Pancreatic Regenerating Gene Overexpression in the Nonobese Diabetic Mouse During Active Diabetogenesis

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The reg gene has previously been shown to be associated with regeneration of pancreatic islets. Strategies for influencing the replication and the growth of the β-cell mass may be important for prevention and/or treatment of type I diabetes. In this study, we have examined the level of reg gene expression at various degrees of diabetogenesis in the pancreas of the NOD mouse (male, female, and cyclophosphamide-treated male) using both human reg cDNA as the probe and dot blot analysis. The expression of the reg gene was found to be significantly increased in female mice compared with male mice, and in both cases, the expression level was not influenced by age. Nondiabetic female mice have a significantly higher expression of the gene than diabetic female mice, and there was a positive correlation between the age of diabetes onset and the reg mRNA level. In addition, overexpression of the reg gene was found in male mice treated with cyclophosphamide, an agent known to be a potent inducer of diabetes in male NOD mice. None of these results were found in the diabetes-resistant control OF1 mice, in which pancreatic reg gene expression did not differ between female and male mice treated or untreated with cyclophosphamide. All of these data suggest that there is a strong correlation between reg gene expression in the pancreas of the NOD mouse and the likelihood of developing diabetes. Diabetes 45:67-70, 1996

Type I diabetes is caused by a specific autoimmune destruction of the insulin-secreting β-cells of the islets of Langerhans (1). Numerous studies have been undertaken to prevent and arrest type I diabetes using immunosuppressive drugs, but another interesting aspect of this undertaking, although still hypothetical, might be the induction of islet regeneration.

A pioneer study showed that the administration of poly(ADP-ribose) synthetase inhibitors such as nicotinamide in 90% depancreatized rats induced islet regeneration and improved glucose tolerance (2). While screening a regenerating islet-derived cDNA library, a new gene was discovered, designated reg (for regenerating); it was found to be expressed in regenerating islets but not in normal pancreatic islets, insulinomas, or the regenerating liver (3). The increase in the expression of this gene was temporally correlated with the increase in size of regenerating islets, suggesting possible roles for the reg gene in replication, growth, and maturation of islet β-cells. The presence of a gene homologous to the rat reg gene was demonstrated in humans (3).

The majority of studies concerning the expression of the reg gene and diabetogenesis have been performed on surgical and pharmacological models of diabetes. In type I diabetics and autoimmune models in rodents, β-cells are subjected to aggressive activity by autoreactive immune mechanisms, leading to an irreversible loss of insulin-secreting cells.

Nevertheless, it is uncertain whether some degree of β-cell replication occurs during these processes. This hypothesis has been substantiated by certain pathological observations made by Gepts (4). This author found rare hyperplastic islets of Langerhans in the pancreas of young diabetic patients who died shortly after clinical onset. In several cases, β-cell regeneration was represented by a proliferation of centroacinar cells and in other cases by an endocrine differentiation of ductal cells.

To elucidate the possible implication of a replication phenomenon in type I diabetes, we studied the expression of the reg gene in the pancreas of the NOD mouse, using dot blot analysis with the human reg cDNA as the probe, which hybridizes with the two mouse genes, reg I and reg II (5). The NOD model allowed the study of three groups of animals: the first group comprised female NOD mice that progressively developed type I diabetes between 100 and 200 days; the second included male NOD mice, which are protected; and the third group included male NOD mice treated with cyclophosphamide to induce an accelerated form of diabetes (6). Finally, we studied female mice with established diabetes. As a control, we studied reg gene expression in the pancreas of OF1 mice: female, saline-injected female, saline-injected male, and cyclophosphamide-treated male.

RESEARCH DESIGN AND METHODS

Animals and treatments. NOD mice were bred from a parental stock provided by Dr. C. Boitard (Department of Immunology, INSERM U25, Necker Hospital, Paris, France). In our colony, the prevalence of diabetes at day 210 was 43% in female and <1% in male mice. Glocosuria was detected using labsticks (Reodiastrx, Ames-Bayer Diagnostica, France). Cyclophosphamide (Endoxan, Lucien Laboratory, France), in aqueous solution and at a dose of 300 mg/kg body wt was injected...
subcutaneously into 100-day-old male NOD mice. We found that the percentage of overt diabetes in male NOD mice was 10% after one injection and 80% after a second injection. In our experiment, the pancreas was removed 10 days after the first injection while in an active phase of diabetogenesis. We studied 16 female, 24 male, 13 diabetic female, and 6 cyclophosphamide-treated male NOD mice.

Control mice (IOPS OF1 strain) were supplied by IFA Credo (France). Nineteen mice (15 weeks old) were studied: 9 female (including 4 receiving a saline injection) and 11 male receiving the same volume of either saline injection (n = 5) or cyclophosphamide injection (n = 6). As described for NOD mice, the pancreas was removed 10 days after the injection.

RNA extraction and Northern blot analysis. After removal of the pancreas, total RNA was extracted immediately according to the method of Chomczynski et al. (7) using RNAzol (Bioprobe, France) and quantified by absorbance at 260 nm (optical density = 40 µg/ml). To demonstrate nondegraded RNA, total RNA was analyzed by electrophoresis on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) according to the procedure described by Maniatis and associates (8). Filters were baked for 2 h at 80°C before hybridization. Filters were prehybridized in a solution containing 50% formamide, 5× sodium chloride–sodium citrate (0.75 mol/l NaCl, 0.075 mol/l sodium citrate, pH 7.0), 5× Denhardt's reagent, 50 mmol/l NaH2PO4/Na2HPO4, pH 7.0, 5 mmol/l EDTA, and 250 µg/ml denatured salmon sperm DNA for 5 h at 42°C. Hybridization was performed for 16 h at 42°C in the above solution containing 0.1% sodium dodecyl sulfate, using 1× Denhardt's reagent instead of 5× and containing the 5P-labeled cDNA probe labeled with [a-32P]dCTP (3000 Ci/mm, Amersham) using a nick translation kit (Gibco-BRL, France). The human reg cDNA was a 600-bp fragment cloned into pBluescript vector. After hybridization, filters were washed, and the results were visualized by autoradiography.

Quantification by dot blot analysis. Sequential dilutions of total RNA (5–0.156 µg) were spotted on nitrocellulose membranes using a manifold apparatus (Minifold I, Schleicher & Schuell). Quantification of reg mRNA was performed after hybridization with a 5P-labeled reg cDNA probe. For the hybridization with the 28 S cDNA probe (ATCC, Rockville, MD), we used a “00-fold lower amount of total RNA and, therefore, the sequential dilutions were from 50 to 1.56 ng of total pancreatic RNA. Filters were then exposed to X-ray films (Hyperfihn MR, Amersham). After autoradiography, the filter-bound radioactivity was determined by scanning autoradiograms at 400 nm using an optical densitometer (Dynatech MR 5000), and mRNA concentrations were estimated from the slopes of the linear regression curves of dot scannings. The regression coefficient, r, was always >0.9. The densitometrically scanned readings for reg were corrected to the 28 S signal to eliminate possible differences in total RNA loading. Results were calculated as the ratio reg/28 S and expressed in arbitrary units (AU).

Statistical analysis. Results were expressed as means ± SE; differences between male and female, diabetic and nondiabetic NOD female, and cyclophosphamide-treated and nontreated male mice were evaluated by Student’s unpaired t test.

RESULTS

Qualitative analysis of mRNA and specificity of the reg cDNA probe. A Northern blot analysis of electrophoretically separated total pancreatic RNA was performed to ensure the specific hybridization of the human reg cDNA probe and to confirm the size of the mRNA. As shown in Fig. 1, reg mRNA was identified as a single band at the expected size (0.9 kb).

Overexpression of the pancreatic reg gene in the female NOD mice. The patterns of reg gene expression during aging of nonobese diabetic female and male mice are presented in Fig. 2. Despite the fact that reg mRNA levels show a large variation of values, there was significantly more reg mRNA in female mice (7.45 ± 1.34 AU, n = 16) than in male mice (0.70 ± 0.14 AU, n = 24) (P < 0.001). There was no change of reg gene expression with age for either male or female mice. To check the specificity of this phenomenon, control OF1 mice were studied. In this diabetes-resistant strain, the pancreatic expression of the reg gene did not differ between female (injected or not with saline) and male (injected with saline) mice. The values were respectively 0.44 ± 0.08 AU for all female and 0.61 ± 0.11 AU for male mice. These values were low and were similar to those found in male NOD mice.

To determine whether the level of reg gene expression in female NOD mice can be modified with the onset of diabetes, we compared the level of reg mRNA of 13 diabetic female mice with the level found in the nondiabetic female mice. The mRNA level was significantly lower for the diabetic female mice (3.38 ± 0.67 AU, P < 0.001). Moreover, as shown in Fig. 3, there was a positive correlation (r = 0.70) between reg gene expression and the age of onset of diabetes (P < 0.001).

Cyclophosphamide induces overexpression of reg gene in the pancreas of male NOD mice. To confirm that overexpression of the reg gene in female NOD mice was associated with the active autoimmune process, we rendered female (injected or not with saline) and male (injected with saline) mice. The values were respectively 0.44 ± 0.08 AU for all female and 0.61 ± 0.11 AU for male mice. These values were low and were similar to those found in male NOD mice.
male NOD mice prone to diabetes using conventional cyclophosphamide treatment. At the time of killing, only one animal of six was overtly diabetic. The results are presented in Fig. 4. For the cyclophosphamide-treated male mice (n = 6), the *reg* mRNA level (4.02 ± 0.76 AU) was significantly higher than the *reg* mRNA level found in nontreated male mice (0.70 ± 0.14 AU, P < 0.001) and, thus, was similar to that found in diabetic female mice. In contrast, in OF1 male mice, the cyclophosphamide injection, which is not diabetogenic in this strain, did not induce any increase in pancreatic *reg* gene expression (0.65 ± 0.37 vs. 0.61 ± 0.11 AU). The difference between cyclophosphamide-injected male NOD mice and cyclophosphamide-injected male OF1 mice was highly significant (P < 0.001).

**DISCUSSION**

The NOD mouse is considered as a model of type I diabetes. The main clinical difference is the sex-linked predisposition to diabetes in this strain. Female mice are prone to develop diabetes early in life, whereas male mice are relatively protected. Our current data reveal for the first time an overexpression of a pancreatic gene in female NOD mice compared with that in male mice. This gene, named *reg* for regenerating, is normally expressed by acinar cells and was found in regenerating islets but not in normal islets. This effect is apparently specific for the diabetogenic strain since no difference was observed between male and female diabetes-resistant mice (OF1). The fact that we also observed an overexpression of the *reg* gene in male NOD mice treated with cyclophosphamide, which is known to be a potent inducer of immune processes leading to overt diabetes (6), reinforces the hypothesis that there is a strong correlation between *reg* gene expression and diabetic risk. Again, there is no difference between OF1 male mice treated or untreated with cyclophosphamide. This result is consistent with the hypothesis that the effect of the drug is dependent on its diabetogenic effect.

Our results are in agreement with recent data obtained with another animal model of autoimmune diabetes, the BB rat (9). In the diabetic-prone BB rat, there was a significant increase in *reg* mRNA levels at 90 days of age (which is close to the current mean age at onset of diabetes for the strain we studied) compared with values in diabetes-resistant BB rats at the same age. These results indicate, as we observed for NOD mice, an enhancement of *reg* gene transcription around the time of development of overt diabetes. In the overtly diabetic female NOD mice, we observed a significantly lower expression of the *reg* gene. In addition, we found a positive correlation between the *reg* mRNA level and the age of the mouse at the onset of diabetes, with a higher level of *reg* mRNA corresponding to a later appearance of the disease. Results suggest that certain female mice are relatively protected against diabetes by an overexpression of the *reg* gene and could explain the fact that some female mice escape diabetes despite the presence of insulitis and their ability to adoptively transfer diabetes. The large variation in levels of *reg* in individual female NOD mice compared with the values found in OF1 mice corroborates this hypothesis.

NOD mice did not exhibit a decrease of *reg* gene expression with age, contrary to what was found by Perfetti et al. (10) for C57BL/6J mice. This difference could be due to the fact that the authors used 30-month-old mice, whereas our study concerns NOD mice up to 8 months old. Another possibility is that a persisting stimulation due to various degrees of insulitis slows down the normal loss of *reg* gene expression in both male and female NOD mice. All of these data suggest a possible role of the *reg* gene in an islet cell-adaptive defense to a cell-mediated immune attack, but a role of the gene in the repair/regeneration of the islet may also be evoked. A transient correlation between an increase in the expression of this gene and the increase in size of regenerating islets has been previously demonstrated (3). In a later work, evidence of a close association between *reg* gene expression and islet cell replication in vitro was re-
ported (11), and recent data obtained with recombinant rat reg protein have shown that the protein stimulates the regeneration and/or growth of pancreatic β-cells, thereby ameliorating the diabetes in depancreatized rats (12). All of these findings suggest that the Reg protein may act on pancreatic β-cells as an autocrine growth factor. Since the protein is expressed in normal pancreatic acinar cells and is also shown to be overexpressed in acinar tissue during β-cell regeneration (11), an additional role of paracrine factor must be considered. The persistence of a relatively high level of reg gene expression in pancreas of overtly diabetic female NOD mice, which have a low residual β-cell mass, is in agreement with this hypothesis. This paracrine action may be necessary to maintain β-cell mass even in the normal pancreas. Our observation of an overexpression of reg gene during the active phase of diabeticogenesis in a mouse strain prone to autoimmune diabetes reveals a novel approach in the investigation of regeneration during the preclinical phase of the disease. Further studies on reg gene expression in pancreatic islets in the female NOD mouse, which corresponds closely to the human model, may help to resolve this issue.

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REFERENCES