differences in the incidence of diabetes between PBS- and dexamethasone-treated mice were analyzed by the Wilcoxon signed-rank test.

RESULTS
To determine whether steroid hormones affect the expression of GAD in mouse β-cells in vitro, we incubated MIN6N8a cells for 6 h with 1 μmol/l testosterone, cortisol, estradiol, or progesterone and determined the expression of GAD67 mRNA by semiquantitative RT-PCR. We found that only cortisol enhanced the transcription of GAD67 in the MIN6N8a cells, whereas the other steroid hormones showed no effect on the expression of GAD67 (Fig. 1A). Non–β-cells, such as NOD MEF cells (Fig. 1B) and NIH3T3 cells (Fig. 1C), did not express any detectable GAD67.

To determine whether glucocorticoid also affects the expression of other β-cell autoantigens, we treated MIN6N8a cells with 100 nmol/l dexamethasone and examined the expression of GAD65, insulin, and HSP60 by RT-PCR. We found that the expression of these antigens was not significantly changed by dexamethasone treatment (Fig. 2).

To determine whether the upregulation of GAD67 by cortisol is truly a glucocorticoid effect, we incubated MIN6N8a cells with 100 nmol/l dexamethasone, a synthetic glucocorticoid, for various time periods. We found that the expression of GAD67 mRNA was upregulated at 1 h and gradually increased thereafter, showing an increase of >10-fold at 6 h (Fig. 3A). This result suggests that the GAD67 gene is an early response gene to glucocorticoids. To examine whether there is a dose-dependent effect of dexamethasone on GAD67 expression, we treated MIN6N8a cells with 0.1–1,000 nmol/l dexamethasone for 6 h and found that the expression of GAD67 increased in a dose-dependent manner (Fig. 3B).

To confirm that the upregulation of GAD67 is a specific effect of dexamethasone, we incubated MIN6N8a cells in the presence of 100 nmol/l dexamethasone; withdrew the dexamethasone 12 h later; and examined the expression of GAD67 mRNA 1, 2, 4, 6, and 24 h after hormone withdrawal. We found that the expression of GAD67 mRNA gradually decreased after the removal of dexamethasone, whereas high levels were maintained in cells continuously exposed to dexamethasone (Fig. 4A). In addition, treatment of MIN6N8a cells with RU486, a GR blocker (36), antagonized the dexamethasone-mediated upregulation of GAD67 mRNA in a dose-dependent manner. The dexamethasone-enhanced GAD67 mRNA expression was reduced to untreated levels by 2 h of incubation with 1 μmol/l RU486 (Fig. 4B). This result indicates that the transcription of GAD67 is specifically regulated by this glucocorticoid.

To determine whether the increase in GAD67 mRNA is correlated with an increase in GAD67 protein, we performed Western blotting on cellular extracts after dexamethasone treatment. We found little immunoreactivity after Western blotting of the MIN6N8a cell lysate but found a strong 67-kDa immunoreactive band after concentration of GAD67 protein from 1 × 10⁶ cells by immunoprecipitation with anti-GAD67 antibody before loading. In contrast, protein from untreated cells precipitated with anti-GAD67 antibody or protein precipitated with preimmune rabbit sera did not show this distinct band (Fig. 5A). In addition, flow cytometric analysis of anti-GAD67 binding in MIN6N8a cells treated with dexamethasone showed that GAD67 expression was increased in dexamethasone-treated cells as compared with PBS-treated cells (Fig. 5B). This result indicates that treatment with dexamethasone results in the increase in GAD67 protein.

To determine whether the response to dexamethasone was an indirect result of the effect of dexamethasone on cell growth or death, we incubated MIN6N8a cells with 0.1 or 1.0 μmol/l dexamethasone for 24 or 72 h and determined cell proliferation and death by MTT assay. We found that the incubation of MIN6N8a cells in 1 μmol/l dexamethasone for 72 h did not result in cell death, whereas incubation even in 0.1 nmol/l sodium nitroprusside, a nitric oxide donor, had a cytotoxic effect of ~40%
on the cells (Fig. 6). This result indicates that dexamethasone, at the concentration used, did not affect the viability of MIN6N8a cells.

To determine whether the enhancement of GAD67 expression by dexamethasone was through the transcriptional activation of the GAD67 promoter, we measured the effect of dexamethasone on the transcriptional activity of the GAD67 promoter using a luciferase reporter assay. The GAD67 promoter was amplified by PCR and subcloned into luciferase assay vector pGL2-basic to construct pGL2-GAD67P. MIN6N8a cells were transfected with pGL2-GAD67P, and the transfected cells were treated with 100 nmol/l dexamethasone for 9 h. The cells were lysed, and the luciferase activity was determined. We found that dexamethasone treatment of pGL2-GAD67P-transfected cells increased luciferase activity approximately threefold over that of untreated pGL2-GAD67P-transfected cells (Fig. 7). This result indicates that the upregulation of GAD67 expression by dexamethasone is due to the transcriptional activation of the GAD67 promoter.

To determine whether dexamethasone can upregulate the expression of GAD67 in β-cells in vivo, we injected dexamethasone (0.1 mg/kg body wt) into neonatal and 6-week-old NOD mice and measured GAD67 mRNA in the pancreas 6 h after injection. We found that dexamethasone-treated 6-week-old NOD mice did not show a significant increase in GAD67 mRNA expression, whereas dexamethasone-treated neonatal NOD mice showed a two- to threefold increase in GAD67 transcription (Fig. 8). This result indicates that this glucocorticoid can enhance the expression of GAD67 in NOD mouse β-cells in vivo, depending on the age of the NOD mice.

To determine whether the dexamethasone-enhanced GAD67 production affected the development of diabetes, we injected dexamethasone into neonatal NOD mice for 5 days and examined the development of diabetes and insulitis at 8 weeks of age. We found that 61.3% of the islets from dexamethasone-treated NOD male mice showed early to intermediate insulitis and that 19.4% showed late to end-stage insulitis at 8 weeks of age. In contrast, 61.5% of the islets from PBS-treated NOD male mice showed intact islets, 34.6% showed early insulitis, and only 3.8% showed intermediate insulitis at the same age (Fig. 9, Table 1). In addition, we found that 40% (4 of 10) of dexamethasone-treated male NOD mice developed diabetes, whereas 20% (3 of 15) of PBS-treated male NOD mice developed diabetes by 30 weeks of age (Table 2). The earliest age of diabetes onset in NOD male mice was 20 weeks for PBS-treated mice and 16 weeks for dexamethasone-treated mice. The age at onset and frequency of diabetes was significantly increased in both male and female mice treated with dexamethasone (Table 2).

**DISCUSSION**

Cumulative experimental evidence in animal models suggests that GAD plays an important role in the development of T-cell-mediated autoimmune diabetes. First, immuniza-
tion with purified GAD protein either intrathymically or intravenously at an early age can tolerize the T-cell–mediated immune response against pancreatic β-cells in NOD mice, thus preventing insulitis and diabetes (6,7). Moreover, tolerization with GAD could also prevent the development of other immune reactions that usually occur in NOD mice, such as those against heat shock protein 65 and carboxypeptidase H. In addition, oral administration of GAD-expressing transgenic plants and nasal administration of a mixture of GAD peptides prevented autoimmune diabetes in NOD mice (37,38). Second, there is direct evidence that GAD-reactive T-cells are diabetogenic in NOD mice (12). Third, the β-cell–specific suppression of GAD expression results in the prevention of autoimmune diabetes in NOD mice (19). However, the precise nature of its role remains unclear. It is speculated that GAD expression on pancreatic β-cells may be involved in the modulation of β-cell–specific autoimmune responses and/or modulation of the functional state of the β-cell. Steroid hormones, particularly sex steroids, are known to influence autoimmune diseases, such as type 1 diabetes (26,27,39,40). In NOD mice, the incidence of diabetes is more prevalent in females. Treatment of female mice with testosterone decreased the incidence of diabetes (27), and castration accelerated the development of diabetes in NOD mice (26). Although it has been suggested that steroid hormones modulate the development of autoimmune diabetes by exerting effects on the immune system, the precise mechanism by which these hormones influence type 1 diabetes is not known.

In this study, we investigated the possibility that steroid hormones may affect the expression of GAD on pancreatic β-cells, resulting in the modulation of β-cell–specific auto-

![FIG. 5. Western blot and flow cytometric analysis of GAD67 protein after dexamethasone treatment. A: After treatment with (Dex [+]) or without (Dex [−]) 100 nmol/l dexamethasone for 12 h, MIN6N8a cell lysates were immunoprecipitated with anti-GAD67 antibody or preimmune serum as a control. The precipitated proteins were fractionated by 10% SDS-PAGE, and the GAD67 protein was detected by immunoblotting with anti-GAD67 antibody. IP, immunoprecipitation. B: MIN6N8a cells treated with (solid line) or without (dotted line) 100 nmol/l dexamethasone for 24 h were stained with anti-GAD67 antibody and analyzed by FACScan.]

![FIG. 6. Effect of dexamethasone on MIN6N8a cell growth and viability. MIN6N8a cells were incubated in the presence of 0 (Control), 0.1, or 1 μmol/l dexamethasone (Dex). Twenty-four or 72 h after treatment, the cells were treated with 0.5 mg/ml MTT for 1 h. The color intensity was measured at 540 nm. As a cell damaging agent, 0.1 or 0.5 mmol/l of sodium nitroprusside (SNP), a nitric oxide donor, was used to treat MIN6N8a cells. Relative cell viability was calculated as the optical density of each treatment as a percentage of the optical density of control MTT-treated MIN6N8a cells. Data are representative of three independent experiments and are expressed as mean ± SD.]

![FIG. 7. Transcriptional activation of the GAD67 gene by dexamethasone. The luciferase gene containing the GAD67 promoter (pGL2-GAD67P) was cotransfected with pSV-β-galactosidase into MIN6N8a cells. Thirty-six hours after transfection, the cells were stimulated with (Dex [+]) or without (Dex [−]) 100 nmol/l dexamethasone for 9 h. The cells were lysed, and the luciferase activity was determined with a Promega luciferase assay kit. The transfection efficiency was monitored by β-galactosidase activity. The luciferase activity was calculated as a percentage of the activity for control pGL2 transfection in the absence of dexamethasone. Each bar gives the mean ± SD from three experiments.]

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immunity, leading to type 1 diabetes. We first examined the effects of sex steroids and a glucocorticoid on the expression of GAD67 mRNA in an NOD mouse pancreas. We chose to examine GAD67 mRNA, rather than GAD65 mRNA, as GAD67 is the major isoform produced only a twofold increase in GAD67 mRNA (22,41). We induced GAD67 expression by incubation with dexamethasone, removed the dexamethasone, and examined GAD67 expression at various times thereafter. We found that withdrawal of dexamethasone completely abolished GAD67 mRNA expression. In addition, RU486, a GR blocker, clearly antagonized the effect of dexamethasone on GAD67 expression. This result suggests that dexamethasone specifically regulates the expression of GAD67 mRNA. As glucocorticoids can induce cell differentiation or death, depending on the cell type (42–44), we examined whether the induction of GAD67 mRNA expression of dexamethasone-treated MIN6N8a cells might be a result of cell proliferation. We found no changes in cell division or viability as a result of dexamethasone incubation; therefore, the dexamethasone-induced increase of GAD67 mRNA is not a secondary result of cell division or death.

Second, we asked whether dexamethasone can also caused by dexamethasone, as exposure of MIN6N8a cells to glucose, glutamic acid, or to the cytotoxic cytokine interleukin-1 resulted in only a twofold increase in GAD67 mRNA (data not shown). Similarly, other studies found that exposure of MIN6N8a cells to nutrients or cytokines produced only a twofold increase in GAD67 mRNA (22,41).

To determine whether the increase of GAD67 mRNA expression in MIN6N8a cells was a specific effect of dexamethasone, we induced GAD67 expression by incubation with dexamethasone, removed the dexamethasone, and examined GAD67 expression at various times thereafter. We found that withdrawal of dexamethasone completely abolished GAD67 mRNA expression. In addition, RU486, a GR blocker, clearly antagonized the effect of dexamethasone on GAD67 expression. This result suggests that dexamethasone specifically regulates the expression of GAD67 mRNA. As glucocorticoids can induce cell differentiation or death, depending on the cell type (42–44), we examined whether the induction of GAD67 mRNA expression of dexamethasone-treated MIN6N8a cells might be a result of cell proliferation. We found no changes in cell division or viability as a result of dexamethasone incubation; therefore, the dexamethasone-induced increase of GAD67 mRNA is not a secondary result of cell division or death.

Second, we asked whether dexamethasone can also

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**TABLE 1**

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<th>Treatment</th>
<th>Insulitis grade (%)†</th>
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<tr>
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<tr>
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*Dexamethasone or PBS was injected into neonatal NOD mice (n = 5) as described in RESEARCH DESIGN AND METHODS. †At 8 weeks of age, male NOD mice were killed and the pancreas was removed. Sections of the pancreas were stained with hematoxylin and eosin, and the insulitis grade was determined as described in RESEARCH DESIGN AND METHODS. ‡P < 0.005 as compared with PBS-injected control by the Mann-Whitney U test. §P < 0.05 as compared with PBS-injected control by the Mann-Whitney U test.

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**TABLE 2**

<table>
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<th>Cumulative incidence of diabetes*</th>
<th>Males</th>
<th>Females</th>
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<tr>
<td></td>
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<tr>
<td>Age (weeks)</td>
<td>(%)</td>
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<tr>
<td>16</td>
<td>0/15 (0)</td>
<td>1/10 (10)</td>
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<td>30</td>
<td>3/15 (20)</td>
<td>4/10 (40)</td>
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* Dexamethasone or PBS (control) was injected into neonatal NOD mice (n = 9–15) as described in RESEARCH DESIGN AND METHODS. The cumulative incidence of diabetes was measured up to 30 weeks of age. †P < 0.01 by Wilcoxon signed-rank test.
induce the expression of GAD67 mRNA in pancreatic \( \beta \)-cells in vivo. We found that treatment of neonatal NOD mice (2–3 days old) with dexamethasone resulted in a significant increase of GAD67 expression, whereas treatment of adult NOD mice (5–6 weeks old) did not result in any significant change. The expression level of GAD67 was found to be highest at 5 weeks of age and gradually declined thereafter. Other studies showed that the expression level of GAD correlated with the maturation of the \( \beta \)-cells (45). As the rate of \( \beta \)-cell proliferation in adult mice is much lower than that in neonatal mice, our finding that the expression level of GAD67 in adult islets was not significantly changed by dexamethasone might be due to the already maximized level of GAD expression in adult islets. In humans, the rate of GAD expression is increased in pancreatic \( \beta \)-cells at the postnatal stage but is decreased in adulthood (46). Similarly, the expression of GAD in the islets of adult rats is probably at a maximal level and thus was used as a marker of \( \beta \)-cell maturity in rats (47). Taken together, the rate of GAD expression in \( \beta \)-cells seems to be correlated with age-dependent \( \beta \)-cell maturation.

Third, we asked how dexamethasone specifically regulates the expression of GAD67 mRNA. We examined the effect of dexamethasone on the transcriptional activity of the GAD67 promoter by a luciferase reporter assay and found that dexamethasone treatment of MIN6N8a cells transfected with the reporter gene construct increased the luciferase activity, indicating that dexamethasone activates the GAD67 promoter. It was reported that glucocorticoid showed an effect on cell differentiation or cell death by interaction between glucocorticoid and the GR. The glucocorticoid-GR complex in the cytoplasm translocates to the nucleus, binds to the glucocorticoid responsive element (GRE) of the promoter region of the glucocorticoid responsive gene, and regulates the transcription of the gene (48). However, the glucocorticoid response is not solely mediated by the interaction between GR and GRE. Interaction with coactivators such as cAMP-responsive element binding protein, which binds several other transcription factors and uncoils DNA wound around histone residues by its enzymatic action, seems to affect the transcription of the glucocorticoid responsive gene (48). The mouse GAD67 promoter has three transcription initiation sites (P1, P2, and P3), and the neuronal-restrictive silencer elements account for the neuron- or \( \beta \)-cell-specific expression of the GAD67 gene (34). However, a palindrome region of the glucocorticoid responsive element recognized by GR and GRE. Taken together, the rate of GAD expression in \( \beta \)-cells seems to be correlated with age-dependent \( \beta \)-cell maturation.

Finally, we asked whether dexamethasone-induced GAD expression in \( \beta \)-cells could affect the development of insulin and diabetes in NOD mice. We administered dexamethasone to neonatal NOD mice, rather than adult NOD mice, to induce GAD expression in \( \beta \)-cells, as the expression of GAD in adult \( \beta \)-cells was not significantly increased by dexamethasone treatment. We found that the development of insulin and diabetes was accelerated in dexamethasone-treated NOD mice as compared with PBS-treated control NOD mice. When we examined the presence of GAD-reactive T-cells in dexamethasone-treated NOD mice, we found that the GAD-reactive T-cell population arose earlier as compared with PBS-treated control mice. These GAD-reactive T-cells might, in part, play a role in the development of diabetes in NOD mice treated neonatally with dexamethasone. In contrast, our preliminary study showed that long-term treatment of adult female NOD mice (from 4 weeks of age) with dexamethasone resulted in delayed onset and decreased incidence of diabetes (H.-S.J., B.S., J.-W.Y., unpublished data). The differential effect of dexamethasone on the development of diabetes seems to be age dependent. Suppression of diabetes in dexamethasone-treated adult female NOD mice may be due to immunosuppression, as glucocorticoids are known to have immunosuppressive and anti-inflammatory effects and can promote the Th2 immune response (50). In contrast, the acceleration of diabetes in neonatal male NOD mice by dexamethasone treatment is probably due, in part, to the early activation of GAD-reactive T-cells by the induced expression of GAD, a major \( \beta \)-cell autoantigen. However, we cannot exclude the possibility that other immune effects of dexamethasone, such as induction of thymocytic apoptosis, interactions of dexamethasone with other factors such as cytokines and sex hormones, or induction of insulin resistance and inhibition of insulin secretion through glucocorticoid (51–53), may influence the development of diabetes.

Psychological stress was suspected to influence the expression of autoimmunity diabetes (23,24), as it is known to accelerate the disease (29). As well, severe psychological stress was found to be a risk factor for the development of diabetes in the subsequent year (54), and psychological stress in the first 2 years of life was associated with the onset of autoimmune diabetes in children (55). Glucocorticoids are known to be produced from the adrenal gland in response to stress. In our study, we found that glucocorticoids significantly increased GAD expression in NOD \( \beta \)-cells. GAD is a major \( \beta \)-cell autoantigen that is recognized by humoral and cellular components of the immune system in both humans and animal models of type 1 diabetes and is suggested to play an important role in the pathogenesis of the disease. Taken together, we suggest that an elevation of glucocorticoids by endogenous or exogenous stress in early life may result in the upregulation of the pancreatic \( \beta \)-cell autoantigen GAD, which may promote \( \beta \)-cell autoimmunity and lead to the development of autoimmune type 1 diabetes.

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