Effects of atorvastatin on the regeneration of pancreatic β-cells after streptozotocin treatment in the neonatal rodent

K. C. Marchand,1,2 E. J. Arany,1,3 and D. J. Hill1,2,3,4

1Lawson Health Research Institute, St. Joseph’s Health Care; and Departments of 2Physiology and Pharmacology, 3Medicine, and 4Paediatrics, The University of Western Ontario, London, Ontario, Canada

Submitted 1 March 2010; accepted in final form 9 April 2010

Marchand KC, Arany EJ, Hill DJ. Effects of atorvastatin on the regeneration of pancreatic β-cells after streptozotocin treatment in the neonatal rodent. Am J Physiol Endocrinol Metab 299:E92–E100, 2010. First published April 13, 2010; doi:10.1152/ajpendo.00132.2010.—To investigate the role of statins in β-cell regeneration a model of streptozotocin (STZ)-induced β-cell injury was used in the neonatal rat. We hypothesized that β-cell growth and regeneration would increase following treatment with atorvastatin and that this would be associated with intraislet vasculogenesis. Pregnant Wistar rats were gavaged with 20 or 40 mg/kg atorvastatin for 21 days commencing on gestation day 15. Atorvastatin was detected in the circulation of the offspring. On postnatal day 4, the pups were given either a control or STZ (70 mg/kg ip) injection. β-Cell mass had partially recovered by postnatal day 44 following STZ treatment, and atorvastatin (20 mg/kg) significantly increased β-cell mass in both STZ-treated and control animals. An increase in the numbers of small islets at postnatal day 44 was seen in STZ-treated animals following atorvastatin, suggestive of neogenesis, and glucose tolerance was improved. Treatment with atorvastatin caused an increase in the numbers of intraislet endothelial cells at postnatal day 14 and the percentage of endothelial cells undergoing DNA synthesis, suggesting that angiogenesis had preceded the increase in β-cell mass. The results indicate that functional β-cell mass was expanded with atorvastatin in both control and STZ-treated neonatal rats and suggests a novel effect of a statin in promoting islet plasticity.

Pancreatic β-cell mass is known to change dynamically in response to altered metabolic demand, and a limited regeneration is possible following β-cell destruction (4). Understanding the mechanisms by which β-cell mass can be increased offers potential for targeted β-cell replacement in diabetes. Previously, we showed (43) that in neonatal rats a partial β-cell depletion using streptozotocin (STZ) had largely recovered within 40 days and blood glucose was normalized. The replacement of β-cells was due to both a repopulation from within islets and neogenesis of new endocrine cells at the pancreatic ducts. The vasculature may have a role in this regeneration process, particularly in early life. Lammert et al. (21) established that the blood vessel endothelium induces pancreatic endocrine cell differentiation while also providing key signals via a shared basement membrane for β-cell growth and function (27). Similarly, the increased β-cell mass seen during pregnancy in the rat is dependent on a preceding islet endothelial cell (EC) proliferation (20).

Vasculogenesis depends in part on the contribution of circulating endothelial precursor cells (EPC) derived from bone marrow (3, 11, 13, 31, 38). Transplantation of bone marrow cells or fractions of marrow progenitor cells have been shown by us and others to facilitate the reversal of diabetes in experimental animals and newly diagnosed individuals with type 1 diabetes (T1D) (7, 17, 39, 45). In some studies, a direct transdifferentiation of bone marrow stem cells into insulin-positive β-cells was demonstrated, either in vivo or following in vitro lineage manipulation (19, 25, 49), but the direct contribution of hematopoietic lineage cells to a new β-cell population has generally been found to be low and inconsistent with the resulting increase in insulin secretion and/or normalization of blood glucose (14, 17, 22, 42). One mechanism of β-cell regeneration would appear to result from the differentiation of marrow-derived vascular progenitors into EPC, present either as isolated cells that can infiltrate the islets and pancreatic ducts or which become incorporated as endothelial cells into pancreatic microvasculature during neovascularization (17). Neovascularization was accompanied by an increase in endogenous β-cells by replication or neogenesis of new islets from the pancreatic ducts (14, 17).

The hydroxymethylglutaryl (HMG)-CoA reductase-inhibiting class of drugs, the statins, are widely used to lower cholesterol levels in individuals with or at risk of cardiovascular disease. Recently, several studies demonstrated that statins could contribute to postnatal neovascularization by augmenting the mobilization of bone marrow-derived EPC (8, 23, 44). Vasa et al. (44) demonstrated that the effects of statins included an increase in the number of circulating EPC, reducing their senescence and enhancing proliferation and differentiation. Statin therapy in patients with coronary artery disease improved EPC differentiation into cardiomyogenic cells (33). The therapeutic use of autologous EPC may stimulate cardiomyocyte regeneration and thereby could potentially contribute to organ-specific regeneration generally.

Atorvastatin (AT) was shown to inhibit β-cell-specific CD8 T cell presence, which contributes to autoimmune β-cell destruction in the NOD mouse (24) but did not prevent the onset of diabetes in the NOD or streptozotocin (STZ)-treated mouse following a 12- or 28-day exposure (24, 29). Conversely, it was recently shown that the administration of simvastatin delayed diabetes onset in two different mouse models of T1D, including STZ treatment (35), and that this was independent of inhibition of HMG-CoA reductase (34). Discrepancies in the literature may result from a reported biphasic effect of statins on endothelial cells, with lower doses being proangiogenic while higher doses were antiangiogenic (10).

To investigate a possible therapeutic role of statins specifically in pancreatic β-cell regeneration, we used a neonatal rat model of STZ-induced β-cell injury, and attempted to improve β-cell mass by AT gavage treatment of the dam. AT is known...
to cross the placenta and be transferred via milk to the offspring (16). Our hypothesis was that AT would enhance the density of intraislet capillaries following β-cell damage and that this would be associated with an increased β-cell mass and function.

MATERIALS AND METHODS

Animals. Wistar rats were purchased from Charles River Laboratories (Montreal, PQ, Canada) and were housed in a temperature- and humidity-controlled room with a 12:12-h dark-light cycle. Animals had access to water and standard rat chow ad libitum. Nulliparous female rats weighing 250–300 g were mated on the night of proestrus, and if the presence of sperm or a vaginal plug was confirmed the rat was considered pregnant and at gestation day (GD) 0. Beginning at GD15, pregnant rats were given a daily oral gavage for 3 wk until postnatal day (PD) 14 with either 20 or 40 mg/kg body wt AT (Pizer Canada, Kirkland, PQ, Canada) suspended in 0.5% carboxymethylcellulose, based on the weight of the animal as recorded daily. Control rats received carboxymethylcellulose alone. Maternal food consumption by weight was recorded. Maternal blood was collected from the tail following parturition, and serum alanine aminotransferase levels were measured by enzymatic assay (Biotron Diagnostics, Hemet, CA) as an indicator of liver function.

Litters were reduced to 10 pups at birth, five male and five female. On PD 4, pups were given a single injection of 70 mg/kg ip of STZ freshly prepared in citrate buffer (pH 4.5) or a control injection with citrate buffer alone. Animals treated with 0, 20, or 40 mg/kg AT, with or without STZ, were examined at PD 6, 14, and 44. Blood glucose was measured from the tail vein using the Ascencia BREEZE glucometer (Bayer, Elkhart, IN), and serum was collected for insulin determination. The animals were killed and the pancreata collected and fixed for histology and for pancreatic insulin content. All animal procedures were performed with the approval of the Animal Care Committee of the University of Western Ontario in accordance within the guidelines provided by the Canadian Council for Animal Care.

Glucose tolerance test and insulin determinations. At PD 44, animals were fasted for 5 h and subjected to an intraperitoneal glucose tolerance test (GTT). A bolus of glucose (2 g/kg body wt) was given, and blood glucose was measured from the tail vein at 0, 15, 30, 60, and 90 min. A 100-mg portion of pancreas from the head region was homogenized in 1 ml of acid ethanol (165 mM HCl in 75% EtOH) and left overnight at 4°C. The homogenate was then centrifuged at 2,000 × g for 20 min, and the supernatant was removed and stored at −20°C until radioimmunoassay for insulin. Insulin content in pancreas and serum was measured using a Sensitive Rat Insulin RIA Kit (Linco Research, St Charles, MO) with a sensitivity of 3 pm and inter- and intra-assay coefficients of variation of 4 and 9%, respectively. Pancreatic insulin content was expressed per milligram wet weight of tissue.

HPLC-mass spectroscopy. Plasma from female rats and pups at PD 1 (newborn) and PD 6 treated with or without 20 mg/kg AT were analyzed. Octadeyl (C18) Speedi columns (10 μm, solid-phase extraction [SPE]; VWR International, Mississauga, ON, Canada) were used to purify and reconstitute the samples for analysis. Samples were analyzed using a ThermoFinnigan Surveyor MS PUMP and Autosampler connected to a ThermoFinnigan Triple Stage Quadrupole (TSQ)-7000 Tandem MS using electrospray ionization (ESI) and Xcalibur version 2.0 computer software (Thermo Electron, Mississauga, ON, Canada). In the Luna C18 (2) column (3 μm, 100 Å, 50 × 3.0 mm; Phenomenex, Torrance, CA), the mobile phase consisted of buffer A (54% acetonitrile with 5 mM ammonia formate buffer) and buffer B (75% acetonitrile with 5 mM ammonia formate buffer). Components were eluted for 6 min beginning with 100% buffer A, increasing to 100% B at 2.01 min and then decreasing to original concentration at 3.01 min, at a constant flow rate of 0.3 ml/min and injection volume of 10 μl. The retention time for AT was 2.6 min, and the internal standard, rosuvastatin (1,000 ng/ml) was 1.5 min.

Immunohistochemistry. Pancreatic tissue was fixed in 10% neutral buffered formalin for 24–36 h and embedded in paraffin. Tissue sections of 5 μm were cut from the head region of the pancreas and mounted on SuperFrost Plus glass slides (Fisher Scientific, Ottawa, ON, Canada) and incubated at 50°C for 24–36 h. Tissues were rehydrated through a series of graded alcohols. Dual staining was performed on pancreas tissue sections to localize the presence of α-cells with mouse monoclonal anti-glucagon (Sigma-Aldrich, St. Louis, MO) and β-cells by using a rabbit polyclonal anti-insulin (Santa Cruz Biotechnology, Santa Cruz, CA) by a modified avidin-biotin-peroxidase method (18). All antisera were diluted in DakoCytomation Antibody Diluent (DA) (DakoCytomation, Mississauga, ON, Canada). Tissue sections were blocked with 5% horse serum (Cedarlane Laboratories, Hornby, ON, Canada) before application of anti-glucagon (Sigma; 1:2,000 dilution). Biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA) was used as the secondary antibody (1:30 dilution). Slides were incubated with ExtrAvidin peroxidase (Sigma), and color was developed in fresh diaminobenzedine (BioGenex, San Ramon, CA). Goat serum (5%, Cedarlane) was then applied followed by incubation with anti-insulin antiserum (Santa Cruz; 1:200 dilution). Biotinylated goat anti-rabbit (Vector Laboratories) was used as a secondary antibody (1:30 dilution). Vectastatin ABC-AP Kit and then Vector Red Alkaline Phosphatase Substrate Kit I (Vector) were used to visualize insulin-positive β-cells. Tissue sections were counterstained with Carazzi’s hematoxylin, and the slides were dehydrated in a series of graded alcohols and then mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

Dual staining was similarly performed on pancreas tissue sections to localize the presence of islet EC using mouse monoclonal anti-Flk1 (Santa Cruz Biotechnology) and biotinylated lectin from the bacteria Bandeiraea simplicifolia (BS-1; Vector) as previously described (26). All slides were subjected to heat-induced epitope retrieval with 0.01 M citrate buffer (pH 6.0) at 95–100°C for 20 min. Tris-buffered saline-Tween (1×) 20 was used as a wash after each step excluding the blocking serum. A 5% horse serum was applied, followed by anti-Flk1 (Santa Cruz Biotechnology; 1:50 dilution). Biotinylated horse anti-mouse was used as the secondary antibody. The Vectastatin Elite ABC Kit and then Vector SG Substrate Kit were used to visualize Flk1 (Vector Laboratories). Biotinylated BS-1 lectin (1:30 dilution) was used to visualize EC using the Vectastatin ABC-AP Kit and Vector Red Alkaline Phosphatase Substrate Kit I. Tissue sections were counterstained with methyl green solution (Sigma). Dual staining was similarly performed using BS-1 lectin and proliferating cell nuclear antigen (PCNA; mouse anti-PCNA 1:100 dilution, Sigma-Aldrich) to observe those EC undergoing DNA synthesis and for insulin and PCNA to identify the incidence of β-cell proliferation. To
establish specificity of the antibodies, controls were performed in which the primary antisera were replaced by nonimmune serum, or the secondary antisera were omitted.

Morphometric analysis. Analysis of the pancreata was performed by light microscopy, and the images were analyzed with Northern Eclipse version 7.0 software (Empix Imaging, Mississauga, ON, Canada) by an observer blinded to the study arms. An islet was defined as containing at least two insulin-positive cells. Islets were arbitrarily separated by area into small (200–5,000 μm²), medium (5,000–10,000 μm²), or large (>10,000 μm²). An α-cell cluster was defined as containing at least two glucagon-positive cells and no β-cells. For each pancreatic section, the following were determined: total area of the pancreatic section, area occupied by each islet, and area occupied by the glucagon-positive or insulin-positive cells within each islet, the total number of islets, and the area occupied by each α-cell cluster. β-Cell mass was calculated as the total β-cell area/total pancreatic tissue area × pancreas weight. Islet density (total, small, medium, or large) was calculated from the number of islets per total pancreatic tissue area. The α-cell cluster area was calculated from total α-cell cluster area/total pancreatic tissue area × 100. Three nonconsecutive tissue sections cut 50 μm apart were used to calculate the area occupied by β-cells for each pancreas, and the β-cell mass. On the basis of these data, the between-section coefficient of variation was found to be 8%; therefore, subsequent analyses were performed with single representative sections from each pancreas. To estimate the amount of Flk1- and BS-1 lectin-positive EC, two blinded observers selected random islets from each pancreatic section and counted the BS-1 lectin-positive cells and

<table>
<thead>
<tr>
<th>AT Dose, mg/kg</th>
<th>PD 6</th>
<th>PD 14</th>
<th>PD 44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.9 ± 0.3</td>
<td>7.4 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>STZ</td>
<td>11.7 ± 1.3</td>
<td>12.4 ± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 5–6/group. PD, postnatal day; AT, atorvastatin; STZ, streptozotocin. Glucose following STZ treatment was significantly greater than control, using 2-way ANOVA (P < 0.001).
Table 2. Body and pancreatic weight at PD 6, 14, and 44 in rats treated with AT with or without STZ

<table>
<thead>
<tr>
<th>Body weight, g</th>
<th>AT Dose, mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PD 6</td>
<td>15 ± 0.5</td>
</tr>
<tr>
<td>STZ PD 6</td>
<td>13 ± 0.4</td>
</tr>
<tr>
<td>Control PD 14</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>STZ PD 14</td>
<td>31 ± 1</td>
</tr>
<tr>
<td>Control PD 44</td>
<td>199 ± 16</td>
</tr>
<tr>
<td>STZ PD 44</td>
<td>180 ± 13</td>
</tr>
<tr>
<td>Pancreatic weight, mg</td>
<td></td>
</tr>
<tr>
<td>Control PD 6</td>
<td>68 ± 4</td>
</tr>
<tr>
<td>STZ PD 6</td>
<td>66 ± 4</td>
</tr>
<tr>
<td>Control PD 14</td>
<td>218 ± 20</td>
</tr>
<tr>
<td>STZ PD 14</td>
<td>241 ± 17</td>
</tr>
<tr>
<td>Control PD 44</td>
<td>1106 ± 60</td>
</tr>
<tr>
<td>STZ PD 44</td>
<td>1194 ± 93</td>
</tr>
</tbody>
</table>

Values represent means ± SE; n = 5–6/group. *P < 0.01 vs. control + 0 or 20 mg/kg; †P < 0.05 vs. STZ + 0 or 20 mg/kg, using 2-way ANOVA with Newman-Keuls multiple comparison test.

RESULTS

Administration of AT. To understand the pharmacokinetics of AT in the adult female rat, a dose of 20 mg/kg AT was administered to nonpregnant females who were age and weight matched to the pregnant rats in this study. The peak concentration of AT was seen 60 min postgavage (Fig. 1). Maternal serum alanine aminotransferase levels in maternal serum following AT were similar to those of control animals at ~5 IU/l, indicating no adverse effects of AT on liver function. To determine whether the AT administered to the mother during gestation crossed the placenta and was present in the pups, we measured the parent compound following administration of 20 mg/kg. Offspring that did not receive AT had no drug present in their plasma, whereas the offspring of dams who received AT had 11.1 ± 3.1 ng/ml AT present at PD 1 (n = 10). Similar findings were seen at PD 14. AT treatment did not alter maternal weight gain during pregnancy or mean maternal weight at any age up to PD 14. Maternal daily food consumption was not altered following statin treatment.

Islet morphology and metabolism after STZ treatment. β-Cell mass was reduced after STZ-induced damage at PD 6 (2 d post-STZ) (Fig. 2, A and B) compared with control animals. The degree of β-cell loss was similar for animals that received AT [β-cell mass for STZ alone, 0.52 ± 0.04 mg (means ± SE), n = 6; STZ + AT 20 mg/kg, 0.49 ± 0.4 mg; STZ + AT 40 mg/kg, 0.50 ± 0.6 mg]. At PD 14 (10 days post-STZ), small insulin-positive cell clusters were observed, often in association with pancreatic ducts (Fig. 2C). By PD 44, no histological evidence of β-cell damage remained within the islets (Fig. 2D). The architecture of the islet had recovered with a regular shaped core of β-cells and an outer mantle of α-cells, with no visible fibrosis or inflammation. β-Cell mass was significantly decreased in the STZ-treated animals (P < 0.001) at PD 6, 14, and 44 compared with controls at the same age (Fig. 2E). Although the STZ-treated animals showed a larger incremental gain in β-cell mass from PD 6 to 44, this was only half that of the control by PD 44 (Fig. 2E). Nonfasting blood glucose was analyzed on PD 6 and was significantly elevated in STZ-treated animals, with or without AT, relative to controls (Table 1).

Pancreatic weight and β-cell mass. There were no differences in body weight at PD 6, 14, or 44 between animals that received STZ and controls, with or without AT (Table 2). Similarly, there were no differences in pancreatic weight at PD 6 or 14. The pancreatic weight at PD 44 in the control rats given 40 mg/kg AT was significantly higher than for the control animals without AT or receiving 20 mg/kg, respectively (P < 0.01) (Table 2). In animals receiving STZ + 40 mg/kg AT, the pancreatic weight was also significantly larger compared with the STZ-treated animals without AT or with 20 mg/kg AT (P < 0.05).
Table 3. Total, small (200–5,000 \( \mu \text{m}^2 \)), medium (5,000–10,000 \( \mu \text{m}^2 \)) and large (>10,000 \( \mu \text{m}^2 \)) islet number per mm\(^2\) of pancreas at PD 14 and 44 in rats treated with AT with or without STZ.

<table>
<thead>
<tr>
<th>Islets</th>
<th>AT Dose, mg/kg</th>
<th>PD 14</th>
<th>PD 44</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>STZ</td>
<td>Control</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9.1 ± 0.77</td>
<td>6.6 ± 0.39</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>20</td>
<td>7.7 ± 1.29</td>
<td>8.2 ± 0.57</td>
<td>1.4 ± 0.11(^*)</td>
</tr>
<tr>
<td>40</td>
<td>9.4 ± 1.00</td>
<td>7.4 ± 0.75</td>
<td>1.3 ± 0.06(^*)</td>
</tr>
<tr>
<td>Small</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.2 ± 0.72</td>
<td>6.0 ± 0.34</td>
<td>0.7 ± 0.04</td>
</tr>
<tr>
<td>20</td>
<td>6.6 ± 1.20</td>
<td>7.7 ± 0.55</td>
<td>1.1 ± 0.07(^*)</td>
</tr>
<tr>
<td>40</td>
<td>8.0 ± 0.90</td>
<td>7.0 ± 0.76</td>
<td>1.0 ± 0.05(^*)</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.5 ± 0.08</td>
<td>0.4 ± 0.08</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>20</td>
<td>0.5 ± 0.08</td>
<td>0.4 ± 0.08</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>40</td>
<td>0.6 ± 0.12</td>
<td>0.3 ± 0.06</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>Large</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.4 ± 0.04</td>
<td>0.3 ± 0.12</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>20</td>
<td>0.6 ± 0.10</td>
<td>0.1 ± 0.06</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>40</td>
<td>0.8 ± 0.11</td>
<td>0.1 ± 0.04</td>
<td>0.2 ± 0.01</td>
</tr>
</tbody>
</table>

Values represent means ± SE; \( n = 5–6/\text{group} \). \(^*\)\( P < 0.05 \) vs. control + 0 mg/kg; \(^\dagger\)\( P < 0.05 \) vs. STZ + 0 mg/kg, using 1-way ANOVA with Newman-Keuls multiple comparison test.

No change in \( \beta \)-cell mass was seen in control animals following treatment with AT by PD 14 (Fig. 3A). However, the animals that received STZ + 40 mg/kg AT showed a significant decrease in \( \beta \)-cell mass at PD 14 compared with rats treated with STZ alone (\( P < 0.05 \)). By PD 44, AT-treated animals showed evidence of an increased \( \beta \)-cell mass both with and without prior administration of STZ (Fig. 3B). When the size of individual \( \beta \)-cells was examined, no differences were found following treatment with 20 mg/kg AT at PD 14 or 44, but a relative hypertrophy of \( \beta \)-cells was seen with the 40 mg/kg dose in STZ-treated animals at PD 44 (82 ± 2 \( \mu \text{m}^2 \)) compared with an absence of AT (69 ± 2 \( \mu \text{m}^2 \)) (\( P < 0.01 \)). This suggests that the increased \( \beta \)-cell mass seen with the higher dose of AT may have resulted mainly from hypertrophy of existing \( \beta \)-cells rather than the gain of new cells. When the size of individual acinar cells was examined, this did not change after exposure to 40 mg/kg AT, showing that the hypertrophic effects on \( \beta \)-cells was specific.

**Islet size distribution.** Islet size distribution was expressed as numbers of total, small (200–5,000 \( \mu \text{m}^2 \)), medium (5,000–10,000 \( \mu \text{m}^2 \)), and large (>10,000 \( \mu \text{m}^2 \)) islets. At PD 14, there were no significant changes in islet distribution with AT, although there tended to be fewer large islets in the STZ groups compared than in the controls (Table 3). By PD 44, there was a significant increase in the total number of islets in the animals that received either dose of AT compared with controls (Table 3). There was a similar increase in STZ-treated rats that received 20 mg/kg AT, but not the higher dose. The changes in islet numbers following AT treatment were predominantly due to

---

Fig. 4. Immunohistochemical localization of \( \alpha \)-cell clusters (200–5,000 \( \mu \text{m}^2 \)) in rat pancreas (A) and budding from ducts (B) from animals treated with STZ + 20 mg/kg AT on PD 14. \( \alpha \)-Cell clusters are indicated by arrows. Scale bar, 50 \( \mu \text{m} \). Area of pancreas occupied by \( \alpha \)-cell clusters is shown at PD 14 (C) and PD 44 (D) for control and STZ-treated animals receiving AT at 0, 20, or 40 mg/kg. Control and STZ-treated animals were analyzed separately. Values represent means ± SE; \( n = 5–6/\text{group} \).
changes in the numbers of smaller islets, with no changes observed in the numbers of medium sized or large islets (Table 3).

α-Cell clusters were visible within the pancreas (Fig. 4A) and apparently budding from ducts (Fig. 4B). At PD 14, there was a much larger percentage of α-cell clusters per pancreatic section than at PD 44, where their occurrence was rare (Fig. 4, C and D). However, no significant changes in cluster numbers or size were seen following treatment with STZ, with or without AT.

Glucose tolerance. Before a GTT was undertaken at PD 44, fasting blood glucose values were measured in the animals at 0 min. The STZ + 40 mg/kg AT-treated rats had a higher mean glucose value (10.0 ± 0.9 mM) than the control + 40 mg/kg AT group (6.9 ± 0.3 mM) (P < 0.05), but other treatment groups had similar values around 7 mM (Fig. 5A). Blood glucose 30 min postinjection was significantly higher in the STZ-treated animals than in the controls (P < 0.001), although the animals that received STZ + 20 mg/kg AT (24.5 ± 1.6 mM) had significantly lower blood glucose than the animals treated with STZ without AT (30.7 ± 1.3 mM, P < 0.01; Fig. 5A). The STZ-treated animals showed elevated blood glucose levels throughout the test period compared with control groups (Fig. 5A), demonstrating that impaired glucose tolerance was still present at PD 44. The area under the curve (AUC) or the STZ-treated groups was significantly higher than for control animals (P < 0.001), although within the STZ treatment, the AUC for STZ + 20 mg/kg AT was significantly less than the that for STZ alone (P < 0.01), indicating a significantly improved glucose tolerance (Fig. 5B).

Fasting serum insulin levels on PD 44 did not differ between control (5.4 ± 0.2 ng/ml) and STZ-treated animals (5.4 ± 0.4 ng/ml) or after 20 mg/kg AT. When the pancreatic content of insulin was measured at PD 44, this did not differ in control rats treated (8.1 ± 1.4 ng/mg pancreas) or not treated (8.5 ± 1.5 ng/mg pancreas) with 20 mg/kg AT. In animals that had received STZ, the insulin content in those treated with AT (3.0 ± 0.6 ng/mg pancreas) was slightly greater than in those not treated (1.6 ± 0.1 ng/mg pancreas, P < 0.05).

Intraislet EC. BS-1 lectin and Flk1 were used to localize the abundance of intraislet EC. BS-1 lectin binds specifically to EC, whereas Flk1 is one of the earliest differentiation markers for EC thought to be associated with proliferative cells (37). Their colocalization would indicate the likely presence of EPC or immature EC. BS-1 lectin was visualized as red cytoplasmic staining (Fig. 6, A and B), and Flk1 was visualized as a grey plasma membranal staining (Fig. 6B). The majority of the EC were found to be integrally associated with islet capillaries. The amount of dual positive BS-1 lectin/Flk1 cells was greatest at PD 14 (Fig. 6D), whereas few such cells were detectable at PD 44. AT treatment was associated with the significant increase in the number of BS-1 lectin-positive cells (P < 0.05; Fig. 6C) and BS-1 lectin/Flk1-positive cells (P < 0.01; Fig. 6D) at PD 14, regardless of STZ treatment. The incidence of EC proliferation was examined by the costaining of pancreas sections with PCNA and BS-1 lectin at PD14 prior to the partial recovery of β-cell mass (Fig. 7A). Animals treated with STZ showed no change in the percentage of proliferating EC at that time, but those receiving AT (20 mg/kg) showed increased EC proliferation whether treated with STZ or control. These findings were compared with the incidence of β-cell proliferation within the same islets (Fig. 7B). At PD 14, animals that had been treated with STZ showed an increased number of β-cells undergoing DNA synthesis compared with control treated. AT did not significantly alter β-cell proliferation in control animals at that time, but a significant increase was found with AT after STZ administration. β-Cell proliferation was observed within small, medium, and large islets. The findings support an angiogenic effect of AT within the islet vasculature that precedes an increase in β-cell mass.

DISCUSSION

The objective of this study was to examine the effect of AT on the regeneration of β-cell mass following STZ-induced loss. Our findings indicate that β-cell mass was expanded with AT and that this occurred by PD 44 in control and STZ-treated animals. In the rat model used, β-cell regeneration is achievable only in early life; therefore, animals had to be exposed to AT starting in utero and extending until weaning through treatment of the dams. A previous study by Henck et al. (16) showed that a single dose of 10 mg/kg AT given to female rats on GD 19 was transferred across the placenta and on PD 13 was excreted into the milk. We provided AT by oral gavage of the dam once daily using 20 or 40 mg/kg. The pharmacokinetics showed that the native drug had reached...
Subsequent to AT treatment, there was a relative increase in \( \beta \)-cell mass with AT treatment, which was most effective with the 20 mg/kg dose. This could have resulted from an increase in the size of existing islets, including those remaining after STZ, or the appearance of new islets through neogenesis from precursor cells, thought to exist both in pancreatic ducts and within islets (47). Islet size analysis showed that the increase in \( \beta \)-cell mass resulted predominantly from an increase in the numbers of smaller islets, suggesting that AT possibly potentiated the appearance of new islets by neogenesis. Studies by Brand et al. (6) and Rosenberg et al. (32) indicate that, in the STZ rat model, islet neogenesis is a significant contributor to \( \beta \)-cell mass expansion. The use of a higher dose of AT, 40 mg/kg, also resulted in increased \( \beta \)-cell mass at PD 44 following STZ treatment. However, it is likely that this did not result from the addition of new \( \beta \)-cells but from the hypertrophy of existing cells. This possibility is supported by the lack of improvement in glucose tolerance in animals given STZ and 40 mg/kg AT by PD 44.

We previously showed (43) that after STZ treatment in the neonatal rat there was increased cell proliferation in both
insulin- and glucagon-positive cells but that a predominant early feature was α-cell hyperplasia. The α-cells synthesized glucagon-like peptide-1 (GLP-1), a potent growth factor for β-cells and their precursors (40, 46), in addition to glucagon. α-Cell aggregates devoid of β-cells, which decline in abundance with age, have been observed in the head of the neonatal rat pancreas (1). It is not known whether these are discrete from islets or represent immature islets that will subsequently generate β-cells. However, they could represent a local source of GLP-1, which could potentiate islet neogenesis. Although the abundance of α-cell clusters was greater at PD 14 than at PD 44 and they tended to be more abundant after STZ treatment, their presence was not altered by AT.

To determine whether the increased β-cell mass seen following AT treatment was functional, we subjected the rats to a GTT. Control animals had similar responses to the glucose load regardless of the AT treatment, as would be expected from an optimal population of β-cells. However, animals that received STZ and 20 mg/kg AT showed an improved glucose tolerance compared with animals without AT or those receiving 40 mg/kg AT, suggesting that the improved β-cell mass was associated with greater glucose-stimulated insulin release. In a study by Satoh et al. (36), glucose tolerance following an oral challenge and glucose-induced insulin secretory in adult Wistar rats was enhanced by statin treatment in excess of 6 wk. Similarly, Suzuki et al. (41) used an oral 30 mg/kg dose of AT for 4 wk in KK/Ay mice, an animal model of obesity-associated type 2 diabetes with insulin resistance. After administration of a GTT, plasma glucose levels were significantly lower at 30, 60, and 120 min in the AT-treated animals.

Tissue regeneration can occur following the mobilization and colonization of tissues by bone marrow-derived stem cells that have multiple effects, including the vascularization of damaged tissue through the differentiation of EPC into functional EC (30). β-Cell proliferation can be facilitated by angiogenic factors released from EC such as hepatocyte growth factor (28). β-Cells, in turn, may potentiate EC proliferation through the release of vascular endothelial growth factor (VEGF) (28). We examined changes in EC presence within islets using two protein markers, BS-1 lectin and Flik1. BS-1 lectin has been used previously to detect endothelium within the islets (26), and Flik1 is one of the earliest differentiation markers for EC thought to be associated with proliferative cells (37). Treatment with AT caused an increase in the number of BS-1 lectin-positive EC and dual positive BS-1 lectin/Flik1 EC at PD 14, but not at PD 44. This may indicate an early activation of intraislet angiogenesis by AT that subsequently supported an increase in β-cell mass, particularly in smaller islets, and this was further supported by an increased percentage of proliferating EC after AT. An alternative explanation is that AT protected EC from developmental or STZ-associated apoptosis and would be supported by the reported ability of pravastatin to promote human pancreatic EC survival following a hyperglycemic challenge in vitro through an activation of the Akt survival pathway (12). Similarly, statin treatment has been shown to increase the survival and function of islet transplants (2).

It is plausible that strategies to enhance pancreatic neovascularization could lead to β-cell neogenesis, proliferation, or improved function, although the present data show only an association between microvascular change and an increase in β-cells. A causal relationship could be proved in the present model through inhibition of intraislet angiogenesis by using a selective inhibitor such as angiostatin. Further studies could examine an optimization of the AT dose, timing, or route of EPC delivery and/or coadministration of growth factors (15, 48). To determine whether EPC are contributing to the increase in EC and affecting the increase in β-cell mass, it would be important to investigate changes in the number and mobilization of EPC after AT treatment. Nevertheless, our findings suggest a novel effect of atorvastatin and its potential role in islet regeneration.

ACKNOWLEDGMENTS

We thank Scott Bailey, Dr. David Freeman and Dr. Terry Paul for their advice and help, and Brenda Strutt for technical assistance.

GRANTS

These studies were made possible by funding granted to D. Hill by the Canadian Institutes of Health Research.

DISCLOSURES

No conflicts of interest are reported by the authors.

REFERENCES


