Expression of a Novel Murine Type I IFN in the Pancreatic Islets Induces Diabetes in Mice

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Expression of a Novel Murine Type I IFN in the Pancreatic Islets Induces Diabetes in Mice

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Interferons are a family of cytokines with antiviral, antiproliferative, antitumor, and immunomodulatory activity (1–7). According to their structural and functional properties, IFNs are divided into two major classes: type I (acid-stable) and type II (acid-labile). Type I and type II IFNs have no molecular homology but share several biological activities. Type I IFNs include several subclasses: IFN-α, IFN-β, limitin (in mice), IFN-κ, IFN-ω (in humans), and IFN-τ (in sheep and cattle). In humans, there are at least 14 structural IFN-α genes encoding active proteins, and single IFN-β, IFN-ω, and IFN-κ genes. Similarly, in mice, there are multiple IFN-α genes (at least 12), and single IFN-β and limitin genes. Mice lack an IFN-ω gene but possess a novel subtype, limitin, that is absent in humans. IFN-κ represents a novel subclass of type I IFNs (9). The general structure of murine type I IFN genes is comparable to that of the corresponding human genes. All type I IFN genes other than IFN-κ lack introns. Murine IFN-α genes encode mature proteins of 166 or 167 aa and IFN-β protein consists of 161 residues. The murine IFN-α genes and the IFN-β gene are clustered on chromosome 4 (10) in a region syntenic with the human IFN cluster on chromosome 9 (11). All type I IFN genes function through a common heterodimeric receptor, IFN-αβ receptor (IFNAR), (12–14) although they bind the receptor in different orientations (15).

Recently, LaFleur et al. (9) reported the identification of a novel human cytokine belonging to the type I IFN family, IFN-κ, that displays ~30% homology to IFN-α, IFN-β, and IFN-ω. In this report, we describe the cloning and preliminary biological characterization of the murine homologue of this gene. We generated transgenic mice expressing murine IFN-κ in the pancreatic islets of Langerhans and observed that the transgenic mice develop hyperglycemia and inflammatory infiltrates in the islets of Langerhans. Interestingly, pancreatic expression of other murine type I IFN transgenes results in a similar diabetic phenotype (16–18). Our results suggest that murine IFN-κ is not only structurally, but also biologically, related to other IFNs.

Materials and Methods

Cloning of mouse IFN-κ gene

A basic local alignment search tool (BLAST) for DNA search of murine expressed sequence tags (ESTs) with the cDNA sequence of human IFN-κ (GenBank entry AF38048) identified a 175-bp 3’ EST from a mouse uterine library (GenBank entry AI155872). Using this sequence, we designed PCR primers and identified an 80-kb BAC clone from a 129/ola mouse library containing the gene (Incyte Genomics, Palo Alto, CA). A PCR-amplified fragment from the EST sequence was used as a hybridization probe, labeled with [32P]-dCTP (3000 Ci/mmol; Amersham, Piscataway, NJ) by random priming (Megaprime DNA Labeling System; Amersham), and two overlapping fragments (8-kb HindIII and 3-kb PstI) containing the mouse gene were identified by Southern blotting of restriction enzyme-digested BAC plasmid DNA. These fragments were subcloned into pBlueScript (Stratagene, La Jolla, CA). Sequence analysis of the two genomic clones revealed that the new member of the mouse IFN family consisted of two exons and one intron (see Fig. 1). The sequence information derived from the genomic clones was used to localize the transcription start site and a predicted open reading frame (ORF) of 600 nucleotides encoding a 200-aa protein. New PCR primers spanning the whole length of the ORF, GV452 5’-agtactcagaaatgggtctgt-3’ (forward), and GV453 5’-ctatttaagtttaaatggtc-3’ (reverse), were used to amplify the full-length cDNA from a mouse peritoneal macrophage cDNA library. This 600-bp ORF fragment was subcloned into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA) and sequenced using the PE Automated Sequencer ABI 377 (Applied Biosystems, Foster City, CA). Chromosomal localization was performed by Incyte Genomics.
Isolation of mouse peritoneal macrophage cultures and treatment with dsRNA and IFN-γ

Mice were injected i.p. with 1.5 ml of thioglycolate (Microbiology Systems, Cockeysville, MD). At 72 h postinjection, the contents of the peritoneum were lavaged by injection of 8 ml of PBS. Peritoneal macrophages (peritoneal exudate cells) were plated at a concentration of 2 × 10⁶/ml PBS and incubated for 1 h at 37°C. Nonadherent cells were removed by washing with PBS and the adherent macrophages were collected for RNA isolation. For the induction studies, the adherent macrophages were scraped off in PBS, resuspended at 2 × 10⁵/ml in RPMI 1640 with 10% FCS with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine, cultured for 24 and 72 h in the presence of 150 U/ml murine IFN (mIFN)-γ (R&D Systems, Minneapolis, MN) and 100 μg/ml poly I:C (Sigma-Aldrich, St. Louis, MO), and collected for RNA isolation.

Isolation of pancreatic islets

Islets of Langerhans were isolated as previously described (19). Briefly, the common bile duct was clamped distal to the pancreatic duct junction at its hepatic insertion. The proximal common bile duct was then cannulated using a 27-gauge needle, and the pancreas was infused by retrograde injection of 2 ml of ice-cold collagenase solution (1.0 mg/ml; Sigma-Aldrich) in HBSS (Invitrogen, Carlsbad, CA). Pancreatic tissue was recovered and subjected to a 20-min digestion at 37°C. Subsequently, ice-cold HBSS was added and the suspension was vortexed at full speed for 10 s. Released islets were filtered through a sterile 0.5-mm pore mesh. Islets in the digested pancreata were enriched by Percoll centrifugation (density 1.089/1.062). After a 10-min centrifugation at 800 × g the islets were recovered from the gradient interface and hand-picked under a dissection microscope.

Islets were cultured in RPMI 1640 supplemented with 10% FBS, 20 μM HEPES, 1% penicillin-streptomycin, 1% l-glutamine, and 2.5 ml 7.5% NaHCO₃ (all from Invitrogen) at 37°C, 5% CO₂. The viability of the islets in culture was examined by trypan blue dye exclusion.

Quantitative PCR analysis

RNAs from various tissues and immune cells were extracted using the TRI reagent RNA isolation kit from MRC (Cincinnati, OH) following specifications from the manufacturer. cDNAs were generated by reverse transcription using random hexamers (Promega, Madison, WI) and oligo-dT primers (Life Technologies, Gaithersburg, MD). Quantitative PCR analysis (TaqMan) was performed on an ABI 7700 (Applied Biosystems) sequence-detection instrument following manufacturer’s instructions. For TaqMan analysis, 20 ng of tissue cDNA or 100 ng of cell cDNA was used together with primers at a 0.9 μM final concentration, and a FAM-labeled diagnostic probe at a final concentration of 0.25 μM. Primer/probe sequences were designed using Primers Express software, version 1.5 (Applied Biosystems) as follows: TM339 5′-tcccccgggcctatttaagttcttgctt-3′ (forward), TM430 5′-ggatatgttttaaatgatttgccctccc-3′ (reverse), probe 5′-tcctgtcgatctggtgcacatggtaaa-3′. Ribosomal RNA primers/probe (Applied Biosystems) were used as an internal control. Quantitative PCR conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 2 min; 95°C for 15 s. Amplification of mouse tail DNA using recognition primers for the transgene was performed following the guidelines of the Schering-Plough Animal Care and Use Committee.

Western analysis

One hundred and fifty islets each from RTIRI transgenic mice and wild-type littermates were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, 20 mM HEPES, 1% penicillin-streptomycin, 1% l-glutamine, and 2.5 ml 7.5% NaHCO₃ (all from Invitrogen) at 37°C, 5% CO₂. The next day, they were resuspended in 30 μl of 1 × loading buffer (Invitrogen), heated at 70°C for 15 min, and centrifuged at 19,000 × g for 5 min. Fifteen-microliter lysates from each genotype were loaded on 4–12% NuPage Bis-Tris gel (Invitrogen) with MES buffer under reduced conditions. After electrophoresis, samples were transferred onto a polyvinylidene difluoride membrane using a semi-dry blotting system (Bio-Rad, Hercules, CA) by discontinuous buffer system. Murine IFN-κ expression in transgenic islets was detected with rabbit anti-mIFN-κ peptide Ab (Cambridge Research Biochemicals, Cambridge, U.K.) using a BM Chemiluminescence Blotting Kit (Roche Molecular Biochemicals, Indianapolis, IN).

Histology

Pancreatic tissues for histology were either fresh-frozen for cryosection, or fixed in phosphate-buffered formalin, processed, and stained with H&E. For immunohistochemical staining, paraffin sections were used for anti-insulin and fresh-frozen sections for the rest of the staining. Briefly, paraffin sections were deparaffinized, rehydrated, and then stained following the same procedure as the frozen sections. Fresh-frozen sections were fixed with ice-cold acetone and air-dried. The sections were then stained by incubation with primary Ab for 1 h at room temperature followed by incubation with appropriate biotinylated secondary Ab for 30 min. After incubation with an avidin-biotin-HP3 complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 30 min, the tissue sections were stained with NovaRed (Vector Laboratories) and counterstained with hematoxylin. The Abs used were anti-insulin (DAKO, Carpinteria, CA), anti-CD45, anti-Mac1, and anti-TCR (BD Pharmingen, San Diego, CA).

Hormone and metabolite assays

Blood glucose levels were measured by using a Glucometer analyzer (Bayer, Wuppertal, Germany) following specifications from the manufacturer. Serum insulin levels were measured using a Linco’s Rat Insulin RIA kit (Anlytics, Gaithersburg, MD). Serum triglyceride levels were determined using a Triglycerides/GPO kit (Roche Molecular Biochemicals).

Results

Identification of the mouse homologue of the human IFN-κ gene

The mouse IFN-κ gene was cloned as described in Materials and Methods. Sequence analysis of the mouse genomic clones revealed that the gene consisted of two exons and one intron (Fig. 1). The sequence information derived from the genomic clones was used to locate the transcription start site and a predicted ORF of 600 nucleotides encoding a 200-aa protein. Unlike human and rat homologues, a portion of the C terminus of mouse IFN-κ protein is coded on the second exon of the gene. New PCR primers spanning the whole length of the ORF were used to amplify the full-length cDNA from a mouse peritoneal macrophage cDNA library (Fig. 1).

Murine IFN-κ gene represents a novel subclass of type I IFNs

Pairwise BLAST analysis revealed that murine IFN-κ gene is 38.5% homologous to the recently cloned human IFN-κ gene (Ref. 9; Fig. 2A). The rat homologue was identified by conducting a BLAST for DNA searching with the mouse cDNA sequence against rat whole genome shotgun traces available at the GenBank database. Portions of the rat IFN-κ gene are contained in GenBank.
FIGURE 1. Genomic structure of the mouse IFN-κ gene. Sequence of a 2480 nucleotide genomic DNA fragment incorporating the IFN-κ gene (GenBank Accession number AF547990). The ORF of 600 nucleotides is interrupted by a single intron of 1145 nucleotides. The positions of the putative TATA box (nucleotides 407–411) and the polyadenylation signal (nucleotides 2405–2410) are underlined. Potential IFN regulatory factor promoter binding regions conforming to the GAAANN consensus sequence are double-underlined (41).

Trace entries 19866922438882/ti82332253, TUWer1E52877A/ti65859934, 19866919893112/ti69695837, and 19866915599319/ti63588204. The rat gene is 75.4% identical to the murine gene (Fig. 2). The leader sequence of human, mouse, and rat IFN-\(\beta\) are of identical length (21 residues) as indicated by the PSORT program, and the mature N terminus was confirmed by cloning, expression, and Edman sequence analysis for human and mouse IFN-\(\beta\) (data not shown). All mature species forms of IFN-\(\beta\) contain five cysteines (see Fig. 2). Mouse IFN-\(\alpha\) displays homology to the other two subclasses of type I mouse IFN genes, 31.2% to IFN-\(\alpha_2\) and 25.3% to IFN-\(\beta\). The first four cysteines would be expected to have a 1–3, 2–4 pairing as seen in IFN-\(\alpha\) genes (25). Comparison to the three-dimensional structures of murine IFN-\(\beta\) (26) and human IFN-\(\alpha_2\) (27) suggests the fifth cysteine would be buried in the helix bundle core.

The murine IFN-\(\kappa\) gene was localized to chromosome 4 (4A5), in an area where other type I murine IFN genes are clustered (28). This region is syntenic with human chromosome 9p21 which contains human IFN-\(\kappa\) and a type I IFN gene cluster (9, 11). The analysis of the entire 80-kb genomic DNA fragment from the BAC clone upstream of the IFN-\(\kappa\) did not reveal the presence of other IFN-related genes. The gene was located 500 bp from the 3' end of the linearized BAC clone. We did not find an overlapping BAC clone and therefore were unable to examine the downstream region for adjacent genes.

IFN-\(\kappa\) is expressed in peritoneal macrophages and induced by dsRNA and IFN-\(\gamma\)

The expression pattern of murine IFN-\(\kappa\) was analyzed by quantitative PCR using total RNA isolated from 24 tissues and 9 immune cell subsets (Fig. 4). Low level of expression (0.06–0.16 fg per 20 ng cDNA) was found in the uterus, Peyer’s patch, ovary and liver, and in peritoneal macrophages (0.8 fg per 100 ng of cDNA). All other tissues and cells did not show detectable expression of the gene (below 0.03 fg of cDNA per 20 or 100 ng of cDNA). To examine whether IFN-\(\kappa\) is up-regulated by IFN-\(\gamma\) and dsRNA, we incubated mouse peritoneal macrophages with 150 U/ml IFN-\(\gamma\) and 100 \(\mu\)g/ml poly I:C for 24 and 72 h and determined the IFN-\(\kappa\) mRNA level by TaqMan. Similar to human IFN-\(\kappa\) (9), the expression of the mouse homologue was increased 2- to 3-fold at 24 h and 5- to 10-fold at 72 h (Fig. 4).

Transgenic mice overexpressing IFN-\(\kappa\) in the pancreas develop insulin-dependent (type 1) diabetes

It has been observed that mice expressing IFNs (IFN-\(\alpha\), IFN-\(\beta\), and IFN-\(\gamma\)) in the \(\beta\) cells of the pancreas develop diabetes (16–18). To get an insight into the functional homology between IFN-\(\kappa\) and other IFN subclasses, we generated transgenic mice expressing IFN-\(\kappa\) in the \(\beta\) cells of the pancreas. To this end, we used the rat insulin promoter (RIP) that targets expression of transgenes to the insulin-producing \(\beta\) cells of the islets of Langerhans in the pancreas (Fig. 5A). A total of five transgenic founders were generated.

FIGURE 3. Alignment of mouse IFN type I IFNs. A. Amino acid sequence comparison between mouse IFN-\(\alpha_2\), limitin, IFN-\(\kappa\), and IFN-\(\beta\) (GenBank Entries P01573, BAA83749, AF547990, and NP_034640, respectively). Secondary structure assignments taken from the crystal structure of mouse IFN-\(\beta\) (26). B. Percent identities of mouse type I IFNs.

FIGURE 4. Expression profile of mouse IFN-\(\kappa\). Total RNAs isolated from different tissues and cell populations derived from B6D2 F1 mice were PCR amplified using gene-specific primers as described in Materials and Methods. The amount of total cellular RNA was calculated and normalized to the 18s ribosomal RNA used as an internal standard. A. Mouse tissue panel. B. Mouse immune cells and fibroblasts. C. Effects of mIFN-\(\gamma\) and poly I:C on IFN-\(\kappa\) expression. BM, bone marrow; PLN, peripheral lymph node; PP, Peyer’s patch; DC, dendritic cell.
as determined by PCR genotyping. These transgenic mice are referred to as RTRI mice. The mice developed normally and did not show any visible pathological abnormalities.

To evaluate the expression of the transgene, we isolated pancreatic islets of F1-transgenic mice and quantified IFN-κ/H9260 message by TaqMan analysis. Mice from all four transgenic lines analyzed showed high level of expression of the transgene (Fig. 5B). No detectable expression was found in islets from a wild-type control mouse. Two independent transgenic lines (nos. 53 and 57) were propagated for further analysis. To investigate whether expression of the transgene was restricted to the pancreas, we quantitated expression of IFN-κ/H9260 in several tissues (heart, liver, lung, mesenteric lymph node (MLN), ovary, spleen, thymus, and uterus). Levels of IFN-κ in these tissues were comparable between wild-type and transgenic mice, suggesting that transgene expression was restricted to the pancreas (Fig. 5C).

To confirm the expression of IFN-κ at the protein level, we analyzed cellular lysates from pancreatic islets from line no. 57 by Western blotting. A single intense band corresponding to the mature murine IFN-κ protein (expected size ~21 kDa) was observed in the transgenic, but not in the wild-type, sample (Fig. 5D).

RTRI transgenic mice from line nos. 53 and 57 and their wild-type littermates were followed for 20 wk. The mice from these two lines appeared normal and did not show any measurable deviations from their wild-type littermates. However, their blood glucose exceeded 200 mg/dL, which is typical for diabetes. RTRI transgenic mice from both lines developed hyperglycemia with >60% of them showing blood glucose level above 200 mg/dL by 14 wk of age (Fig. 6A). Higher than normal blood glucose levels were observed as early as 4 wk of age. Mice from higher expressing line no. 57 showed a higher frequency of diabetes than lower expressing line no. 53. At the same time, the blood glucose level of the wild-type littermates did not exceed 200 mg/dL. Transgenic mice from line no. 57 displayed nearly 2-fold lower serum concentration of insulin than the control wild-type littermates (Fig. 6B), and 3-fold higher levels of serum triglycerides (Fig. 6C). The alterations in the above serum parameters suggested that transgenic mice expressing IFN-κ in pancreas had spontaneously developed diabetes.

Histological analysis of the transgenic pancreata revealed different degrees of islet destruction with inflammatory infiltration around the islets (Fig. 7, A and B). Immunohistochemical analysis showed clearly reduced numbers of insulin-producing cells (Fig. 7, C and D). To further characterize the nature of the infiltrating cells,
we performed immunohistochemical analysis of the islets. As shown on Fig. 8, the majority of the cells around the islets were positive for leukocyte marker CD45. Part of them were found to be T cells because they reacted with the TCRβ Ab, and part of them stained for Mac-1, a marker for macrophages and polymorphonuclear cells.
Discussion
IFN-κ is a novel mouse gene with 38.5% homology to the newly discovered human IFN-κ. Several lines of evidence suggest that it is the mouse homologue of the human IFN-κ subclass. First, it displays a strong family relationship among its rat and human counterparts (see Fig. 2). Second, in addition to the four conserved cysteine pairs for all members of the IFN family, it contains a fifth cysteine, which is conserved between the mouse, the rat, and the human IFN-κ. Third, both human and mouse IFN-κ genes encode proteins of ~200 aa, different from the rest of the IFN subclasses both in humans and mice. IFN-κ has a longer loop between helices C and D than that seen for IFN-α genes; this is especially evident in the human and rat forms (see Figs. 2 and 3).

In addition, IFN-κ displays some of the key functional properties of the IFN family. Like all known IFNs, it is expressed at a very low or undetectable level in all examined mouse tissues. It can be induced by dsRNA, viral infection, IFN-β, and IFN-γ (9). Also, human IFN-κ uses type I homodimeric IFNAR1/IFNAR2 as its actions are blocked by anti-receptor Abs (9).

The finding that transgenic mice overexpressing IFN-γ (17), IFN-α (16), and IFN-β (18) in the β cells of the pancreas can develop diabetes, and the discovery of elevated IFN-α expression in diabetic lesions (29, 30) led to the speculation that IFN may be involved in the etiology of diabetes. As part of the functional characterization of the putative new mouse IFN, we generated transgenic mice overexpressing the gene in the pancreas. Targeted overexpression of mouse IFN-κ in the β cells of the pancreatic islets resulted in hyperglycemia, due to an altered insulin production. Histological analysis of the pancreatic islets showed the presence of inflammatory cells around the islets and destruction of the β cells. This phenomenon was observed with differing penetrance in both transgenic lines studied, suggesting that the disease observed was not caused by mutations induced by integration of the transgene. It is likely that expression of IFN-κ in islets induces secondary events that favor recruitment and or activation of macrophages and T cells and that these cells are responsible for islet destruction. A substantial body of evidence implicates both macrophages and T cells in the pathogenesis of diabetes and in islet dysfunction, apoptosis, and islet graft loss that is observed after transplantation (31–34). Macrophages are the first cells that infiltrate the islets in animal models of diabetes (streptozotocin-treated mice, BB rats, and nonobese diabetic (NOD) mice) and transplantation (35), and are highly toxic to islet cells in vitro. Inactivation of macrophage function in the NOD mice and BB rats results in the near prevention of insulitis and diabetes (32). Similarly, T cells are critically important (reviewed in Adorini et al. (31)). Apathic, as well as T cell-depleted, BB and NOD mice, do not develop diabetes. Further, adoptive transfer of T cells reacting to islet autoantigens actively promotes disease in animal models. Finally, agents targeting T cells or inducing immune tolerance prevent diabetes in animal models, while immunosuppressive drugs increase the remission rate of diabetes in newly diagnosed patients. In summary, inflammatory and autoimmune mechanisms participate in the etiology of diabetes, and it is likely that IFNs contribute to it (36). At this point it is unclear how IFN-κ production by the islets could influence recruitment of both macrophages and T cells and initiation of the disease. The disease described in this study may be different from the autoimmune disease described in humans. Type I diabetes is an autoimmune disease characterized by the selective destruction of pancreatic β cells by autoreactive T lymphocytes leading to decreased production of insulin (37, 38). Although we cannot rule out at this stage that IFN-κ leads to generation of autoreactive T cells, we suggest that the main mechanism of diabetes induction in the mice reported in this study may be the destruction of β cells by macrophages. Activation of bystander and infiltrating macrophages by IFN-κ produced by the transgenic islets could lead to production of cytokines, chemokines, and other agents, such as nitric oxide, which could increase the cellular infiltrates and lead to islet destruction (39, 40).

Taken together, our results suggest that IFN-κ is a novel member of the IFN family sharing not only structural but also biological properties with the other family members. Our current studies are aimed at defining how IFN-κ induces development of diabetes in mice and at determining if it has a role in the development of type I diabetes in humans.

Acknowledgments

References


