Transitional cells in the regenerating pancreas

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SUMMARY

We examined the spectrum of intermediate cell types in the regenerating pancreas as duct epithelial cells progressed through their differentiation pathway to become mature endocrine cells. The model used was transgenic mice in which the pancreatic islets continue to grow during adulthood, unlike normal mice whose islet cell formation ceases early in life. Because the intermediate cells migrated into islet-like clusters at specific locations, we propose a specific pathway for islet development. Endocrine cells are derived from duct cells co-expressing a duct cell antigen, carbonic anhydrase II (CA II) and an exocrine enzyme, amylase. The CA II/amylase cells become amylase/endocrine intermediate cells as they exited from their luminal location. The abluminal amylase/endocrine cells continue to differentiate to multihormone-bearing young endocrine cells, which migrated to form clusters with other differentiating endocrine cells.

Key words: amylase, carbonic anhydrase II (CA II), intermediate cell, pancreatic duct, islet regeneration, transgenic mouse

INTRODUCTION

Endocrine cells in the pancreas are believed to originate from its duct epithelial cells, which derive from two outpocketings of the primitive gut (Pictet and Rutter, 1972; Wessells and Cohen, 1967; Wessells and Evans, 1968). In mice, such endocrine cells initially appear individually in the duct wall, later form small clusters in the interstitial tissue, and finally organize into well-defined islet structures with centrally located beta cells surrounded by the other endocrine cell types (Deltour et al., 1991; Dubois, 1989; Orci, 1988). Each islet consists of endocrine cells arising from several epithelial progenitors (Deltour et al., 1991). This neogenesis in the islets of Langerhans continues throughout the neonatal period and ceases shortly after weaning (Deltour et al., 1991; Githens, 1988). Thereafter, no additional islets form, and the islet cells do not exhibit appreciable growth (Hellerstrom et al., 1988). The general inability of the pancreatic duct cells to proliferate and differentiate in adults is reflected by the fact that the beta cell mass, which is lost in insulin dependent diabetes mellitus, is not replenished by regeneration of islet cells.

Despite the cessation of islet cell growth, differentiated endocrine cells are known to be capable of undergoing mitosis even in adult animals under certain conditions (Davidson et al., 1988; Green et al., 1981; Korcakova, 1971; Nielsen et al., 1989). However, the pattern by which the ductal precursors generate endocrine cells in adults is unknown.

We previously described duct epithelial cell proliferation in adult transgenic mice carrying the interferon-g (IFN-g) gene linked to an insulin promoter (Gu and Sarvetnick, 1993). The pancreata of these mice underwent ductal hyperplasia and destruction of islets in association with lymphocytic infiltration. Simultaneously these mice exhibited extremely high proliferative activity in their pancreatic ducts, with a mitotic index sometimes reaching as high as 73% in a 12-hour period. Within the duct walls, single endocrine cells and clusters were found (Gu and Sarvetnick, 1993). We have hypothesized that the new islet cells originated from the proliferating duct cells and that the islet regeneration in this mouse strain might recapitulate embryonic islet neogenesis.

MATERIALS AND METHODS

Light and electron microscopy

For light microscopic examination, samples of tissue were fixed in
4% zinc-formalin or Bouin’s fixative, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Paraffin sections were cut at 5 µm thickness, deparaffinized, and stained with H & E for routine observation.

Samples for electron microscopy were initially fixed in 1.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, postfixed in 1% OsO₄, dehydrated in graded ethanol, cleared in propylene oxide and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Hitachi HU 12A electron microscope (Gu and Sarvetnick, 1993).

Immunocytochemistry
The procedures for immunolabeling and identification of proliferating cells by BrdU incorporation, colocalization of pancreatic hormones, and colocalization of insulin, carbonic anhydrase II (CA II), amylase, and BrdU were essentially the same as described by Gu and Sarvetnick (1993), except that the immunofluorescent markers FITC and rhodamine were also used. No frozen sections were used in the present study. For double immunofluorescent staining, the sequential staining technique was used. The first primary antibody was either anti-glucagon, anti-somatostatin, anti-pancreatic polypeptide, or anti-amylase. The first secondary antibody was fluorescence (DTAF, Dichloro-triazinyl amino) conjugated goat anti-rabbit. The second primary antibody, anti-insulin was applied. The second secondary antibody was rhodamine (TRITC) conjugated goat anti-guinea pig. For comparison, BALB/c pancreatic tissue served as a control and was used and processed like that from transgenic mice. To check the specificity of antibodies, control experiments consisted of replacement of each primary antiserum with a preimmune serum or with PBS/bovine serum albumin (BSA). Background staining was minimal, and no spectral overlap was observed. Furthermore, the presence of both single- and double-positive cells indicated the absence of cross-reactivity between the different hormone antibodies. Antibodies were obtained from the following sources: guinea pig antibodies against insulin were purchased from Dakopatts. Of the rabbit antibodies, those against glucagon came from Chemicon, those against somatostatin from The Binding Site. Monoclonal rat antibodies against BrdU were purchased from Sera Lab and Chemicon. The biotinylated goat anti-guinea pig IgG, biotinylated goat anti-rabbit IgG, biotinylated rabbit anti-sheep IgG, and biotinylated rabbit anti-rat IgG were purchased from Vector Labs. In addition, rhodamine conjugated goat anti-guinea pig IgG and fluorescence conjugated goat anti-rabbit IgG were purchased from Chemicon.

BrdU pulse-labeled chase experiment
27 mice were injected intraperitoneally with 100 µg/g body weight of BrdU. The maximum availability time of BrdU is 40-60 minutes (Boswald et al., 1990). The fates of the BrdU incorporated by duct cells, beta cells and amylase positive cells were counted at 6, 12 and 18 hours after BrdU administration.

Quantification of labeled cells and statistical analysis
The immunolabeled cells were scored on a 400× microscope field. At least 20 ducts or islet structures from 5 animals were examined for each test group. Each section was systematically scanned to avoid overlapping of the fields. The percentage of positive cells was expressed as the number of labeled cells divided by the total number of cells counted times 100. The statistical analyses were performed by Student’s t-test.

RESULTS

Evidence for primitive endocrine cells
Because multiple hormone-bearing cells are indicative of newly differentiated endocrine cells on their path to the single hormone of their mature state, we performed double immunolabeling experiments with antibodies to the four islet hormones, i.e., insulin, glucagon, somatostatin, and pancreatic polypeptide (B, A, D, and PP). We chose 4-month old transgenic mice for this co-expression of hormones studies, since the existing islets were destroyed and the regeneration of new islet structures was vigorous. The double-positive cells in the ducts and islet structures were then scored (Fig. 1; Tables 1, 2). These experiments demonstrated that approximately 16% of the insulin-containing duct cells in pancreata of the transgenic mice expressed an additional islet hormone. Of these double-positive cells, a high proportion coexpressed insulin and glucagon (see Table 1). On the contrary, in pancreata of control, non-transgenic mice, no endocrine cells expressed more than one detectable hormone in this test.

All double-positive cells, which were considered intermediate cells in the maturation process, were found in both the duct walls and the islet structures. However, the proportion of islet cells coexpressing two hormones was much higher in the ductal location. Since this characteristic is associated with immature endocrine cells, these findings suggested that the duct endocrine cells were in the early stages of differentiation and the islet structure contained a high proportion of newly formed endocrine cells.

Evidence for the ductal origin of islet cells
To study the origin of the young endocrine cells, we performed BrdU labeling experiments. The changes in percentage of BrdU-positive duct cells and islet cells were compared and analyzed at different time intervals. Mice were given injections of BrdU and killed at the time intervals indicated in Table 3. The number of BrdU-positive cells in the duct increased 15.3% by 6 hours after injection and reached a peak of 21.32% at 12 hours (a 1.4-fold increase). Clearly, mitotic activity of the duct cells was extremely high in this transgenic mouse strain. In contrast, the islet structures contained only 1.89% BrdU-positive cells after the first 6 hours of incubation. However, the number of labeled islet cells increased nearly 4.3-fold to 8.03% at the end of a 12-hour incubation. Thus, the rate of increase in the labeling index of the islet cells during the 6- to 12-hour period was significantly higher than that of duct cells (P<0.001). Since no additional stimulatory factors were present or administered to the mice to cause duct cells or islet cells to synthesize DNA at a later stage, this dramatic increase in the labeling index of islet cells during this 6- to 12-hour period suggests that the dividing duct cells could be the source of the extra BrdU-labeled cells in the islets. The percentage of BrdU-labeled cells dropped sharply after 18 hours in both ducts and islets, indicating that many cells containing BrdU were not detectable as the total cell population increased, thereby diluting the signal (data not shown).

To ascertain that the newly arrived islet cells indeed were differentiated endocrine cells, and not lymphocytes or fibroblasts, etc., we performed double labeling experiments to identify BrdU-incorporating endocrine cells in the ducts and in islet structures adjacent to the ducts. We determined that these BrdU and endocrine double positive cells represented 95% (40/42) and 97% (117/120) of the total BrdU incorporated cells in islets at the 6- and 12-hour incubation times, respectively. Table 3 depicts the changes in the percentage of the BrdU and
Table 1. A survey of intermediate beta cells in the duct wall

<table>
<thead>
<tr>
<th>Intermediate type</th>
<th>Insulin-glucagon (No.)</th>
<th>Insulin-somatostatin (No.)</th>
<th>Insulin-PP (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double positive</td>
<td>41</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Total positive</td>
<td>412</td>
<td>474</td>
<td>272</td>
</tr>
<tr>
<td>% double positive</td>
<td>9.95</td>
<td>8.65</td>
<td>4.41</td>
</tr>
</tbody>
</table>

Double indirect immunofluorescence stainings were performed on paraffin sections of pancreata from 4-month old transgenic mice with red color for insulin in combination with green for each of three other islet hormones. Single and double cell-layered duct walls were considered as duct cells and scored for positively stained cells. The values represent pooled data from 5 animals. Note the high proportion of insulin-containing cells and glucagon-containing cells that coexpressed both hormones.

Table 2. A survey of intermediate beta cells in the islet structure

<table>
<thead>
<tr>
<th>Intermediate type</th>
<th>Insulin-glucagon (No.)</th>
<th>Insulin-somatostatin (No.)</th>
<th>Insulin-PP (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double positive</td>
<td>13</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Total positive</td>
<td>1091</td>
<td>392</td>
<td>1292</td>
</tr>
<tr>
<td>% double positive</td>
<td>1.19</td>
<td>3.32</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Double indirect immunofluorescence stainings were performed on paraffin sections with red for insulin and green for each of three other islet hormones. The buds and clusters of more than 5 cells in association with the duct wall were considered islet structures. The values represent pooled data from 5 animals.

Table 3. Time course of proportion of total BrdU labeled cells and BrdU/endocrine double positive cells in ducts and islet structures

<table>
<thead>
<tr>
<th>BrdU labeling time (Positive cells/total cells counted)</th>
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<tbody>
<tr>
<td>(%) 6 hour 12 hour 18 hour</td>
</tr>
<tr>
<td>BrdU positive</td>
</tr>
<tr>
<td>Duct cells/ (281/1837)</td>
</tr>
<tr>
<td>Total cells in ducts</td>
</tr>
<tr>
<td>BrdU positive</td>
</tr>
<tr>
<td>Islet cells/ (34/1799)</td>
</tr>
<tr>
<td>Total cells in islets</td>
</tr>
<tr>
<td>BrdU and endocrine double double</td>
</tr>
<tr>
<td>Positive cells/ (69/680)</td>
</tr>
<tr>
<td>Total endocrine cells in ducts</td>
</tr>
<tr>
<td>BrdU and endocrine double double</td>
</tr>
<tr>
<td>Positive cells/ (40/1712)</td>
</tr>
<tr>
<td>Total endocrine cells in islets</td>
</tr>
</tbody>
</table>

Single and double-label indirect immunoperoxidase staining experiments were performed on paraffin sections of pancreata from 4-month old transgenic mice using red color for BrdU and purple color for endocrine cells. The single labeling data were for the detection of proliferating cells (BrdU positive) in the ducts or in the islets. The endocrine-positive cells were identified by the use of pooled antibodies to glucagon, insulin, somatostatin and pancreatic polypeptide. The double-labeling experiments (BrdU and endocrine double-positive cells) were designed to calculate the number of double-labelled dividing endocrine cells as a proportion of the total endocrine cells in the ducts or in the islets. The values represent pooled data from 4 animals.
endocrine double positive cells, including A, B, D and PP cell types at 6, 12 and 18 hours after BrdU administration. At the 6 hour time point, 2.34% of the islet endocrine cells were BrdU-incorporating endocrine cells, a percentage similar to 1.89% of BrdU single-labeled positive cells from total islet cell numbers (P>0.05). The labeling index of double-labeled endocrine cells in islet structures increased 3.7-fold in the 6- to 12-hour period, which is significantly higher than the 1.4-fold increase in the ducts (P<0.001). This increase was slightly lower than the nearly 4.3-fold increase registered by single-labeled islet cells, possibly because some newly differentiated cells contained low levels of hormone and were not detected by the method used. Nevertheless, the large number of double-positive cells that appeared in the islet structures during this period most likely represented recent arrivals from the differentiating duct cells.

Evidence for the existence of ductal/exocrine intermediate cells

Interestingly, we frequently observed cells containing exocrine granules in the lumen of ducts in the transgenic mice (Fig. 2), but not in non-transgenic mice. To characterize these potential transitional cells, we performed immunomarking experiments employing antibodies to CA II and amylase. The CA II antigen is expressed in interlobular duct epithelial cells of both normal and transgenic mice, whereas the exocrine enzyme, amylase, was chosen because of its characteristically acinar localization.

Immunolabeling studies with CA II revealed that all the positive cells resided within the duct walls, mainly in small ducts corresponding to the intercalated pancreatic ducts in the transgenic mice. Immunostaining with amylase showed that, in addition to acinar cells, numerous duct cells of the transgenic mice also contained amylase, as might have been predicted from our ultrastructural analysis. In comparison, only very few amylase-containing duct cells were observed in BALB/c mice. To identify transitional cells, we then studied the coexpression of the CA II antigen with amylase. Quite frequently, double-labeled cells were found within the ducts of our transgenic mice, apparently bordering the lumen. Since cells with these characteristics were not found in normal non-transgenic mice, we conjectured that they could represent transitional cells in our model of differentiation.

Since transitional cells in intermediate stages of this process should undergo mitosis, considering that epithelial cells of the pancreatic duct do proliferate in the adult transgenic mice we use, this possibility was tested further by double labeling for amylase and BrdU. The resulting mitotic index of these ductal amylase-positive cells was 2.3% at the 6 hour time point and increased to 6.8% at 12 hours, thereafter declining to 4.4% after 18 hours of incubation. A subsequent triple-labeling experiment, to determine whether CA II/amylase double-
positive cells incorporated BrdU, again showed that this intermediate cell type was mitotically active (Fig. 3).

Evidence for the existence of exocrine/endocrine intermediate cells
Electron microscopy (EM) previously revealed that the ductal endocrine cells do not border the lumen but are separated from it by duct cell cytoplasm (Gu and Sarvetnick, 1993; Higuchi et al., 1992). Consequently, the appearance of exocrine/endocrine intermediate cells would suggest that the differentiating cells had begun to exit from the lumen lining. Therefore, we immunostained duct cells for insulin and amylase to identify cells possessing both exocrine and endocrine characteristics. In control BALB/c mice, no cells co-expressed amylase and insulin at a detectable level. However, in pancreata of the transgenic mice, 1.6% (30/1838) of the amylase positive cells reacted with the insulin antibody. These cells frequently appeared within ducts and periductal regions and occasionally were visible in islets. In apparent agreement with EM observations, all of the double-positive amylase/insulin intermediate cells were not lining the lumen, while nearly all of the single stained amylase cells were at the lumen’s lining (Fig. 4). We considered that the double-positive cells might represent an intermediate stage between ductal cells and islet cells in our model.

To determine if the endocrine/exocrine intermediate cells exhibited mitotic activity, triple labelings for amylase, insulin and BrdU were performed (Fig. 5). As Fig. 5 shows, this intermediate cell type manifested mitotic activity.

Although in EM observations, no endocrine granules were found in cells lining the lumen we studied the coexpression of CA II and insulin to find ductal/endocrine intermediate cells. However, this dual labeling failed to locate any duct/endocrine intermediate. Evidently no cells of this type were present, at least not at a detectable level.

Evidence for the existence of exocrine/endocrine intermediate cells in the fetal pancreas
To determine whether the cells in the rudimentary pancreas in embryos also contain exocrine/endocrine intermediate cells, we immunostained BALB/c and transgenic E13-E18 fetal pancreata for both amylase and insulin. First, we carried out single antibody labeling using antibodies to either amylase or insulin. The results of these single antibody staining experiments from BALB/c and transgenic pancreata were quite similar. The earliest stage we could detect insulin-containing cells was E13, in agreement with the published report by Alpert et al. (1988). We observed that amylase reactive cells first appeared on E14.5, in agreement with previous reports (Teitelman et al., 1987; Alpert et al., 1988). Interestingly, in double-labeling experiments, we could detect coexpression of amylase and insulin in the pancreas of transgenic embryos on E14.5 (Fig. 6). The double-stained cells were relatively rare, however, they could be clearly identified. We could not detect any double-labeled cells of this type in the pancreas of BALB/c embryos.

DISCUSSION
We have demonstrated that the pancreatic islet cells, which regenerate uniquely in adult IFN-g transgenic mice, originate
Fig. 4. Exocrine/endocrine intermediate cells. Double stainings performed by indirect sequential immunofluorescence staining are for insulin (red) and amylase (green). Double-positive cells (long arrows) in the duct wall do not border the lumen. Also note insulin containing cells (short arrow) and single amylase positive cells (open arrowhead). Magnification, 560×.
from duct epithelial cells. This conclusion rests on two experimental results. First, the ductal endocrine cells from these mice expressed multiple hormones, a trait attributed to the newly differentiated endocrine cells. Second, the dividing ductal endocrine cells migrated to join the newly formed islet structures. That is, a disproportionately large number of BrdU-labeled endocrine cells homed to the islet structures, and intermediate endocrine cell types were identified within these islet structures. These combined results led us to conclude that the new islet cells arose from duct cells.

In this model, the differentiation process appears to initiate within duct structures and terminate within islet structures. Although we observed ductal cells and ductal/acinar intermediate cells on the duct lumen, the exocrine/endocrine and endocrine cells were found exclusively in the ‘periductal’ location. Such compartmentalization of cell type and locality suggests that movement from a ductal to periductal location occurs as a developmental progression. Furthermore, our analysis of the transitional cells identified in this study demonstrated the presence of duct/acinar and acinar/endocrine cells but not duct/endocrine cells. Therefore, duct cells evidently progress to duct/acinar to acinar/endocrine to endocrine cells. Indeed, occasional cells within islet structures contained amylase, suggesting the down regulation of this enzyme during the migration of the developing islet cells into islet structures.

Some of the ductal cells examined here contained both insulin and amylase. Thus, these cells exhibited phenotypes of both acinar and endocrine cells. Cells having these characteristics have been reported in both normal animals and in the animals under certain metabolic stress (Melmed et al., 1972; Melmed, 1979). However, in our transgenic mice, these acinar/endocrine cells were located primarily in the duct wall and were mitotically active. The presence of ductal intermediate acinar/beta cells raises the possibility that beta cells and acinar cells may have a common progenitor, at least in this system. A noteworthy observation was that these acinar/beta intermediate cells were destined to become endocrine cells, not acinar cells, since we found no evidence for the neogenesis of acinar tissue in the pancreata of transgenic mice. This conclusion is in agreement with the general belief that restoration of the exocrine gland is achieved by replication of acinar cells not by differentiation of duct cells (De Lisle et al., 1990; Elsasser et al., 1986; Githens, 1988; Rao et al., 1990). We do not know whether the exocrine/endocrine intermediate cells occur during fetal life. The presence of such cells has been hypothesized, but not directly demonstrated (Alpert et al., 1988; Teitelman, 1987). Our own efforts to demonstrate such cells in the normal fetal pancreas of non-transgenic mice have proved unsuccessful, but we have identified putative exocrine/endocrine cells in our transgenic embryos. This allows us to speculate that exposure to the cytokine during fetal development induces the expansion of primitive dedifferentiated cells. If this theory proves to be true, our model will be useful for identification and characterization of these primitive cells.

The origin of the duct cells is also a subject of speculation.

**Fig. 5.** Acinar/endocrine cells and their incorporation of BrdU. Indirect sequential immunofluorescence staining for amylase (green), insulin (purple), and BrdU (red), of paraffin embedded pancreas to reveal intermediate acinar/endocrine cells. (A) Bright-field micrograph of an islet structure confirmed its location at the duct lumen. An insulin and BrdU double-stained cell (long arrow) appears in the duct wall but not at the lumen’s lining. Single-stained BrdU and single insulin containing cells are also present (arrowhead and short arrow). (B) Immunofluorescence micrograph of the same field as A. Note that the double-stained insulin/BrdU cell shown in A is also stained with amylase (long arrow). An amylase single-stained cell (short arrow) is evident immediately above the triple-stained cell. Magnification, 1,000x.
Owing to the mitotic activity exhibited by our duct cells, we concluded that the dividing duct cells can give rise to differentiated duct/exocrine cells, as discussed above. Alternatively, the duct cells can be derived from acinar cells. In fact, the transformation of acinar cells to duct cells has been reported in acinar carcinoma derived cell lines (De Lisle et al., 1990; De Lisle and Logsdon, 1990; Pettengill et al., 1993). We have observed acinar cells in small ducts that appeared fully differentiated. Possibly these represent the transformation of acinar cells to duct cells in the exocrine tissue near the islet lesions. These acinar cells could dedifferentiate to duct/acinar cells and then eventually redifferentiate to islet cells.

Islet cell regeneration in the pancreas of adult transgenic mice progresses through the following steps. The duct wall contains duct/acinar cells with extremely high mitotic activity. When duct cells divide at a right angle to the duct wall, one daughter cell exits from the lumen, coexpressing amylase and hormone (insulin). The acinar/endocrine cells eventually stop expressing amylase while still retaining multihormone phenotypes as they move further away from the lumen. The newly differentiated endocrine cells initially appear as single cells dotting the duct wall and then migrate to form clusters in the islets. This regeneration process parallels islet neogenesis in embryos and provides a model system for studying factors influencing islet cell differentiation.

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