Molecular Investigation of Age-Related Changes in Mouse Endocrine Pancreas

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Aging is an etiologic factor in non-insulin-dependent diabetes mellitus. While the effect of aging on insulin secretion has been described by several classic studies, the characterization of the molecular basis of β-cell abnormalities is still under way. We recently demonstrated in rats that aging is associated not only with a reduction in insulin secretion but also with diminished levels of intracellular insulin content and the mRNA for insulin. In this study, we investigated whether the molecular abnormalities previously described in the rat β cell were also present in the mouse (C57BL/6J).

Samples were subjected to slot-blot analysis by using homologous probes for insulin, glucagon, somatostatin, glucose transporter-2 (glut-2), glucokinase, elastase-1, and β-actin. We observed a progressive age-dependent decrease in insulin mRNA levels: insulin mRNA levels decreased by 40% with age (p = .007). This paralleled decreases in glut-2 (p = .001) mRNA levels, but it was in contrast with glucokinase mRNA levels which increased markedly (p = .0003).

Somatostatin mRNA levels were unchanged, glucagon mRNA levels decreased modestly (p = .001), and mRNA levels for elastase-1 and β-actin increased with age (p = .0001 for either one). In summary, it appears that in the mouse a progressive decline in the activity of the endocrine pancreas occurs with aging. This phenomenon seems to affect only the β cells and not the α or δ cells of the islet of Langerhans or the exocrine pancreas. This progressive decline may represent the biological features of the age-dependent risk for the development of diabetes.

DEVELOPMENT of non-insulin-dependent diabetes mellitus (NIDDM) is characterized by an impairment of pancreatic β-cell function as well as a diminished effectiveness of insulin action at its target tissues (Maneatis et al., 1983; Unger and Foster, 1990). Increasing age is associated with decreasing glucose tolerance (De Fronzo, 1984) and represents a major risk factor for the development of NIDDM (Andres and Tobin, 1975). The age-dependent decline in glucose tolerance which leads to insulin resistance of skeletal muscle appears, to a large extent, to be due to changes in body composition (i.e., decrease in lean body mass, increase in adiposity) and a decrease in physical activity (Zavaroni et al., 1986). The presence of acute or chronic illness and medications that affect glucose tolerance represent additional risk factors (Shimonata et al., 1991). However, age itself is an additional and independent risk factor for glucose intolerance (Andres and Tobin, 1975), and factors other than insulin resistance play a large role in the glucose intolerance that may occur with age. Pure insulinopenia, without insulin resistance, accounts for glucose intolerance in as many as 30% of diabetic subjects over 65 years of age (Arver et al., 1991). Of the remaining subjects, it is held that the onset of diabetes occurs when β cells fail to produce sufficient amounts of insulin to overcome insulin resistance (Cerasi, 1995).

In so-called "normal" aging, abnormalities in glucose tolerance, when compared to young people, can also be detected (Jackson, 1990). Population studies indicate that in normal subjects, plasma glucose measured 2 h after an oral glucose tolerance test is approximately 6–9 mg/dl higher for each decade of age (Jackson, 1990). Furthermore, fasting plasma glucose increases 1–2 mg/dl per decade without a concomitant increase in fasting insulin levels (Andres and Tobin, 1975). Reaven et al. (1983) have shown that insulin secretion decreases with age, even if the animal is calorie restricted and does not become obese. Elahi et al. (1985) demonstrated that older rats had a significantly lower insulin response to rising glucose than younger ones. Using islets isolated from rats, we have recently shown that aging is associated with altered insulin secretion after glucose stimulation and diminution of both intracellular insulin content and insulin mRNA levels (Perfetti et al., 1995).

It seems important, therefore, to study the nature of these age-related changes, as they are likely the "setting" for the development of diabetes. It appears also important to show that these changes may occur in other aging animals and are not unique to the Wistar rats. In the present study, we measured the mRNA for insulin and for other genes of the endocrine and exocrine pancreas in order to better comprehend some of the changes that occur in the β-cell physiology with age.

MATERIALS AND METHODS

Animals. — Three-, 9-, and 30-month-old C57BL/6J male mice (n = 6 per age group) were housed at the Gerontology Research Center, National Institute on Aging. They were fed a stock diet and maintained in a controlled environment (12-h light/dark cycle; 22 °C). Prior to sacrifice by decapitation, they had free access to water but were fasted overnight. Their pancreata were quickly removed and used for subsequent experiments. All procedures were in accord-
ance with National Institutes of Health guidelines and were approved by the Animal Resource Section of the National Institute on Aging.

Oligonucleotide primers and probes. — Oligonucleotides, synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer, were cleaved from the resin with concentrated ammonium hydroxide at room temperature, deprotected by heating to 55 °C overnight, and purified over Nensorp Prep columns (New England Nuclear, Boston, MA). Polymerase chain reaction (PCR) primers were designed according to the published mouse or rat sequences for insulin, glucagon, somatostatin, glucose transporter-2 (glut-2), glucokinase, β-actin, and elastase-I cDNAs (MacDonald et al., 1982; Heinrich et al., 1984; Bodoya et al., 1986; Tokunaga et al., 1986; Wentworth et al., 1986; Magnuson et al., 1989; Suzue et al., 1989; Fuhrmann et al., 1990) (Table 1). Homologous cDNA probes were generated by reverse transcription–PCR (RT-PCR). Oligonucleotide and cDNA probes were radioabeled using [γ-32P]ATP (polynucleotide kinase) and [α-32P]dCTP (random priming), respectively (Amersham, Arlington Heights, IL).

RT–PCR and synthesis of pancreatic cDNA probes. — One microgram of mouse pancreatic RNA extracted from a 3-month-old male was used as template for RT which was performed at 37 °C in a final volume of 20 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25 °C), 1.5 mM MgCl2, 0.01 mg/ml gelatin, 0.2 mM each dNTP, 40 units/tube RNasin (Promega, Madison, WI), 0.5 μM gene-specific downstream primer (Table 1), and 7 units/tube avian myeloblastosis virus reverse transcriptase (Promega) (Saiki et al., 1988; Shuldiner and Perfetti, 1993).

The second DNA strand was synthesized during the first cycle of PCR in which 5 μl of the RT reaction mixture was used in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25 °C), 1.5 mM MgCl2, 0.01 mg/ml gelatin, 0.2 mM each dNTP, 0.5 μM gene-specific upstream primer, 0.5 μM gene-specific downstream primer, 1 unit/tube Taq polymerase (Perkin–Elmer/Cetus, Norwalk, CT), and approximately 50 μl of paraffin oil. For insulin, somatostatin, β-actin, and glut-2, which were amplified by using homologous mouse primers based on the known mouse sequences (Tokunaga et al., 1986; Wentworth et al., 1986; Suzue et al., 1989; Fuhrmann et al., 1990), 39 cycles of PCR were performed. Each cycle consisted of denaturation (1 min at 94 °C), annealing (1 min at 55 °C), and extension (1 min at 72 °C), except the first cycle in which the denaturation time was increased to 5 min, and the last cycle in which the extension time was increased to 10 min. Glucagon, elastase-I, and glucokinase were amplified from mouse pancreatic RNA by using primers that corresponded to the rat sequences (MacDonald et al., 1982; Heinrich et al., 1984; Bodoya et al., 1986). The conditions for the amplification were identical to those described above except that the annealing temperature was decreased to 50 °C. RT–PCR products were subjected to electrophoresis on a composite gel consisting of 1% of agarose (Bethesda Research Laboratories, Gaithersburg, MD) and 2% Nusieve (FMC Byproducts, Rockland, ME). The gel was stained with ethidium bromide and photographed to verify that the amplified products corresponded to the predicted size. Hybridization of pancreatic RNA extract with cDNA probe obtained by RT–PCR as described above further demonstrated the specificity of those probes.

Table 1. Sequence of Primers Used to Generate Mouse cDNA Probes

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>Up 5'-GTCCCGCGGTGAAGTGAGG-3'</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>Down 5'-GGTGCAATGTTCTCAG-3'</td>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
<td>Up 5'-GACAAACGCCATTCACAGGGCACA-3'</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Down 5'-GTCTCTTCTTCGTTCAAA-3'</td>
<td></td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Up 5'-ATGCTGTCCCTGCCGTCACAG-3'</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td>Down 5'-GTTCTTGGCAAGACCTGGG-3'</td>
<td></td>
</tr>
<tr>
<td>Glut-2</td>
<td>Up 5'-GCCCTAGGTGTTCCTCCT-3'</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>Down 5'-CCTCTTCTGAGACCCAGG-3'</td>
<td></td>
</tr>
<tr>
<td>Glucokinase</td>
<td>Up 5'-GGGAAACAACATCGTAGGACTT-3'</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td>Down 5'-TCATCCACCATCCGGTCATAC-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Up 5'-ATGCGATATCGTGCGCTGTG-3'</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>Down 5'-CATAGGATCTCCTCTGCACC-3'</td>
<td></td>
</tr>
<tr>
<td>Elastase-I</td>
<td>Up 5'-ATGCTGGCGCTCCTGTC-3'</td>
<td>393</td>
</tr>
<tr>
<td></td>
<td>Down 5'-AGAATCTGGGCGGTCACCTT-3'</td>
<td></td>
</tr>
</tbody>
</table>
slot-blot minifold. The wells were washed three times with 400 µl cold 10 × SSC, allowed to air dry, and then baked in a vacuum oven at 80 °C for 2 h. The membranes were hybridized with 0.5–1 × 10^6 cpm of 32P radiolabeled probe per ml of hybridization solution. Hybridization conditions were the same as those described for Northern blot analysis. The blots were washed twice in 0.2 × SSPE and 0.5% SDS for 15 min at 55 °C when hybridized with cDNA probes, or twice in 2 × SSPE and 0.5% SDS for 15 min at 42 °C when hybridized with oligonucleotide probes, then subjected to autoradiography.

The relative levels of mRNA for insulin, somatostatin, glucagon, glut-2, glucokinase, elastase-I, and β-actin between mice of different ages were quantified by using a Betascope 703 blot analyzer (Betagen, Waltham, MA). After quantification, the blots were stripped by washing twice with 0.01 × SSPE and 0.1% SDS at 80 °C. To correct for possible differences in loading of RNA, data were normalized by rehybridizing the same blots with 32P-oligo-dT$_{20}$ (5'-GATGGATCCTGCAGAAGCTTTTTTTTTTTTTTTTTT-3').

Statistical analyses. — The data were expressed as mean ± SEM of three to five replicates and analyzed by one-way analysis of variance (ANOVA). Regression analysis was performed for all data presented.

RESULTS

Northern blot analysis of pancreatic RNA. — Northern blot analysis was performed for all samples to verify the integrity of the RNA by ethidium-bromide staining and visualization of the 28S and 18S ribosomal bands. In addition, after transfer onto nylon filters, RNA samples were hybridized with an oligonucleotide probe homologous to the 18S ribosomal subunit, which revealed the presence of a single sharp band indicating that no RNA degradation was present (Figure 1).

Expression of genes of the endocrine pancreas. — Insulin mRNA levels were measured by slot-blot analysis of RNA extracts obtained from 3-, 9-, and 30-month-old mice. Insulin mRNA levels declined dramatically as the age increased. Nine- and 30-month-old mice showed mRNA levels corresponding respectively to 56.1 ± 6.6% (p = .005) and 39.2 ± 12.9% (p = .01) of the levels detected in 3-month-old pancreas (p = .01) (Figure 2). Regression analysis of insulin mRNA levels showed a constant decrease with age (p = .007) (Figure 2).

In order to investigate if the age-dependent decrease in the amount of insulin mRNA was a generalized phenomenon affecting all islet cells, we determined the mRNA levels for glucagon α cells and somatostatin δ cells. Glucagon and somatostatin mRNA levels varied less dramatically in the different age groups, showing that α and δ cells were less affected by age. Glucagon mRNA levels slightly but significantly decreased in old mice when compared to young animals (p = .01) (Figure 3), but they were never lower than 61.9 ± 10.9% of the levels observed in pancreata isolated from 3-month-old mice (p = .01). The levels of somatostatin mRNA were even more stable, and no significant variations were observed among the different age groups (Figure 3). Regression analysis of glucagon and somatostatin mRNA levels showed that while there was a modest but constant decrease of glucagon with age (p = .01), there was not a definite trend for the variations in somatostatin mRNA levels (Figure 3).

Expression of other exocrine and endocrine genes during normal aging. — The mRNA levels for elastase-I and β-actin (two major products of the exocrine pancreas) increased with age (Figure 4). Elastase-I mRNA levels in 30-
Figure 3. Quantitative slot-blot analysis for glucagon and somatostatin mRNAs. Five micrograms of pancreatic RNA from 3-, 9-, and 30-month-old mice were subjected to slot-blot analysis and hybridized with probes for mouse glucagon (A) and somatostatin (B). The data were normalized by hybridizing the same membrane with oligo-dT\(^\_\) (data not shown) and were expressed as a percentage of the value observed in the 3-month-old group (C, glucagon; D, somatostatin). A and B represent one individual experiment, while the values shown in C and D indicate the means of three slot-blot analyses. Regression analysis of the data showed that glucagon mRNA levels significantly decreased per each month of age \((p = .01)\); variations of somatostatin mRNA levels did not show a clear trend with age. While the blots show an individual experiment, statistical analysis of the data was performed on all data collected. For graphic purposes, the age scale on the x axis was drawn using an arbitrary space between bars.

Figure 4. Quantitative slot-blot analysis for elastase-I and \(\beta\)-actin mRNAs. Five micrograms of pancreatic RNA from 3-, 9-, and 30-month-old mice were subjected to slot-blot analysis and hybridized with probes for mouse elastase-I (A) and \(\beta\)-actin (B). The data were normalized by hybridizing the same membrane with oligo-dT\(^\_\) (data not shown) and were expressed as a percentage of the value observed in the 3-month-old group (C, elastase-I; D, \(\beta\)-actin). A and B represent one individual experiment, while the values shown in C and D indicate the means of three slot-blot analyses. Regression analysis of the data indicating either elastase-I and \(\beta\)-actin mRNA levels showed that both elastase-I and \(\beta\)-actin mRNA levels increased progressively with age \((p < .0001\) for both elastase-I and \(\beta\)-actin) (Figure 4). This was in contrast with the decline of insulin mRNA levels, a product of the \(\beta\) cell.

Expression of \(\beta\)-cell-specific genes during normal aging.

To further investigate the specificity of the age-related changes of \(\beta\)-cell transcription activity, we studied the gene expression of glut-2 and glucokinase, two other products of the \(\beta\) cell. Glut-2 mRNA levels decreased progressively with age \((p = .0003)\), while glucokinase mRNA levels increased markedly \((p = .0003)\) (Figure 5). At 9 and 30 months of age, glut-2 mRNA levels were respectively 69.5 ± 25% \((p = .05)\) and 39.2 ± 10% \((p = .001)\) of the levels detected in 3-month-old mice. Showing a different age-related regulation, glucokinase mRNA levels at 9 and 30 months of age were 221 ± 24% \((p = .02)\) and 210 ± 12% \((p = .0001)\) of the values detected in the 3-month-old mice. Regression analysis of glut-2 and glucokinase mRNA levels showed that while there was a constant decrease of glut-2 mRNA with age \((p = .001)\), glucokinase mRNA levels significantly increased per each month of age \((p = .0003)\) (Figure 5).

Figure 5. Quantitative slot-blot analysis for glut-2 and glucokinase mRNAs. Five micrograms of pancreatic RNA from 3-, 9-, and 30-month-old mice were subjected to slot-blot analysis and hybridized with probes for mouse glut-2 (A) and glucokinase (B). The data were normalized by hybridizing the same membrane with oligo-dT\(^\_\) (data not shown) and were expressed as a percentage of the value observed in the 3-month-old group (C, glut-2; D, glucokinase). A and B represent one individual experiment, while the values shown in C and D indicate the mean of three slot-blot analyses. Regression analysis of the data for glut-2 and glucokinase mRNA levels showed that while glut-2 mRNA levels significantly decreased per each month of age \((p = .001)\), glucokinase mRNA levels significantly increased per each month of age \((p = .0003)\). While the blots show an individual experiment, statistical analysis of the data was performed on all data collected. For graphic purposes, the age scale on the x axis was drawn using an arbitrary space between bars.
DISCUSSION

NIDDM is one of the most common diseases that affect people as they age. In normal aging there is a decline in β-cell responsiveness to glucose, a rise in blood glucose levels, and a defect in β-cell adaptation to insulin resistance (Jackson, 1990). This renders the aging individual more susceptible to NIDDM as demands for insulin release with age increase due to (1) changes in fat distribution and increasing obesity; (2) decreased exercise; (3) decrease in muscle mass; (4) age-related increases in insulin resistance, probably due to a postreceptor defect, independent of the preceding causes; and (5) medications. Therefore, an understanding of the changes which occur with age might be helpful in elucidating why β-cell function declines with age.

We previously demonstrated that in isolated rat islets there was a consistent diminution of the insulin mRNA levels throughout the lifespan of the rat; the total pancreatic insulin content declined in a parallel age-dependent fashion; and the number of glucose-responsive cells as well as the amount of insulin released per individual β cell also diminished (Perfetti et al., 1995). Prior to our study on isolated rat islets (Perfetti et al., 1995), Reaven et al. (1979, 1983) and Matschinsky and coworkers (Burch et al., 1984) showed diminished glucose-induced insulin release from isolated islets of Langerhans of elderly Wistar rats. Harris et al. (1994), using aging mice, demonstrated using ad libitum-fed animals that 80% of older mice showed evidence of decreased glucose tolerance with respect to young animals. Elahi et al. (1985), using the isolated perfused pancreatic model, also showed impaired glucose-dependent insulin secretion with age in Wistar rats. In the study by Elahi et al. (1985) there was a markedly attenuated first-phase insulin release as well as total insulin release by aged animal in response to glucose. Nadiv et al. (1992) demonstrated that after a glucose tolerance test (glucose, 1 g/kg) there were significantly higher blood glucose levels at 15, 30, and 45 min in older Wistar rats. While the demonstration of an impaired age-associated defect in insulin secretion has been shown by many (Reaven et al., 1979, 1983; Burch et al., 1984; Elahi et al., 1985), a defect in insulin mRNA synthesis is a more recent finding (Perfetti et al., 1995).

In this study, we demonstrated that normal aging is associated with a progressive decline in insulin mRNA levels (p = .007). In order to discern whether this phenomenon was associated with aging of the whole organ or if it represented a more specific event selectively affecting the β cell, we measured the mRNA levels for other pancreatic proteins. Studies of the gene product of the exocrine pancreas showed that the exocrine function of the pancreas was not impaired with age. Investigating the mRNA for genes of the endocrine pancreas, we demonstrated that somatostatin mRNA levels did not change with age, while glucagon mRNA showed a modest but constant decrease with age (p = .01).

In order to determine if the decrease in insulin mRNA levels represented a loss of β-cell mass, we further investigated the expression of two other genes produced by β cells: glut-2 and glucokinase. While glut-2 mRNA levels decreased with age (p = .001), glucokinase mRNA levels increased significantly (p = .0003). This finding is unique considering it has been long believed that hexokinase and glucose transporter regulation were coordinate (Bell et al., 1993; Pilkins et al., 1994). The observed decline in glut-2 mRNA levels further suggests selective dysfunction of the β cell with age. Increases in glucokinase mRNA levels may represent a compensatory mechanism for decreased glut-2. These data, together with a previously demonstrated age-related increase of glucokinase activity and glucose utilization (Burch et al., 1984), suggest that with age a compensatory mechanism for decreases in insulin and glut-2 levels may be operating through a "resetting" of the pancreatic islet metabolism.

In summary, we have shown that in the normal mouse: (1) a decrease in insulin gene expression occurs with aging, which parallels a decline in glut-2 mRNA levels; (2) as insulin mRNA levels decline, the mRNA for glucokinase increases; and (3) mRNA for other genes of the endocrine and the exocrine pancreas do not exhibit any decline with aging.

In conclusion, our findings are consistent with the hypothesis that aging is associated with a dysfunction of the islets of Langerhans which selectively affects the β cell, a defect that in NIDDM is most likely magnified. It appears, therefore, important to locate the biochemical and molecular mechanisms that underlie the physiological age adaptation of β-cell activity, as it is on this aging background that NIDDM may occur.

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GERON CORPORATION–SAMUEL GOLDFSTEIN
DISTINGUISHED PUBLICATION AWARD

The Geron Corporation–Samuel Goldstein Distinguished Publication Award lecture will be presented at 3:30 p.m. Wednesday, November 20, 1996 at the Annual Meeting of The Gerontological Society of America in Washington, DC, by Gwen S. Adrian, PhD, co-author of the article selected by the Editorial Board as the best published in The Journal of Gerontology: Biological Sciences in the six issues starting May 1995 and ending March 1996.

The article chosen was:

Adrian, Gwen S.; Seto, Edward; Fischbach, Kathryn S.; Rivera, Edna V.; Adrian, Erle K.; Herbert, Damon C.; Walter, Christi A.; Weaker, Frank J.; and Bowman, Barbara H. YY1 and Spl Transcription Factors Bind the Human Transferrin Gene in an Age-Related Manner. J. Gerontol. Biol. Sci. 51A:B66–75; 1996.

An award will again be given at the 1997 Annual Meeting for the best article published in the journal starting May 1996 and ending March 1997.