Intermediate endocrine-acinar pancreatic cells in duct ligation conditions

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Bertelli, Eugenio, and Moïse Bendayan. Intermediate endocrine-acinar pancreatic cells in duct ligation conditions. Am. J. Physiol. 273 (Cell Physiol. 42): C1641–C1649, 1997.—When tissues were subjected to 24 h of duct ligation, intermediate pancreatic cells simultaneously displaying endocrine and exocrine phenotypes appeared. Immunocytochemistry by laser scanning confocal microscopy revealed the appearance of a large number of these cells coexpressing insulin and amylase. These cells were located within the islets of Langerhans as well as in the acinar parenchyma. They were also detected in a culture system of isolated pancreatic cells. With the use of immuno-electron microscopy, two types of secretory granules were identified in these cells. One was insulin immunoreactive, whereas the other, resembling zymogen granules, contained amylase. Occasionally, some small granules displayed a double labeling for both secretory proteins. Numerous crinophagic bodies and autophagosomes containing insulin and/or amylase were also present. In situ hybridization, applied with the specific probes, confirmed the presence of both insulin and amylase mRNAs in these cells. Because duct ligation is known to induce insulin cell proliferation, the present results confirm that endocrine-acinar cells do appear in such condition and may represent intermediate steps in a transdifferentiating process.

pancreas; transdifferentiation; confocal microscopy; immunocytochemistry; in situ hybridization

IN MULTICELLULAR ORGANISMS, cells are exposed to specific sets of signals arising from the surrounding environment. These signals may include hormones, cytokines, growth factors, and interactions with the extracellular matrix and among adjacent cells. According to the specific set of signals and to their own phenotype, cells may respond by differentiating, proliferating, fulfilling their own specialized functions, undergoing a programmed cell death (apoptosis), or simply maintaining their differentiated state (2). Some cells, however, have been found to respond to changes in the surrounding environment by transdifferentiating, i.e., by switching between differentiated phenotypes (11). Transdifferentiation is a complex aspect of cell physiology that has been recently identified as taking place in many cell types. The switch in cell phenotype may require several steps, including dedifferentiation, proliferation, and redifferentiation (11), but direct transdifferentiation, without cell division, has also been described as occurring in several occasions (7, 25, 38). In the latter case, the crucial evidence for transdifferentiation has been the demonstration of mixed cells, i.e., cells expressing intermediate phenotypes (7, 25, 32).

Transdifferentiation has been induced in several cell types in vitro (1, 11, 32) but has also been reported to occur in vivo (24, 25). In the pancreas, for example, transdifferentiation of acinar and islet cells toward ductal cells has been documented in vitro (3, 10, 38), whereas pancreatic cells have been reported to transdifferentiate into hepatocytes in rats temporarily submitted to a copper-depleted diet (28). Transdifferentiation of acinar cells has been reported to occur in chronic pancreatitis and hypothesized as the source of ductal proliferation (8). The capability of pancreatic cells to transdifferentiate seems, therefore, remarkable and could offer a basis for new strategies in the treatment of insulin-dependent diabetes mellitus (IDDM), characterized by the selective loss of β-cells. Because of the existence of common precursors for acinar and islet cells (17, 26, 27), an original hypothesis has been put forward for β-cell neogenesis presupposing transdifferentiation of acinar into islet cells (9). This eventuality, however, has never been explored. The demonstration of transitional cells, i.e., cells displaying a phenotype common to both exocrine and endocrine cells, should represent an important clue in such a transdifferentiating pathway. Cells simultaneously displaying acinar and endocrine phenotypes have indeed been reported repeatedly since 1902 (19) under different nomenclatures (see Refs. 9 and 23 for reviews). In addition to intermediate cells, these cells have been recently identified as mixed cells (12) and transitional cells (15). Their existence, however, has not found general recognition, their presence being sometimes considered as the artifactual result of nonoptimal tissue preparation (13, 18, 20). Current opinions on transdifferentiation, however, may give a novel support to these cells, and, despite previous opposition, renewed attention has recently been given to these pancreatic mixed cells (12, 15, 38).

In the present study, duct ligation experiments were carried out that triggered the appearance of a large number of cells coexpressing both exocrine and endocrine phenotypes. Characterization of these cells was carried out by light and electron microscopy using various cytochemical approaches, including immunocytochemistry and in situ hybridization. Two distinct populations of such intermediate cells were detected in pancreatic tissue when subjected to 24 h of duct ligation. One, located within the islets of Langerhans, was characterized by a predominance in insulin- vs. amylase-containing granules, whereas the other, situated within the exocrine tissue, was represented by cells that had a large number of amylase- and insulin-containing granules. In addition, such cells were also detected in a culture system of isolated pancreatic cells.

The presence of both endocrine and exocrine secretory granules in the same cells, in situ as well as in cell
culture, the existence of granules containing insulin and amylase, and the expression within the same cells of insulin and amylase mRNAs suggest that, when given a particular stimulation, cells displaying both endocrine and exocrine phenotypes appear and could represent transitional steps in a transdifferentiating process.

METHODS

Animals. Twelve male Sprague-Dawley rats weighing 100–110 g (Charles River, St. Constant, PQ, Canada) were maintained under standard conditions with free access to food and water. Six animals underwent pancreatic duct ligation, whereas the remaining rats were used as controls.
Surgical procedures and tissue processing. The animals subjected to the obstruction of the main pancreatic duct were anesthetized with a freshly prepared solution of Nembutal (40 mg/ml), and the abdominal cavity was opened with a midline incision. The reversion of the stomach allowed for the optimal exposure of the splenic portion of the pancreas and the identification of its duct, which was promptly ligated close to its joining with the bile duct. The animals were killed 1 and 4 days after surgery. The control animals underwent the same procedures, with the exception of the ligation of the pancreatic duct. The splenic portion of the pancreas was sampled, fixed in formalin for 48 h at 4°C or in Bouin’s fluid for 24 h at room temperature, dehydrated in ethanol, and embedded in paraffin. For electron microscopy, small pieces of tissue were fixed in 0.1 M phosphate-buffered (pH 7.4) 1% glutaraldehyde for 2 h at room temperature, postfixed in 1% OsO4 for 1 h at 4°C, dehydrated in ethanol, and embedded in Epon. Areas of interest for electron microscopic analysis were chosen from semithin sections. Consecutive ultrathin sections were stained with uranyl acetate and lead citrate and observed with a 410 LS Philips electron microscope. The animals were killed 1 and 30 days after surgery and observed with a 410 LS Philips electron microscope.

Detection of red and green stainings within the same cell suggests the presence of different compartments storing amylase and insulin. Magnification \( \times 1,300 \). c: Intermediate endocrine-acinar cell coexpressing amylase (green) and insulin (red). Detection of red and green stainings within the same cell suggests the presence of different compartments storing amylase and insulin. Magnification \( \times 1,900 \). d: Intermediate endocrine-acinar cell coexpressing amylase (green) and insulin (red). Detection of red and green stainings within the same cell suggests the presence of different compartments storing amylase and insulin. Magnification \( \times 1,300 \). e: Double immunofluorescence (yellow) revealing strong immunoreactivity for amylase and insulin within 2 cells located in an acinus. Within the same acinus, an insulin-immunoreactive cell (red) is also present, whereas the rest of the pancreas is strongly positive for amylase alone (green). Magnification \( \times 1,300 \). f and g: Cell culture system, illustrating the same single isolated pancreatic cell maintained in culture and displaying simultaneously stainings for amylase (green), insulin (red), and combined (yellow). In f, full contrast is shown, whereas g shows specific stainings. Magnifications = \( \times 1,500 \).
(DEPC)-treated water. The sections were then processed in 2× standard saline citrate (SSC) for 10 min at 60°C, DEPC-treated water, 0.05 M Tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.6), and 10 mg/ml proteinase K diluted in the same buffer for 10–30 min, depending on tissue fixation, at 37°C in a humid chamber. To stop the hydrolytic action of the enzyme, the sections were briefly washed with PBS, fixed with 0.4% paraformaldehyde in PBS at 4°C for 20 min, and rinsed in DEPC-treated water. The slides were then incubated for 1 h at 37°C in the prehybridization buffer (0.6 M NaCl, 30% deionized formamide, and 150 mg/ml salmon sperm DNA) and transferred to the preproinsulin probe (200 ng/ml) or to the amylase probe (400 ng/ml) prepared in the same buffer. Incubation was carried out overnight at 37°C in a wet chamber. After hybridization, the sections were rinsed twice at 37°C for 10 min in mixtures of decreasing concentrations of SSC (4, 2, and 0.2×) containing 30% formamide (vol/vol) and once in 0.05 M TBG (Tris buffer (pH 7.6), 0.1 M NaCl, and 2 mM MgCl2) containing 0.1% Triton X-100 and 0.1% bovine serum albumin (BSA) for 15 min. Afterward, the sections were incubated for 60 min with alkaline phosphatase-conjugated anti-DIG antibody diluted 1:600 in TBS containing 0.1% BSA, washed twice in TBS-0.1% BSA, and finally incubated in the revealing solution [45 ml of nitro blue tetrazolium, 35 ml of X-phosphate in 10 ml of 0.1 M Tris (pH 9.5)–0.1 M NaCl-0.05 M MgCl2]. The specificity of the probes was tested by omitting them in the labeling protocol or by pretreatment of the tissue sections with 100 mg/ml RNase (R&D Systems) diluted in 2× SSC containing 10 mM MgCl2 for 1 h at 37°C. The slides were finally mounted with 50% glycerol in PBS and observed with a Leitz Orthoplan microscope.

**RESULTS**

Laser scanning confocal microscopy. Twenty-four hours after duct ligation, acinar cells were easily recognized in the pancreatic exocrine parenchyma and were stained by the anti-amylase antibodies with particular intensity compared with the controls. On the other hand, 4 days after duct ligation, only a few weakly labeled amylase-containing cells were detected. In contrast, insulin-containing cells were present at all time points, predominantly within the islets of Langherans but also scattered in small clusters within the exocrine tissue. Twenty-four hours after ligation, it was possible to detect cells that simultaneously displayed positive stainings for amylase and insulin either within the islets of Langherans (Fig. 1a–d) or scattered in the exocrine tissue (Fig. 1e). According to staining intensities, some cells were predominantly immunoreactive for insulin (Fig. 1a–c), with low levels of amylase, whereas others showed strong labelings for both antigens (Fig. 1d and e). These cells, coexpressing amylase and insulin, were only rarely encountered in tissues from control animals.

Application of the double-labeling technique on isolated pancreatic cells maintained in culture allowed for the detection of a few cells simultaneously displaying positive signals for amylase and insulin (Fig. 1f and g). These cells were isolated, possessed single nuclei, and exhibited signals for amylase and insulin as well as a combination of both.

Light and electron microscopy. Twenty-four hours after ligation, the pancreas underwent advanced stages of involution of its acinar parenchyma, displaying signs of apoptosis, cellular disruption, accumulation of zymogen granules, swollen rough endoplasmic reticulum, and large autophagic vacuoles. The lumen of the ducts was enlarged, and the duct cells appeared hypertrophied and frequently in mitosis. Many granulocytes and mononuclear cells were detected in the connective tissue. In addition to these alterations, some endocrine β-cells displayed some zymogen-like granules (Figs. 2 and 3). These cells were found in endocrine cells located either at the periphery of the islets or within their core (Fig. 2). Close examination at high magnification of these cells clearly demonstrated that both types of granules were located within the same cells, the borders of which were well delineated by plasma membranes (Fig. 2). The zymogen-like granules of the endocrine cells were located either close to the plasma membrane or in the Golgi area (Figs. 2 and 3). When the immunogold technique was applied with different antibodies, the zymogen-like granules of the endocrine cells were intensely labeled for amylase. On the other hand, only insulin-secreting cells were found to display zymogen-like granules. Indeed, we were unable to detect any glucagon- or somatostatin-secreting cells that contained zymogen-like granules. In addition to zymogen granules, the mixed cells displayed large vacuoles, characteristic of autophagosomes, strongly labeled for amylase (Fig. 3b) and crinophagic bodies that contained amylase and/or insulin (Fig. 3c). The zymogen-like granules present within β-cells were frequently close to the Golgi complex (Fig. 3), which, in some instances, also showed a certain degree of labeling for amylase (Fig. 3a and b). In addition to these islet cells containing zymogen-like granules, cells located in the acinar tissue and apparently belonging to typical acini (Fig. 4a) were provided with an almost equal ratio of amylase-immunoreactive zymogen granules and insulin-immunoreactive β-cell-like granules (Fig. 4b). These cells, belonging to typical acini, were highly polarized, made junctional complexes with adjacent acinar cells (Fig. 4b), and outlined classical acinar lumens that were continuous with excretory ducts. Also, the delineation of these cells by their plasma membrane was prominent in the electron micrographs (Fig. 4). Although few in number, secretory granules simultaneously labeled for amylase and insulin were present in some of these cells. These double-labeled granules displayed the morphological features of mature β-cell granules. They were small in size with a defined dense double-labeled core and a clear halo (Fig. 4c). At day 4 after duct ligation, cells displaying both acinar and β-cell phenotypes were also encountered but with a much lower frequency.

In situ hybridization. To confirm that coexpression of amylase and insulin in the same cells reflects translational activities in these cells, we revealed the presence of amylase and insulin mRNAs by applying the in situ hybridization approach, using specific probes on consecutive tissue sections. The pattern of distribution of amylase and insulin mRNAs in the control pancreas was essentially that previously reported (14) and characterized by the exclusive expression of each messenger in the correspond-
ing acinar or islet cells. In contrast, in tissues from duct-ligated pancreas, such a rigid compartmentalization of the stainings was not respected. Indeed, some cells displayed signals for insulin as well as for amylase mRNAs. Such cells were encountered on tissues from 24-h ligation (Fig. 5) as well as 4 days after the ligation.

**DISCUSSION**

Pancreatic cells displaying both exocrine and endocrine phenotypes have been reported as occurring under physiological as well as experimental and pathological conditions. The general skepticism surrounding these cells has, however, hampered detailed studies that remained limited to simple morphological descriptions (23). The recent findings that, under well-defined stimuli (i.e., activin A, betacellulin, hepatocyte growth factor), a pancreatic tumor cell line (AR42J) normally expressing amylase can be induced to secrete insulin (21, 22) and that cells coexpressing amylase and insulin are present in the pancreas of interferon-\(\gamma\) (IFN-\(\gamma\)) transgenic mice (15) have encouraged the reopening of the "intermediate cell" file. The existence of such cells, challenged for several years, is now also supported by the description of an acinar-endocrine cell tumor (34), as well as by several examples of intermediate phenotypes in various cell types considered to result from transdifferentiation processes (7, 25, 32). Moreover, major improvements in tissue preparation procedures (application of cytochemical approaches such as immunocytochemistry and in situ hybridization as well as development of cell culture systems) have strengthened the reliability of these acinar-endocrine mixed cells. In addition to the immunocytochemical demonstration of insulin and amylase within the same cells, and occasionally even in the same granules, the in situ hybridization approach has revealed that these cells also express both corresponding mRNAs. Such observations reflect the simultaneous translational activity for amylase and insulin within a given cell. The combined morphological and cytochemical data, particularly the demonstration of double-labeled granules, the expression of both mRNAs together with the presence of isolated mixed cells in culture, and the location of mixed cells in typical acini, rule out previous suggestions of membrane fission and/or fusion between adjacent cells or the uptake of amylase granules from neighboring disrupted cells.

The appearance of these intermediate cells was induced, in the present study, by pancreatic duct ligation, an experimental condition known to stimulate pancreatic endocrine neogenesis (35). After 24 h of pancreatic duct ligation, intermediate cells coexpressing insulin and amylase together with their corresponding mRNAs were detected in the islets of Langerhans as well as in the exocrine parenchyma. Ultrastructurally, however, these cells showed differences with respect to the ratio of their amylase- to insulin-containing granules. Intermediate cells located within the islets of Langerhans

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**Fig. 2.** Tissue sections from a 24-h duct-ligated rat pancreas. (a) Semi-thin section illustrating an islet of Langerhans (IL) in which some cells located either at the periphery or in the core of the islet display some zymogen-like granules (arrows). Ac, Adinar cells. Magnification = ×2,800. (b) Electron micrograph of an insulin-secreting cell (Ins) surrounded by 2 acinar cells. Islet cell displays some zymogen-like granules (arrows). Delineation of each cell is particularly conspicuous by differences in electron density of the cytoplasm. DL, duct lumen. Magnification = ×10,000.
resembled normal β-cells due to the predominance of insulin-containing granules, whereas those located in the exocrine parenchyma had relatively equal proportions of insulin-immunoreactive granules and amylase-containing zymogen granules. Their location in the context of acini or islets of Langerhans and their appearance already within 24 h seem to exclude any ductal origin, as previously suggested in conditions of nesidioblastosis (4). On the other hand, it is worth noting that all intermediate cells detected in the pres-

Fig. 3. Electron micrographs of insulin-secreting cells in tissue sections of a 24-h duct-ligated rat pancreas. Double immunogold labeling for amylase (15-nm gold particles) and insulin (5-nm gold particles) was performed. a: Some zymogen-like granules (ZG) in the islet cells are located close to the Golgi complex (G) and are strongly labeled for amylase. Magnification = ×8,000. b: Large amylase-immunoreactive lysosome (L), resembling an autophagosome, located near a Golgi complex, which also displays a labeling for amylase. Magnification = ×12,000. c: Three lysosomes (L1–L3) within a single insulin-secreting cell show different immunocytochemical and morphological features. L1, labeled for insulin, presents the characteristics of a normal crinophagic body, whereas L2 is double labeled for both insulin and amylase and L3 is only amylase immunoreactive. L3 contains an almost intact amylase-positive zymogen-like granule (arrowheads). Magnification = ×34,000.
Fig. 4. Light and electron micrographs of tissue sections of a 24-h duct-ligated pancreas. a: Semithin section that illustrates the good preservation of the tissue. Framed region shows an apparently normal acinus. Magnification = ×500. b and c: Electron micrographs illustrating details of the acinus highlighted in a. Double immunolabeling for amylase (15-nm gold particles) and insulin (5-nm gold particles) was performed. b: Acinus is predominantly formed by cells provided with a mixture of amylase-immunoreactive zymogen (ZG) and insulin-containing granules (IG). L, acinar lumens; RER, rough endoplasmic reticulum. Magnification = ×26,000. c: High magnification of an insulin-like granule (arrow) displaying immunostainings for both amylase and insulin. This granule is surrounded by more classical amylase-immunoreactive zymogen and insulin-containing granules. Magnification = ×59,000.
ent study involved insulin-secreting cells. Together with the previous demonstration that duct ligation induces β-cell proliferation (35), these results suggest that intermediate cells could reflect transitional steps in an endocrine-acinar cell transdifferentiation process. The concept of cells undergoing a switching process between secretory programs is also supported by the presence within some of these cells of numerous autophagosomes containing amylase-immunoreactive secretory granules.

Factors triggering endocrine-acinar cell transdifferentiation, leading to the formation of new β-cells, could have a great impact in the treatment of IDDM. On the other hand, they could also be useful in elucidating the pathogenesis of the syndrome known as "hyperinsulinemic hypoglycemia" because pancreatic intermediate cells seem to be a common feature in patients affected by this syndrome (4, 9). Many factors such as IFN-γ, activin A, hepatocyte growth factor, and betacellulin have previously been reported as capable of influencing pancreatic cell differentiation and/or inducing a switch in their secretory pattern. The finding of intermediate cells with pancreatic duct ligation suggests that these factors are likely to be released under such condition. IFN-γ is an inflammatory cytokine that in particular circumstances can be secreted by epithelia and fibroblasts (29). Activin A, normally expressed in the islets of Langerhans (37), has been shown to be overexpressed after tissue injury (16). Betacellulin, secreted by β-cells (33), has been demonstrated to be required for the PDX-1-induced transcription of insulin by the glucagon-secreting tumor cell line αTC1 clone 6 and could modulate a generalized DNA demethylation (36). Activin A along with betacellulin or hepatocyte growth factor, the latter overexpressed after tissue injury as well (39), can convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells (21, 22). Due to analogy in experimental models, ilotropin (30) may also be one of the factors involved in the appearance of acinar-endocrine intermediate cells. Ilotropin is a factor expressed on chronic partial obstruction of the pancreatic duct and shown to reverse the streptozotocin-induced diabetes in hamsters through the appearance of a new generation of pancreatic β-cells (31).

The process of cell transdifferentiation is largely unknown and could follow several pathways according to cell types. Gene expression in transdifferentiating cells has been characterized step by step in one case only: the transformation of retinal pigmented epithelial cells (RPEC) into lens cells. In this instance, the activation of lens-specific genes occurs only after repression of the RPEC-specific genes, passing, however, through a dedifferentiated state (1). On the other hand, a direct transdifferentiation with the existence of mixed phenotypes has been reported in several cases (7, 25, 32), and pancreatic transdifferentiating cells could follow similar pathways with the simultaneous transcription of amylase and insulin mRNAs during transitional steps. Data from in situ hybridization and the autophagolysosomal activity seem to support this view. In such an eventuality, however, we should assume the capability for cell compartments, such as the Golgi...
complex, to discriminate between secretory products and to generate different types of secretory granules or envision two populations of Golgi complexes, each processing its own secretory product.

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