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Increased Amounts of a High Molecular Weight Insulin-Like Growth Factor II (IGF-II) Peptide and IGF-II Messenger Ribonucleic Acid in Pancreatic Islets of Diabetic Goto-Kakizaki Rats*

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ABSTRACT

Insulin-like growth factor II (IGF-II), a member of the insulin family, regulates cell growth and differentiation. The IGF-II gene is localized close to the insulin gene in man and rat. IGF-II peptide binds weakly to the insulin receptor and exerts insulin-like effects on the blood glucose level. We studied IGF-II in endocrine pancreas in an animal model of noninsulin-dependent diabetes mellitus, the Goto-Kakizaki (GK) rat. At the age of 2 months, these rats have structural islet changes, with fibrosis and irregular configuration, so-called starfish-shaped islets. Immunohistochemical investigation revealed IGF-II immunoreactivity in the β-cells in both GK and control rats. Pancreatic extraction, followed by size separation using gel chromatography, disclosed a high mol wt form of IGF-II in all animals, and RIA measurements revealed a considerably larger amount of the IGF-II peptide in the 2- and 6-month-old GK rats than in the 1-month GK and control rats. *In situ* hybridization of 3-month-old GK rats showed increased IGF-II messenger RNA expression in the starfish-shaped islets of GK rats than in the islets with normal structure in both diabetic and control animals.

The reason for the increased amount of IGF-II is unclear. As the animals are diabetic before the islet changes occur, it might be a compensatory effect in response to hyperglycemia, but could also be a cause of the islet fibrosis. (*Endocrinology* **137:** 2415–2423, 1996)

THE INSULIN-LIKE growth factors (IGFs) are important regulators of cell growth, differentiation, and metabolism (1, 2). They belong to the insulin family; there are four structurally homologous members, *viz.* proinsulin/insulin, relaxin, IGF-I and IGF-II (2, 3). The IGFs are synthesized as proforms, with a further carboxy-terminal extension, the E peptide (1, 4). IGF-II occurs in several variants, and there is no consensus with regard to nomenclature (5–9). We have concentrated our studies on IGF-II and used the simplified term high mol wt (HMW) IGF-II (10).

The IGF-II gene is located on chromosome 11 in the human (1, 11) and on chromosome 1 in the rat (12), close to the insulin genes in both species. This proximity has raised the question of a possible coregulation of insulin and IGF-II expression in the pancreatic β-cells (13). Furthermore, we recently described a HMW IGF-II immunoreactive peptide in extracts from normal adult human and rat pancreases (10) and localized this peptide to the insulin-producing β-cells by using immunohistochemical (IHC) techniques. Our results have been confirmed by Maake and Reinecke (14). A natural subsequent step was then to study the expression of this peptide in a state of impaired function of the pancreatic β-cell. Noninsulin-dependent diabetes mellitus (NIDDM) is characterized by an impaired insulin response to glucose stimulation, but with a generally preserved β-cell content (15). In studies of diabetes, it is difficult to obtain well preserved pancreatic tissue from diabetic patients. Hence, there is a need for representative animal models of diabetes mellitus.

The Goto-Kakizaki (GK) rat is an animal model of NIDDM in which the diabetic state develops spontaneously. The model originated in 1972 through the selective inbreeding of normal Wistar rats with a serum glucose concentration in the upper normal range, as demonstrated by an oral glucose tolerance test (16–18). Even 8-day-old animals exhibit a significantly elevated blood glucose level (19), and in this model, the glucose-induced insulin release is markedly impaired (20, 21). GK rats are lean, in contrast to the obese animals of most other NIDDM animal models. Structural islet changes in GK pancreases are said to appear at the age of 3 months (18), comprising the loss of islet capsule, the appearance of irregular starfish-shaped islets, and the development of fibrous strands within these (18, 22).

In the present study we examined the content of HMW IGF-II at three different ages in the GK rat to allow of observations both before and during the occurrence of struc-

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tural changes in the endocrine parenchyma of the pancreas. This was performed by evaluating quantitatively, by means of RIA, the amount of HMW IGF-II in pancreas from 1-, 2-, and 6-month-old GK rats and normal Wistar rats and by determining the cellular localization of HMW IGF-II by means of immunohistochemical techniques. Lastly, the IHC observations were supplemented by means of *in situ* investigations of the expression of IGF-II messenger RNA (mRNA).

Materials and Methods

Animals and ip glucose tolerance test

Six 1-month-old, six 2-month-old, and six 6-month-old male GK rats were used, together with age-matched nondiabetic Wistar rats. The GK rats were obtained from our own colony (19–21), whereas control rats were purchased from a commercial breeder (B&K Universal, Sollentuna, Sweden). All animals were fed *ad libitum* a standard pelleted diet (B&K Universal) and kept in a room with alternating 12-h periods of light and darkness.

The ip glucose tolerance test was performed on all rats after an overnight fast. Glucose (2 g/kg BW) was injected ip, and blood was sampled by tail snipping before and 15, 30, 60, and 120 min after the injection. Blood glucose was determined with the glucose oxidase technique, using test strips read for absorbance with a reflectance photometer (BM-test glycemia 1–44 and Reflolux-S, Boehringer Mannheim, Mannheim, Germany).

Before death, the animals received thiopental sodium ip (100 mg/kg BW), and the pancreas was removed and sliced transversely into eight pieces. Alternate pieces were frozen for extraction, and the others were fixed in 4% buffered formaldehyde solution for 18–20 h and routinely processed to paraffin.

In cross-reactivity tests of the IGF-II antibody, 10 1-day-old and 2 3-month-old Wistar rats were anesthetized as described, and the sera were collected after clotting and centrifugation. *In situ* hybridization was performed on 3 GK rats and 3 age-matched control Wistar rats, 3 months old, as the starfish-shaped islets are well established in the GK rat at this age. The rats were anesthetized, and pancreas, liver, and muscle tissue samples were quickly frozen on dry ice.

All studies were conducted in accordance with the guidelines of the local ethics committee at the Karolinska Institute. Statistical results are expressed as the mean ± SEM, and significance of variance was assessed by Student's *t* test for unpaired data (Sigmastat for Windows, version 1, Jandel Scientific Software, Erkrath, Germany). $P < 0.05$ was considered statistically significant.

Histopathological and histochemical techniques

For routine histopathological examination, the sections were stained with hematoxylin and eosin and by van Gieson's method to visualize the presence of connective tissue. Congo red staining was used to identify amyloid. For IHC investigations, two monoclonal antibodies against IGF-II were used, one from KabiGen (Stockholm, Sweden; clone 128, mouse IgG; diluted to 2 μg/ml), and the other from Serotec (Oxford, UK; clone W2H1, MCA 830, mouse IgG; dilution, 1:70). The former antibody, useful for both IHC and RIA investigations (10), was raised against human recombinant IGF-II (4) conjugated to ovalbumin with glutaraldehyde. To confirm the specificity of this IGF-II antibody, an absorption test was carried out by overnight incubation at 4–8 C with 0.5, 2, and 10 nmol antigen/ml diluted antibody. The amount of antigen was equivalent to 4–70 μg IGF-II (10). Two insulin antibodies were used, one monoclonal (Biogenex, San Ramon, CA; clone AE9D6, mouse IgG; dilution, 1:1000) and the other polyclonal (a gift from Å. Lernmark, Department of Molecular Medicine, Karolinska Hospital, code 844230, guinea pig; dilution, 1:2000) (10). Other antibodies used were directed against somatostatin (Dakopatts, Glostrup, Denmark; A566, rabbit IgG; dilution, 1:800), glucagon (a gift from M. Ziegler, Zentralinstitut für Diabetes, Karlsburg, Germany; K79bB10, mouse IgG; dilution, 1:3000), and pancreatic polypeptide (Dakopatts; A619, rabbit IgG; dilution, 1:1800). Sections, 4 μm thick, were incubated with primary antibodies for 18–20 h at 4–8 C using the avidin-biotin-peroxidase complex method and following the manufacturer's instructions (Vectastain, Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as chromogen, and Mayer's hematoxylin was used for counterstaining. The distribution pattern of insulin and IGF-II immunoreactive cells was studied in consecutive sections.

Electron microscopy

Pancreatic tissue samples (~1 × 1 × 1 mm) were fixed in 4% glutaraldehyde in PBS buffer (pH 7.3), postfixed in 1% Dalton's chrome-osmium fixative, dehydrated in increasing concentrations of ethanol, immersed in acetone, and embedded in an Araldite plastic (Durcupan, Fluka, Buchs, Switzerland). Semithin sections were cut and stained with toluidine blue, and areas with typical starfish-shaped islets were further investigated at the ultrastructural level. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a JEOL 1200EX electron microscope (JEOL, Peabody, MA) with 80–100 kV accelerating voltage.

Extraction technique, chromatographic methods, and RIA

The pancreatic tissue from each group of rats was homogenized using a Polytron high speed metal homogenizer (Brinkmann Instruments, Westbury, NY) in 5 vol 50 mM Tris buffer (pH 7.7) containing 0.5 mM phenylmethylsulfonylfluoride to inhibit proteolysis. The supernatant obtained after centrifugation at 50,000 × *g* for 15 min (4 C) was dialyzed three times with a Spectrapor 6 dialysis membrane (Spectrum Medical Industries, Houston, TX) (M_r cut-off, 1000) for 48 h against 10 vol 0.1 M acetic acid, concentrated by rotary evaporation, and separated on a Sephadex G-50 fine (Pharmacia Biotech, Uppsala, Sweden) column (1 [*tims*] 100 cm), equilibrated with 0.1 M acetic acid (10, 23). Markers for calibration were blue dextran, [^{125}I]IGF-II, [^{125}I]IGF-IIE$_{1-21}$, and cobalt chloride. The column flow rate was 4 ml/h, and 1-ml fractions were collected, freeze-dried, and resuspended in 750 μl 0.01 M PBS buffer with 1% BSA.

The IGF-II antibody (diluted to 0.15 ng/ml) was used as matrix, and ^{125}I-labeled IGF-II was used as a ligand in a RIA for IGF-II (RIA-IGF-II). The ligand was radioiodinated by the lactoperoxidase method. After overnight incubation at 4 C, bound and free ligands were separated by centrifugation after incubation with solid phase secondary antibody-coated cellulose suspension (Sac-Cel, Wellcome Diagnostics, Washington, UK). The peptides used for cross-reactivity studies on the IGF-II antibody (KabiGen) were human recombinant IGF-I, IGF-II, IGF-IIE$_{1-21}$ (4) (KabiGen, Stockholm), human semisynthetic insulin (Novo, Bagsvaed, Denmark), and human recombinant proinsulin (Sigma Chemical Co., St. Louis, MO). In addition, 6 of the collected fractions of pancreatic extracts with HMW IGF-II reactivity from 6-month-old GK rats were pooled and measured for the ability to react with the antibody. Cross-reactivity of the human IGF-II antibody to rat IGF-II was tested by measuring serum samples from 10 1-day-old and 2 3-month-old Wistar rats. To measure IGF-I in extracts, a polyclonal (rabbit) IGF-I antibody (dilution, 1:60,000) was used, raised against recombinant IGF-I coupled to protein A (K. Hall, Department of Molecular Medicine, Karolinska Hospital, Stockholm, Sweden; code K519). The IGF-I RIA was set up in the same way as that for IGF-II. This antibody was not particularly useful for IHC investigations in the formalin-fixed and paraffin-embedded tissue samples. The RIA for detecting the position of insulin in the fractions was set up using an antibody raised in guinea pig against porcine insulin, and the cross-reactivity to proinsulin was 100%.

Ligand blot analysis of IGF-binding proteins (IGFBPs)

To ascertain whether any IGFBPs remained in the pancreatic tissue after extraction, an ordinary ligand blot analysis with size separation of the IGFBPs was performed. No IGFBP activity was identified, but to be even more certain, a more sensitive dot blot technique without separation was used as well. Material for analysis of the IGFBP content was sampled from different steps in the extraction procedure: 1) extracted, noncentrifuged tissue; 2) the corresponding supernatant; and 3) the supernatant obtained after 2 days of dialysis against acetic acid, followed

by centrifugation. Purified IGFBP-1, the samples, and serum from the animals were dot blotted on a membrane filter (BA85, Schleicher and Schuell, Dassel, Germany), dried, blocked overnight at 4 C in Tris buffer with 3% BSA, and then washed with the blocking solution and 0.01% Tween-20. The membrane was incubated with the same [^{125}I]IGF-II as that used for RIA measurements (400,000 cpm) overnight at 4 C and washed in blocking solution followed by PBS buffer, dried, and exposed on a photographic film for 24 h.

Preparation of probes and in situ hybridization

Oligonucleotide probes with sequences complementary to mRNAs encoding rat IGF-II (nucleotides 18–61) (12), rat IGFBP-2 (nucleotides 609–654) (24), rat IGFBP-4 (nucleotides 488–534) (25), and a mouse/human IGFBP-2 sense probe (nucleotides 692–736, exon 3) (26) were synthesized and purified through NAP-25 columns at Pharmacia Biotech. The oligonucleotides were labeled at the 3'-end with [α-^{35}S]deoxy-ATP (DuPont Medical Scandinavia, Sollentuna, Sweden), using terminal deoxynucleotidyl transferase (Amersham, Aylesbury, UK) in a buffer containing 10 mM cobalt chloride, 1 mM dithiothreitol (DTT), 300 mM Tris, and 1.4 M potassium cacodylate (pH 7.2). Labeled probes were purified with Nensorb-20 columns (DuPont Medical), and DTT was added to a final concentration of 10 mM. The specific activities obtained ranged between 10–40 $\times$ 10^8 cpm/μg oligonucleotide.

Sections (14 μm) were cut on a cryostat microtome (2800 Frigocut, Leica Nussloch, Germany) at −20 C and thaw-mounted onto microscope glass slides (Probe On, Fisher Scientific, Pittsburgh, PA). Consecutive sections were stained with hematoxylin and eosin to identify islets with normal and abnormal microscopical structures. The tissues were hybridized according to previously reported procedures (27). Briefly, sections were air-dried and then covered with a hybridization buffer containing 50% formamide, 4 $\times$ standard sodium saline (SSC) buffer, 1 $\times$ Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% BSA, and 0.02% Ficoll), 1% sarcosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulfate (Pharmacia Biotech), 500 μg/ml heat-denatured salmon sperm DNA (Sigma), 200 mM DTT (Pharmacia Biotech), and 1 $\times$ 10^7 cpm/ml of one of the labeled probes. The slides were placed in a box, humidified with 50% formamide and 4 $\times$ SSC buffer, and incubated for 16–18 h at 42 C. After hybridization, the sections were sequentially rinsed in four changes of 1 $\times$ SSC buffer at 55 C for 60 min and then dehydrated in 60% and 95% ethanol. After air drying, the sections were exposed to Hyperfilm beta-max x-ray film (Amersham) for 6 days, and then dipped in NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY). After exposure at −20 C for 6–8 weeks, slides were developed in Kodak D 19 for 3 min and fixed in Kodak 3000 A for 6 min. After rinsing, the sections were mounted in glycerol phosphate buffer. Sections were analyzed in a microscope equipped for darkfield illumination (Zeiss, Oberkochen, Germany), using TMX 100 black and white film (Kodak). Consecutive sections were stained with hematoxylin and eosin to identify islets with normal or changed microscopical structure.

Results

Animal body weight and ip glucose tolerance test

Although the GK and control rats were age matched, the GK rats weighed significantly less [94 ± 2 g for 1-month-old GK *vs.* 142 ± 6 g for controls ($P < 0.002$), 243 ± 5 g for 2-month-old GK *vs.* 297 ± 7 g for controls ($P < 0.0001$), and 375 ± 11 g for 6-month-old GK *vs.* 410 ± 9 g for controls ($P < 0.03$)]. Compared with control rats, the GK rats in all age groups demonstrated markedly decreased glucose tolerance (Fig. 1) and their fasting blood glucose values were significantly higher ($P < 0.001$).

Histopathological and histochemical observations

Tissue samples from GK and controls rats were without signs of inflammatory changes. The monoclonal IGF-II antibodies from KabiGen gave results easily evaluated with strong immunoreactivity (IR) and low nonspecific binding. The other IGF-II antibody (from Serotec) gave similar quantitative results, but produced a low specific staining combined with a high nonspecific background. The specificity of the IGF-II antibody from KabiGen was tested in GK and normal rat pancreases by means of conventional absorption tests in which the IR was completely abolished.

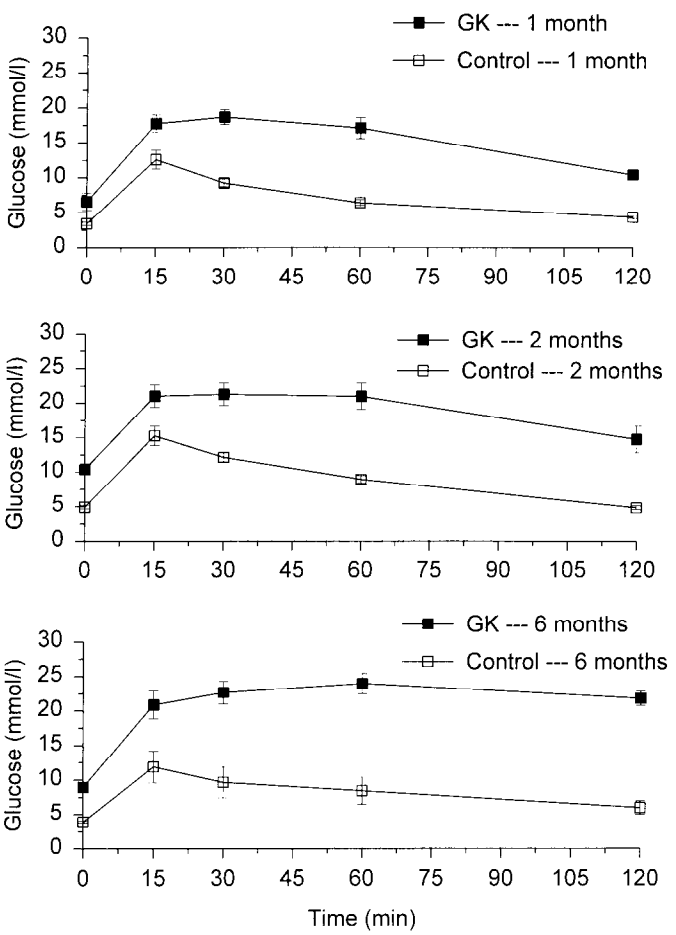

FIG. 1. Blood glucose levels in GK rats (n = 6) and control rats (n = 6) at 1, 2, and 6 months of age. In the ip glucose tolerance test, GK rats demonstrated a consistently diabetic glucose tolerance curve in all age groups compared with controls ($P < 0.001$).

GK rats

In the pancreases from the 1-month-old group, the islets appeared normal. In the pancreatic glands of the 2-month-old rats, five of six rats contained starfish-shaped islets (18), and these constituted 22% of all islets. In the 6-month-old group, as many as 40% of the islets were of the starfish-shaped type (Fig. 2), almost all of large size. However, most of the remaining islets, usually the small ones, were of normal appearance, indistinguishable from those in the control rats. The starfish-shaped islets contained increased quantities of fibroblasts and connective tissue (Fig. 2). The results of the Congo red staining did not indicate the presence of any amyloid. In the starfish-shaped islets, the insulin cells did not show their typical central location; rather, they were more

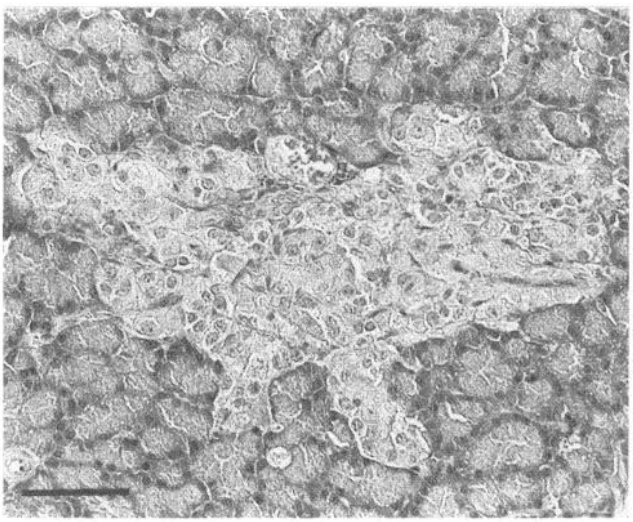

FIG. 2. Typical starfish-shaped islet with irregular shape, lacking a capsule but containing fibrotic strands. These islets occur in diabetic GK rats, 2 and 6 months of age. Hematoxylin and eosin stain; magnification, ×140; *bar* = 100 μm.

evenly distributed. Both insulin IR and IGF-II IR had a pronounced variability in staining intensity (Fig. 3). Most of the cells in the islets showed a granular type of IGF-II IR restricted to the cytoplasm. In consecutive sections, it was observed that the distribution patterns of the insulin and IGF-II immunoreactive cells were similar (Fig. 3).

Normal Wistar rats

In the control Wistar rats, the endocrine pancreas had a normal microscopical structure. The islets were spherical and surrounded by a thin capsule of connective tissue (Fig. 4). They all displayed the typical distribution of endocrine cells, with a majority of insulin cells centrally, glucagon cells at the

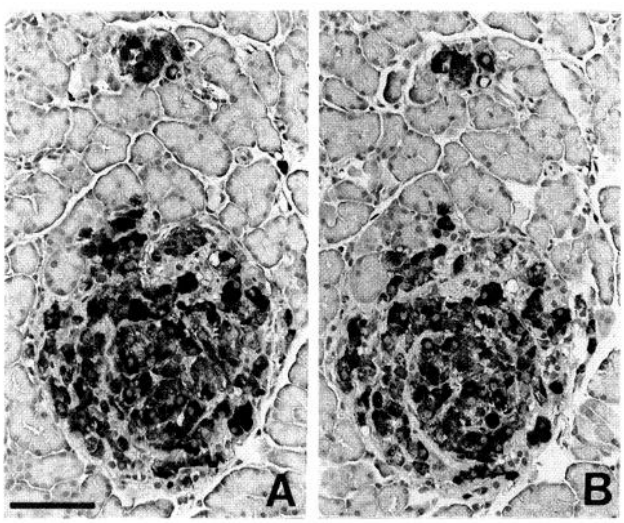

FIG. 3. Islet from a diabetic GK rat, aged 6 months, with increased connective tissue content. Immunohistochemical staining of consecutive sections shows a similar distribution of insulin (black cells in A) and IGF-II immunoreactive cells (black cells in B), indicating colocalization of IGF-II in the insulin-producing β-cells. Magnification, ×110; *bar* = 100 μm.

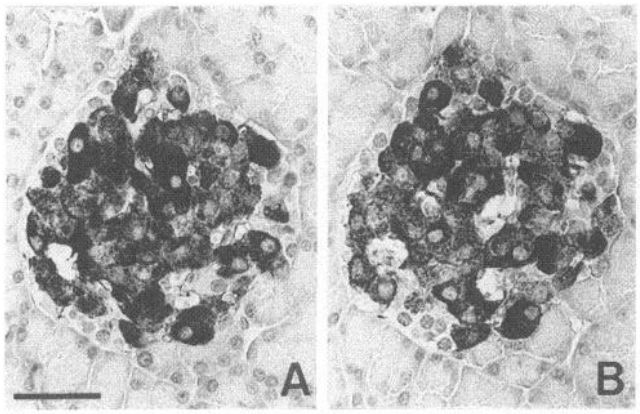

FIG. 4. Consecutive sections of a pancreatic islet from a normal rat. The islet was spherical and had a thin surrounding capsule of collagenous connective tissue. Immunoreactive insulin cells in A (black cells) agree well with the number and distribution of IGF-II immunoreactive cells in B (black cells). The peripheral nonimmunoreactive cells correspond to the glucagon and somatostatin cells. Magnificaiton, ×225; *bar* = 50 μm.

periphery, and somatostatin cells in between. Only a few pancreatic polypeptide cells were observed in the samples. As in the GK rats, IGF-II IR was localized in the cytoplasm only and was found in a majority of the islet cells. The intensity of the IR in the IGF-II cells varied more than that in the insulin immunoreactive cells. The immunostained consecutive paraffin sections showed insulin immunoreactive cells with the same distribution pattern as the IGF-II immunoreactive cells (Fig. 4). Endocrine cells at the periphery of the islets, where glucagon and somatostatin cells were usually located, remained unstained.

Ultrastructural observations

The electron microscopic examinations confirmed that the starfish-shaped islets of GK rats contained several fibroblasts and an abundance of collagen fibrils with typical cross-striations (Fig. 5). There were widely dispersed collagen fibrils within the islets, often separating the endocrine cells. These islets also lacked the normal capsule of connective tissue that separates them from surrounding exocrine tissue. No amyloid deposits were found either within or outside the islets. The secretory β-granules had a dense core with a peripheral halo typical of the insulin cells in rats. Moreover, several of the β-cells displayed pale secretory granules that had a tendency to fuse and become confluent (17).

Results of the extraction technique, the chromatographic methods, and the RIA

The cross-reactivity tests showed that the IGF-II antibody (KabiGen) had 1% of the reactivity to IGF-I in this system, whereas proinsulin and insulin failed to cross-react even at concentrations up to 1000 ng/ml. Increasing concentrations of the separated pancreatic extract reacting with the IGF-II antibody gave a displacement in parallel to that of IGF-II, indicating a similar or identical peptide (data not shown). Our IGF-II RIA measurements of rat serum confirmed known findings (28) that IGF-II levels are high in newborn

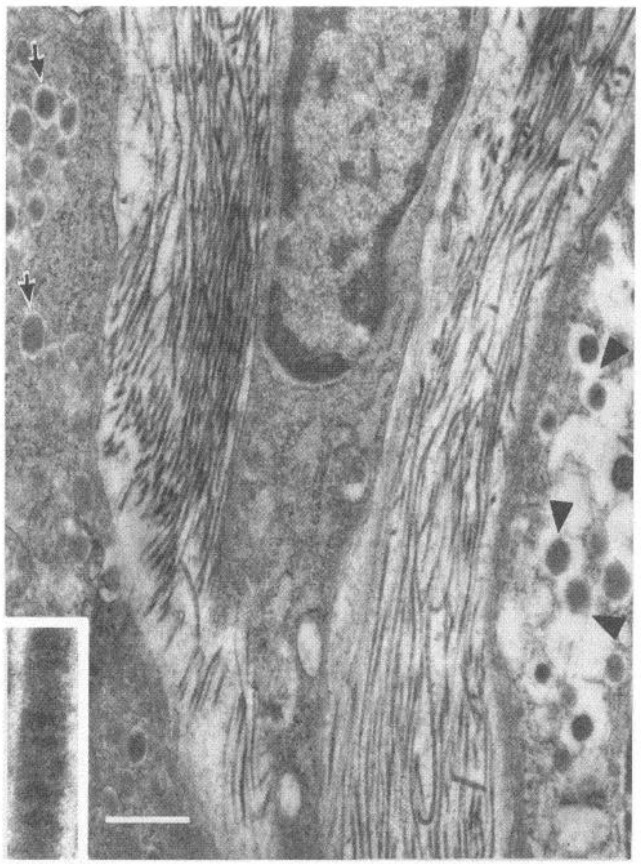

FIG. 5. Electron micrograph of parts of a few cells in a starfish-shaped islet from a 2-month-old GK rat. A fibroblast with elongated nucleus is seen centrally with an abundance of collagen fibrils with typical cross-striations (*inset*), separating two insulin cells from each other. The insulin cell to the *left* has secretory β-granules typical of those in rats (*arrow*). The one to the *right* shows pale fused atypical granules (*arrowhead*), indicating disturbed insulin secretion. Magnification, ×11,000; *bar* = 1 μm; *inset* magnifcation, ×170,000.

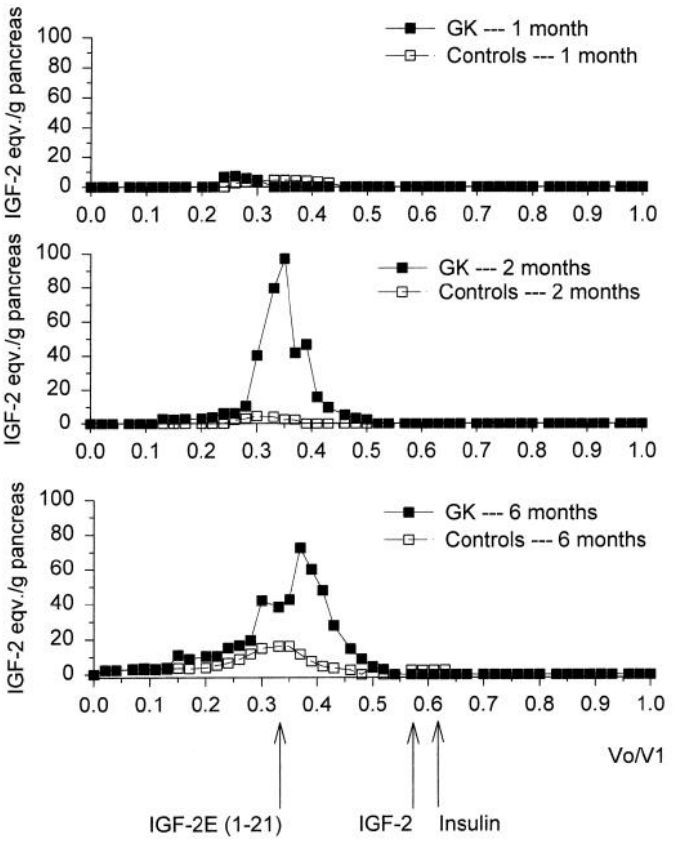

FIG. 6. Pancreatic extracts from GK and control rats were size-separated by gel chromatography; IGF-II peptide was measured by RIA. A HMW IGF-II peptide was found in the extracts, with a greater amount in 2- and 6-month-old rats than in 1-month-old GK rats and controls of all ages. No processed 7K IGF-II peptide was found in the extracts. The positions of IGF-IIE_{1-21} (10K), IGF-II (7K), and insulin (6K) are indicated with *arrows*.

infants and low during adult life, *i.e.* the human IGF-II antibody was useful for investigations in rats (data not shown). In all pancreatic extracts it was possible to identify an IGF-II IR peptide that, after chromatographic separation, eluted at a position indicating a higher mol wt than that of mature processed IGF-II (Fig. 6). Compared with the positions for insulin, [^{125}I]IGF-II, and [^{125}I]IGF-IIE_{1-21} (M_r = 10K), the mol wt of the IGF-II peptide was estimated to be approximately 10K. In the control rats, the pancreatic levels of HMW IGF-II were low at 1 and 2 months age, but slightly higher at the age of 6 months. The pancreatic extracts of 1-month-old GK rats had a low amount of HMW IGF-II, similar to that in control rats; this was considerably increased at 2 months. An increased amount of the peptide was also found at 6 months, with a lower, but more obtuse, peak representing a similar amount of peptide (Fig. 6). No RIA-IGF-II activity was detected in the void volume (V_o) of the column, indicating that IGFBPs with affinity for IGF-II were not present in the rat pancreatic extracts.

The IGF-I RIA values in the extract were consistently around 5% of the IGF-II levels. The values were invariably found at the same positions as the HMW IGF-II eluted; no detectable amounts could be observed at the position of mature IGF-I (data not shown). We conclude that IGF-I peptide was lacking in the pancreatic extracts and that the values found with the IGF-I RIA were the result of cross-reactivity between the IGF-I antibody and IGF-II peptide.

Ligand blot analysis of IGFBPs

The IGF-binding activity measurements indicated IGFBPs in the crude pancreatic extract, but smaller amounts in the supernatant after centrifugation and complete absence after 2 days of dialysis in acetic acid, followed by centrifugation. This proves that the extraction procedure successfully removed IGFBPs from the pancreatic extracts. Serum samples, a well known source of IGFBPs, had a large IGFBP content and served as a positive control together with purified IGFBP-1 (data not shown).

In situ hybridization

Intense labeling of IGF-II mRNA was observed, overlying starfish-shaped islets of GK rats (Fig. 7A). In GK rat islets with normal structure, as in control rat islets, no specific labeling of IGF-II mRNA was observed (Fig. 7, A and D). In GK liver, discrete IGF-II labeling was found overlying small cell groups close to sinusoids, most likely Kupffer cells (Fig.

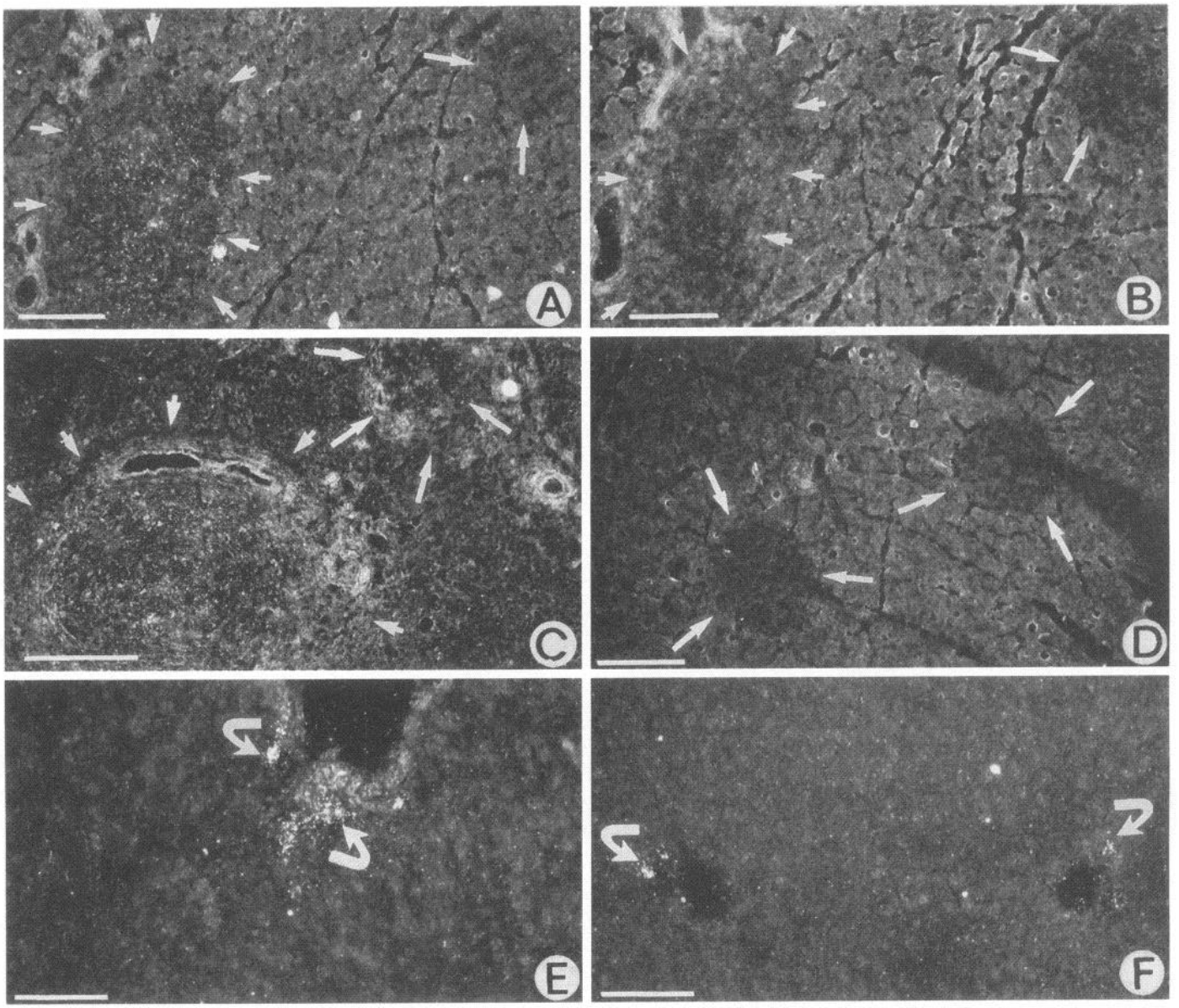

FIG. 7. A–F, Darkfield photomicrographs of pancreas and liver sections from 3-month-old rats hybridized with the IGF-II (A, D, E, and F), IGFBP-2 (B), and IGFBP-4 (C) probes. Intense labeling of IGF-II mRNA was observed in starfish-shaped islets of GK rats (A, *arrowhead*), whereas no specific labeling was observed in islets with a normal structure (A, *arrow*) or in islets from control rats (D). No specific IGFBP-2 expression was observed in either GK or control islets (B). IGFBP-4 mRNA displayed an intense signal in the starfish-shaped islets from GK rats (C, *arrowhead*), whereas no specific labeling was observed in islets with normal structure (C, *arrow*). In GK rat liver, IGF-II mRNA labeling was observed overlying small cell groups close to sinusoids, most likely representing Kupffer cells (E, *curved arrow*). This signal was observed in control animals as well, but at lower intensity (F, *curved arrow*). Magnification: A, B, and D, ×310; C, ×390; E and F, ×630. *Bar* = 50 μm in A-D and 25 μm in E and F.

7E). This signal was observed in control animals as well, although it was of lower intensity (Fig. 7F). No IGF-II mRNA was found in striated muscle from either GK or control rats (data not shown). We found an intense labeling of IGFBP-4 mRNA in the starfish-shaped islets of the GK rats (Fig. 7C), but no labeling in islets of normal structure (Fig. 7C) or in control rat islets. In liver, IGFBP-4 mRNA levels were markedly high, but showed no difference between GK rats and controls, nor was there any measurable IGFBP-4 mRNA in striated muscle (data not shown). No specific IGFBP-2 expression was observed in the pancreas (Fig. 7B), liver, or muscle tissues in this study.

Discussion

Thus, we found greater amounts of a HMW form of IGF-II in pancreatic extracts from 2- and 6-month-old GK rats than in extracts from 1-month-old GK rats and control rats of all ages. The IHC observations showed close similarity in the distribution of insulin and IGF-II immunoreactive cells between all age groups of GK and control rats. By using *in situ* hybridization, IGF-II mRNA expression was localized to the starfish-shaped islets of the GK rats.

Due to the structural homology between proinsulin/insulin and IGFs, cross-reaction between these peptides and

corresponding antibodies is conceivable, and a careful interpretation of the results is, therefore, necessary. However, the cross-reactivity tests performed using RIA and IHC techniques strongly dispute such cross-reactions and confirm the suitability of the human IGF-II antibody used in this study for investigations in rat tissue. When the pancreatic extracts were sized on a Sephadex column, the HMW IGF-II peak was found in samples separated from those of the size of insulin and mature IGF-I and IGF-II. Furthermore, when the six collected fractions of the major peak from the separations were pooled and tested against the IGF-II antibody, a displacement curve was obtained, parallel to that of commercial IGF-II.

Another source of doubt concerning the results obtained is the effects of IGFBPs (1, 29). There are six specific IGFBPs with extremely high affinity (K_d, 10^{-10}-10^{-11} mol/liter), approaching the binding affinities of IGF antibodies, that can affect both the separation and the measurement of these peptides. To remove IGFBPs from the pancreatic extracts, we used well known acid gel chromatography (23, 29). The absence of IGFBP activity in the final samples was also proved by the results of our dot blot analysis and by the absence of RIA-IGF-II activity in the V_o in the separated samples. Of the six IGFBPs, we studied IGFBP-2 and IGFBP-4 because their expression is known to be altered in diabetes mellitus or fasting (30). IGFBP-1 is also altered in diabetes mellitus (31), but was not examined in this study. The *in situ* hybridization affords additional evidence that there is an IGF-II peptide production related to the occurrence of starfish-shaped islets. The fact that no IGF-II mRNA was detected in GK rat islets with normal structure or in control rat islets does not refute the immunohistochemical findings of HMW IGF-II in all islets. If the secretion is scanty, and most of the peptide produced is stored in the cells, the expression of mRNA may be so low that it escapes detection (32).

HMW forms of IGF-II have frequently been reported to occur in tissues and fluids such as those of the central nervous system (23, 33), in various organs in fetuses (34), in spinal fluid (5), in plasma (6) and in tumors and cell lines (9, 35–37). There are several reports of IGF-II in human and rat fetal pancreas (34, 38–41); in contrast, there are few reports of findings in adult pancreas. In adult hamster pancreas, IGF-II was localized by means of mRNA isolation, RIA, and IHC investigations (42). The HMW IGF-II peptide found in adult rat pancreas in the present study had a M_r estimated to be 10K, with no findings of mature 7K IGF-II. Thus, there seems to be an arrest in the further processing of the IGF-II peptide. There is some evidence from studies of IGF-II production in tumors and from transfected cell lines, that *O*-glycosylation is a conceivable determinant of normal propeptide processing (9). Defective glycosylation or a similar mechanism might explain the observation that mature IGF-II is not present in the pancreatic extracts.

It is interesting that the increased content of HMW IGF-II coincides with the structural changes occurring in GK islets, and that these defective islets, *i.e.* starfish-shaped islets, display an increased expression of IGF-II mRNA. That these two phenomena are mutually related is uncertain, but several observations point in that direction. In the nervous system, the contents of IGF-II and IGF-I are increased after various types of damage (43, 44), suggesting a possible role for IGFs in repair processes. Such effects of HMW IGF-II can be supported by the finding that multiplication-stimulating activity, an early term for rat IGF-II (28), stimulates islet β-cell replication in neonatal rat islets and rat pancreatic monolayer culture (45). Additionally, after hamster pancreas was wrapped in cellophane, the production of IGF-II peptide and corresponding mRNA expression was reported, with the formation of new islets (42). Newly formed islets of this kind were functionally capable of reversing diabetes symptoms induced by streptozotocin (46). This also suggests that increased IGF-II levels may be involved in neoformation of islet parenchyma in the pancreas.

Another speculative explanation for the increased content of HMW IGF-II in GK pancreata is its involvement in a compensatory mechanism for the markedly impaired insulin responses to glucose. Circulating IGF-II, although mostly bound to IGFBP's in an inactive form, can cross-react with the insulin receptor and exert some insulin-like effects. As mentioned above, the mutually close localization of the insulin and IGF-II genes has led to speculation concerning a possible coregulation of the two genes (13). Thus, markedly increased levels of the peptide could be expressed as a compensatory mechanism to sustain a form of response to glucose stimulation in GK rats. Preliminary findings, indicating an increased content of HMW IGF-II peptide in the serum of 3-month-old GK-rats compared with that in controls, will be followed up in further studies. This might also explain the insulin independence of the diabetes mellitus in the GK rat. Evidence for such a glucose-induced IGF-II production is the finding that a 4-fold higher level of IGF-II mRNA was expressed at glucose concentrations of 10–20 mM in a highly differentiated glucose-sensitive insulin-producing rat cell line (INS-1) (47) than at low glucose levels in the medium. Clinical hypoglycemia can, in fact, be caused by IGF-II production in a subset of mesenchymal tumors (9, 37). The increased IGF-II content occurs predominantly in the HMW form, without any increase in insulin (9, 37).

Starfish-shaped islets are a typical finding in aging GK rats. Our ultrastructural studies demonstrated an increased number of fibroblasts in these islets and an abundance of collagen fibrils with their typical cross-striations, but without amyloid deposition. Usually, fibrosis is a consequence of inflammatory processes, but in the GK rat pancreas, there were no signs of such changes at any age. High glucose concentrations *per se* could be one explanation for the increased collagen formation (48, 49). Connective tissue cultures exposed to a high glucose concentration express increased amounts of collagen mRNA in rat sciatic nerves (48) and in cultured renal glomerular cells (49). Another cause of collagen formation is an induction by the IGFs themselves. In experiments with four different rat osteoblastic cell lines, both IGF-I and IGF-II were capable of stimulating collagen mRNA production (50). Interestingly, in studies of the effects of IGF-II on human fibroblasts, a HMW IGF-II peptide was even more potent than the mature form in stimulating proliferation (8). Such an induced fibrosis could disrupt the normal arrangement of islet architecture and destroy the thin capsule of connective tissue that separates the endocrine cells from the exocrine part with its potentially harmful enzymes.

The importance of such exposure of islet constituents to the exocrine environment on the functions of endocrine cells remains to be elucidated.

Thus, it is still uncertain whether the increase in HMW IGF-II in the pancreatic glands of diabetic GK rats is a consequence or a cause of the islet changes. If the lesions appear first, the process might be triggered by islet destruction due to a genetically predetermined event, the damaging effects of long term hyperglycemia, or a combination of the two, leading to increased local production of HMW IGF-II as a reparative response and possibly also to the induction of islet neoformation. On the other hand, if the lesions appear after the increase in HMW IGF-II, it could be speculated that the genetic defect is an increased local production of HMW IGF-II by the β-cells, stimulating fibroblast proliferation and collagen deposition in GK islets, with islet damage as a result.

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