Gastrin Stimulates β-Cell Neogenesis and Increases Islet Mass From Transdifferentiated but Not From Normal Exocrine Pancreas Tissue

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It is still unclear which factors regulate pancreatic regeneration and β-cell neogenesis and which precursor cells are involved. We evaluated the role of intravenously infused gastrin in regenerating pancreas of duct-ligated rats. The ligation of exocrine ducts draining the splenic half of the pancreas resulted in acinoductal transdifferentiation within the ligated part but not in the unligated part. We found that infusion of gastrin from day 7 to 10 postligation resulted in a doubling of the β-cell mass in the ligated part as measured by morphometry. This increase in insulin-expressing cells was not associated with increased proliferation, hypertrophy, or reduced cell death of the β-cells. Furthermore, we found an increased percentage of single, extra-insular β-cells and small β-cell clusters induced by gastrin infusion. These changes occurred only in the ligated part of the pancreas, where transdifferentiation of the exocrine acinar cells to ductlike cells (metaplasia) had occurred, and was not found in the normal unaffected pancreatic tissue. In conclusion, we demonstrate that administration of gastrin stimulates β-cell neogenesis and expansion of the β-cell mass from transdifferentiated exocrine pancreas. *Diabetes* 51:686–690, 2002

The limited and insufficient amount of donor β-cells may become a critical factor in the treatment of type 1 diabetic patients, since the islet-transplantation strategy itself has been significantly improved (1). One option for compensating for this limited amount of β-cells is to generate a pool of new β-cells starting from their precursor population. Because there is evidence that these precursor cells reside in the exocrine acinar and ductal cell populations (2), we aimed to study the role of selected growth factors in the regenerative capacity of these exocrine pancreatic cells. Our study is based on the model of pancreatic duct ligation (PDL) in rats (3). In this model, the splenic portion of the rat pancreas is ligated, which results in a transdifferentiation of acinar to ductal cells in the ligated tail, whereas the head part remains unaffected in terms of histology (2,3). The term transdifferentiation is used to denote changes in the differentiation or phenotypic state at the cell level (4), meaning that the obtained phenotype can have adult as well as fetal characteristics. The cells in the ductal complexes consist of a mixture of transdifferentiated acinar cells and ductal cells, and eventually these cells can no longer be distinguished on the basis of morphological or immunophenotypic grounds (5,6). The obtained ductal complexes in the ligated portion of PDL show characteristics of normal adult duct cells, such as the expression of cytokeratin 7 (CK7) and CK20 (3) and flk-1 receptors for vascular endothelial growth factor (7), but also have features in common with the fetal duct cells, such as glut-2 (2,3) and, as we recently demonstrated, gastrin/cholecystokinin (CCK)-B receptors (8). Gastrin infusion in duct-ligated rats results in increased proliferation of the cells in these regenerative foci (8). Gastrin and its receptors are also known to be transiently expressed in the fetal pancreas in a period of pronounced islet neogenesis (8–11). Gastrin has a presumed neogenetic effect, since in double-transgenic mice overexpressing transforming growth factor (TGF)-α and gastrin in the pancreas, a doubling of the islet mass was found that could not be found with overexpression of TGF-α alone (12). Another study reported endocrine differentiation as a result of endogenous hypergastrinemia in a model of subtotal pancreatectomy (13). It was demonstrated that new β-cells are budding from the ductal complexes in PDL to build up endocrine islets (3). This process of neogenesis is most pronounced between 5 and 7 days after the surgical procedure of duct ligation (3). In our model of PDL (3), an internal control is present: the unligated part, which remains unaffected histologically and allows us to discriminate between effects of gastrin on normal exocrine tissue and on transdifferentiated cells. We evaluated the effect of intravenously administered gastrin (8,14) from day 7 postligation; this treatment lasted for 72 h.

RESEARCH DESIGN AND METHODS

Animals. Male Wistar rats (age 10–12 weeks; weight 250–300 g) were used for the pancreatic duct ligations (Proefdierencentrum, Heverlee, Belgium). Experiments were approved by the Free University of Brussels ethics committee.
Pancreatic duct ligation, gastrin infusion, and sampling procedures. The experimental set-up has been described (8). Rats were anesthetized with pentobarbital (Sanofi Sante Animale, Brussels, Belgium) and subjected to duct ligation of the splenic half according to a previously published protocol (3). Starting on day 7 after ligation, one group of the rats was chronically treated with nonsulfated gastrin-I (Sigma Chemical, St. Louis, MO) delivered at 2.5 nmol · kg^{-1} · h^{-1} via an indwelling catheter inserted in the right vena jugularis on the previous day (14). Rats in the control group received physiological

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BrdU, 5-bromo-2′-deoxyuridine; CCK, cholecystokinin; CK, cytokeratin; ODI, optical density of insulin stainings; PDL, pancreatic duct ligation; PGP9.5, protein gene product 9.5; TGF, transforming growth factor.

TABLE 1
Effects of gastrin in the insulin-expressing cell population

		Saline-infused		Gastrin-infused	
	n	Unligated	Ligated	Unligated	Ligated
Pancreatic weight (mg)	6–7	673 ± 37	298 ± 27*	631 ± 34	289 ± 30*
Absolute β-cell mass (mg)	6–7	6.2 ± 0.8	11.0 ± 1.9†	9.0 ± 2.1	21.8 ± 2.3‡
β-Cell size (μm^2)	5–6	163.7 ± 10.7	229.7 ± 14.4§	183.7 ± 12.3	211.2 ± 7.4
Absolute insulin content (μg)	4–5	32 ± 6	91 ± 17	23 ± 4	78 ± 14
ODI (arbitrary units)	3–4	14.0 ± 2.1	9.9 ± 0.5	22.4 ± 3.0	6.5 ± 0.8‖,¶
Small β-cell clusters (%)	3–5	10 ± 3	20 ± 1	11 ± 2	28 ± 5#,**

Results are presented for the unligated pancreatic head and the ligated pancreatic tail of saline- versus gastrin-infused rats. *$P < 0.005$ vs. the corresponding unligated part; †$P < 0.01$ vs. the unligated saline-treated part; ‡$P < 0.01$ vs. the unligated gastrin-treated and the ligated saline-treated part; §$P < 0.05$ vs. the unligated saline-infused part; ‖$P < 0.05$ vs. the ligated saline-treated part; ¶$P < 0.01$ vs. the unligated gastrin-treated part; #$P < 0.05$ vs. the ligated saline-treated part; and **$P < 0.005$ vs. the unligated gastrin-infused part.

saline treatment. The treatment lasted for 72 h, and rats were fasted for 2 h before being killed on day 10 after duct ligation. To study the cell replication, 5-bromo-2′-deoxyuridine (BrdU; Boehringer-Mannheim, Mannheim, Germany) was intraperitoneally injected at a dose of 50 mg/kg 1 h before death. At the time of death, glycemia levels in the tail vein were measured with Glucocard memory strips (A. Menarini Diagnostics, Florence, Italy). Plasma was sampled by heart puncture under pentobarbital anesthesia. Plasma insulin levels were determined as described previously (15). Pancreatic parts on both sides of the ligation were carefully dissected, weighed, and processed separately for paraffin embedding and/or determination of total insulin content (15).

Immunohistochemistry for phenotypic analysis. Ligated and nonligated parts of the pancreas, recognizable by the presence of the ligature, were separately processed for paraffin embedding. Paraffin sections (4 μm thick) were immunostained with the streptavidin-biotin method, as described (16,17). Endocrine β-cells were identified by a polyclonal guinea pig anti-insulin antibody (18). α-Cells were stained with anti-glucagon antibodies raised in rabbit (18). As a marker of acinar cells, we used a polyclonal rabbit antiserum against α-amylase (obtained from Dr. G. Klöppel, University of Kiel, Germany) (19). Duct cells were stained with the mouse monoclonal antibodies anti-CK20 (clone IT-Ks20) and anti-CK7 (clone OV-TL12/30) from Dako (Glostrup, Denmark). We have previously demonstrated the specificity of these antibodies (16,17). Antiserum to protein gene product 9.5 (PGP9.5) and peptide-blocked antiserum were from UltraClone (Isle of Wight, U.K.) (20). For cytokeratin staining, antigen retrieval was performed using 25-min treatment with 15 μg/ml proteinase K (Novocastra Laboratories, Newcastle, U.K.) at 37°C.

Morphometry. The percentage insulin-positive area (insulin immunoreactive area per micrometer squared of pancreatic epithelium in the sections) was measured by computer-assisted stereological morphometry (3,21). Fibrotic tissue present outside the pancreatic lobules was excluded from the measurements. β-Cell mass was calculated by multiplying the percentage insulin-positive area by the weight of the corresponding pancreatic portion. The individual β-cell area was determined as the insulin-positive area divided by the number of nuclei counted in the corresponding insulin-positive structures, which were chosen randomly and corresponded to 125–150 nuclei per sample. The number of single β-cells and small β-cell clusters was determined as the number of measurements with an area arbitrarily set at $<250\ \mu m^2$, expressed as the percentage of the total number of measurements in the section. Measurement of mean optical density of insulin stainings (ODI) was modified after Rahier et al. (22) and was measured by computer-assisted morphometry. Here, the measurements included only medium-sized and large islets, not single β-cells or very small insulin-positive groups (22). All values were corrected for the background staining in the exocrine tissue, and all sections were stained simultaneously with anti-insulin antibody to exclude variation in intensity due to the staining procedure. All analyses were performed at a microscopic magnification of ×200.

Immunohistochemical measurement of cell proliferation. In paraffin sections, double immunohistochemistry was applied for detection of BrdU combined with insulin or glucagon. For BrdU staining, antigen was retrieved with trypsin, DNA was denaturated with 2N HCl, and the reaction was stopped with borate buffer. Mouse monoclonal anti-BrdU was applied (Eurodiagnostics, Apeldoorn, the Netherlands). These immunocytochemical stainings were performed by the streptavidin-biotin method, as described (23).

Analysis of the apoptotic index. The frequency of apoptosis was analyzed as described previously by others (24) by a combination of immunocytochemistry for the appropriate cell marker (insulin) with a propidium iodide staining on RNAse-treated paraffin sections. Cells with nuclei that appeared condensed or fragmented under fluorescent light were considered apoptotic.

Statistics. Results are expressed as means ± SE. Data were analyzed by Student's t test, and statistical significance was considered at the confidence interval of $P < 0.05$.

RESULTS

Gastrin increases the β-cell mass. The weight of the pancreas was significantly lower in the ligated than the unligated parts: 673 ± 4 mg for the unligated part vs. 298 ± 3 mg for the ligated portion in controls, and 631 ± 3 mg for the unligated part vs. 289 ± 3 mg for the ligated part in gastrin-treated rats. When saline-infused rats were compared with gastrin-infused rats, the weight of both the unligated and ligated parts did not differ ($P = 0.429$ and $P = 0.827$, respectively). The total pancreatic weight did not differ between control and gastrin-treated rats (976 ± 4 mg vs. 920 ± 6 mg, respectively; $P = 0.405$). In the ligated part, the insulin-immunoreactive area increased from 3.7 ± 0.7% of the tissue area in saline-infused controls to 7.6 ± 0.8% in gastrin-infused rats (Table 1). The absolute β-cell mass doubled in the ligated part when gastrin was infused, whereas no changes were found in the unligated pancreas of saline-infused rats (Table 1). In the unligated pancreatic parts, no significant differences were found ($P = 0.157$) (Table 1). In control rats, we found that the β-cell size is higher in the ligated part than in the unligated part, so ligation induces hypertrophy of the β-cells. However, this difference was not statistically significant in gastrin-exposed rats ($P = 0.08$). The individual β-cell size in the ligated part from gastrin-infused rats was not significantly different from that of controls (Table 1), meaning that the gastrin treatment did not induce β-cell hypertrophy. Thus, the β-cell mass doubled during a 3-day infusion of gastrin, but only in the ligated portion of the pancreas. This increase in β-cell number was not reflected in the absolute insulin content of the pancreas, which did not differ from saline-infused controls (Table 1). However, the mean ODI differed between the islets from the ligated part of gastrin versus saline-infused rats, namely 6.5 ± 0.8 vs. 9.9 ± 0.5 arbitrary units, respectively (Table 1). This difference indicates that the amount of insulin per β-cell decreased during gastrin treatment, possibly as a mechanism to compensate for the increased number of cells expressing insulin. Significant differences in ODI were also found in unligated versus ligated pancreatic parts of the gastrin-exposed rats (Table 1), where the value was higher in the unligated part.

Morphological evidence of β-cell neogenesis. The rel-

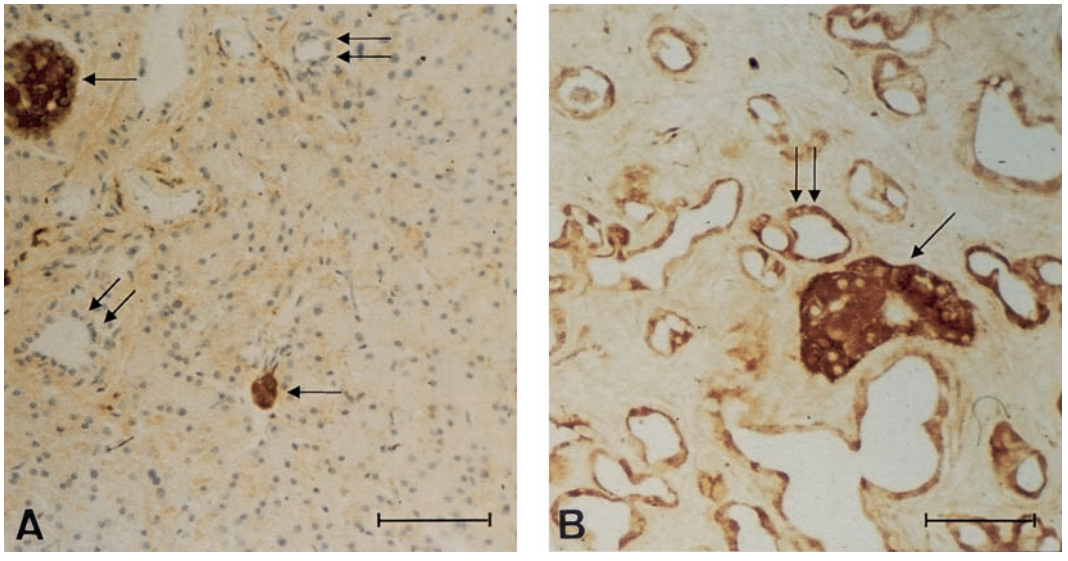

FIG. 1. Immunocytochemical staining for the pan-neuroendocrine marker PGP9.5 in endocrine islets of normal adult rat pancreas (*A*) and the transdifferentiated ductal complexes of the duct-ligated part of the rat pancreas (*B*). Single arrow, islet; double arrow, duct; bar, 100 μm.

ative number of single, extra-insular β-cells and small β-cell clusters was also significantly increased in the ligated part of gastrin-exposed rats (28 ± 5%) compared with the saline controls (20 ± 1%) (Table 1). This is an indication of increased neogenesis in the gastrin-treated rats. These single β-cells and small β-cell clusters were located within the ductal complexes. The transdifferentiated state of these ducts was also indicated by their expression of the pan-neuroendocrine cell marker PGP9.5, which otherwise is only detectable in islets (Fig. 1*A*). In the ligated part of saline- and gastrin-infused rats, practically all duct cells showed PGP9.5 immunoreactivity (Fig. 1*B*). Staining specificity was demonstrated by preabsorption of the antibody with the corresponding antigen (not shown).

Gastrin does not affect apoptosis or proliferation of β-cells. The BrdU-labeling index of the β-cells in the ligated pancreatic part of gastrin-exposed animals was comparable to that in saline-infused rats (0.9 ± 0.2% vs. 0.8 ± 0.2%, respectively; $n = 5$). The frequency of apoptotic β-cells in the ligated part of the gastrin condition (0.63 ± 0.03%) was not different from that of the control condition (0.47 ± 0.13%) ($n = 3$). This means that neither altered β-cell replication nor cell death contributed to the increase in the number of insulin-expressing cells.

Metabolic data. At the end of our study, all rats were found to be normoglycemic. Glycemia levels were 5.5 ± 0.2 mmol/l in gastrin-treated PDL rats vs. 5.3 ± 0.1 mmol/l in saline-infused PDL rats. The plasma insulin levels did not differ significantly (0.250 ± 0.085 vs. 0.185 ± 0.035 pmol/l) (n = 5-6).

DISCUSSION

In the model of pancreatic duct ligation, no histological changes occur in the unligated part; in the ligated part, exocrine acinar lobules are replaced by ductal complexes and new β-cells are generated from the latter (3). In the present study, we report that a 3-day continuous intravenous gastrin administration further increases expansion of the β-cell mass in the ligated pancreas and that this occurs by the process of neogenesis.

Infusion of gastrin increased the percentage of insulin-immunoreactive area versus the total pancreatic area in the sections, while the total tissue weight remained unchanged. This increased β-cell mass was not due to gastrin-induced hypertrophy of the β-cells, since the individual β-cell size remained constant compared with saline-infused rats. No protective effect of gastrin on β-cell death was found, since the frequency of apoptotic β-cells was not altered by gastrin administration. There remain two possible mechanisms to explain the observed β-cell mass expansion, namely, stimulation of β-cell proliferation or neogenesis from precursor cells (2). The present study revealed no mitogenic effect of gastrin on β-cells; in a previously published study using the same rat model, we demonstrated a strong mitogenic effect of gastrin on ductal cells in the regenerative foci (8). A mitogenic effect of gastrin on β-cells would seem unlikely, given that they do not express CCK-B/gastrin receptors, whereas expression of this receptor is induced in the exocrine cells during transdifferentiation, as demonstrated earlier (8). Therefore, increased neogenesis must have been responsible for the gastrin-stimulated expansion of the β-cell number. The observation of an increased frequency of single, extra-insular β-cells and small β-cell clusters in gastrin-treated animals points in this direction. We found that PGP9.5 is expressed in fetal pancreas and becomes restricted to the endocrine cells in the adult. Re-expression of this pan-neuroendocrine marker and of other markers such as glut-2 (3), flk-1 (7), and gastrin receptor (8) in duct-ligated regenerative conditions suggests that the cells in the regenerative foci become fetal-like. This may underline their differentiation potential, which can be similar to the cells in the fetal state, including the differentiation toward insulin-expressing cells. We conclude that gastrin extends the process of neogenesis that was already induced by the procedure of duct ligation (3). Wang et al. (25) reported that there is an upregulation of gastrin expression in the ductal complexes of the ligated part of the pancreas from 3 to 7 days postligation. Administration of exogenous gastrin from day 7 on, as in our experiments, may cause prolongation of the stimulatory effect of endogenous gas-

trin on proliferation (8) and differentiation of the endocrine precursors. So, in contrast to normal pancreatic exocrine cells, the transdifferentiated exocrine cells in duct-ligated tissue acquire the gastrin receptor, whereby they become responsive to the mitogenic (8) and neogenetic (this study) effects of gastrin. Important in this concept is that fully differentiated cells first change their differentiation state and then may become active in regenerative processes. Normally, gastrin exerts no mitogenic or neogenetic effect on the adult rat pancreas, but it may become an important factor when the target tissue undergoes significant remodeling.

Gastrin stimulated a near-doubling of the β-cell number in 3 days. This increase was not, however, reflected in the absolute insulin content of the pancreas, which was not significantly different from that of the control pancreas. However, we observed that the immunohistochemical expression level of insulin (ODI) in islets was lower in the ligated part of gastrin-exposed rats than in saline-exposed rats, whereas in the pancreas of gastrin-treated rats the ODI in the unligated part was higher than in the ligated part. This means that the total cellular insulin content of islets may be adapted to compensate for the increased cell number, resulting in a constant insulin mass. There were no indications of increased secretion of insulin, since no differences in plasma insulin levels were found. Correction of the insulin stores to maintain homeostasis may occur by intracellular degradation of insulin (26). We did notice that in the single β-cells and in the very small islets, the insulin staining was more intense than in the larger islets (data not shown), suggesting that the compensation in insulin content occurred in mature islets. β-Cell degranulation may also be proportional to the level of hypergastrinemia (27). However, stopping the gastrin infusion and waiting another week before evaluating plasma insulin levels and pancreatic insulin content did not show any further changes (not shown). Thus, the increased β-cell number seems to be compensated by a lower granulation or insulin biosynthesis.

Transdifferentiation of acini to regenerative foci of ductal cells appears to be a prerequisite for having gastrin effects, which is likely explained by the observation that these cells acquire the gastrin receptor during their transdifferentiation. Nesidioblastosis, or the apparent hyperplasia of islet cells, has been described in patients with pancreatic or duodenal gastrinomas classified as the Zollinger-Ellison syndrome (28). It was proposed that gastrin secreted from these tumors may exert a trophic effect on islet cells. It is not known, however, which other (growth) factors are produced by these tumors, nor what their effects are on the proliferation and differentiation of the exocrine cells. Interestingly, gastrinomas are also associated with varying degrees of pancreatitis, acinoductal conversion, and inflammation, which may be at least partly caused by duct obstruction due to the presence of tumors in the pancreas (29,30). There may thus exist similarities between these human pathologies and our experimental model. On the other hand, it is also important to note that species differences exist in the expression of CCK receptors, as human exocrine cells do express CCK-B/gastrin receptors and rat exocrine cells normally do not (31). The effects of gastrin on human pancreatic cells remain yet to be unraveled.

Not only during pancreatic regeneration (as in the case of duct ligation [25]) but also during cancer development, gastrin and its receptors become expressed and gastrin exerts its mitogenic effect on the pancreas (32–34). This may represent a recapitulation of embryonic development, because it is known that gastrin and its receptor are expressed in the fetal pancreas (8–11), and a role of gastrin in pancreatic development has been postulated (10,35).

In double-transgenic mice, gastrin was reported to stimulate β-cell neogenesis in the pancreas that overexpressed TGF-α but not in the normal pancreas (12). In the TGF-α–overexpressing pancreas, acinoductal transdifferentiation or metaplasia was shown to occur (5). It is striking that in most experimental models of islet regeneration, the neogenetic process is preceded by the appearance of ductal complexes, from which the newly formed β-cells bud off (3,12,36,37). These ductal complexes represent transdifferentiated tissue, as in the model of duct ligation (2). Thus, we have proposed the hypothesis that adult exocrine cells, both acinar and ductal, can transdifferentiate to a fetal state (de-differentiation), wherein they may reacquire multipotential precursor capacity. In vitro, we have shown that adult acinar cells can be considered at least as bipotential precursor cells, since they can obtain a phenotypic state from which they are able to transdifferentiate to either duct cells or acinar cells (38). There is evidence from in vivo studies that acinar cells can transdifferentiate into β-cells (39,40), and the acinar cell line AR42-J can be stimulated to transdifferentiate into β-cells (41–43). In vitro, rat and human exocrine cells were shown to transdifferentiate to ductlike cells in a period of 3–5 days (38,44). The resulting cells had a phenotype that is quite similar to what is observed in the regenerative complexes in vivo after duct ligation. Therefore, in vitro experiments that try to reproduce this phenomenon seem to be promising. By infusing 2.5 nmol · kg^{-1} · h^{-1}, it was reported that a level of ~0.15 nmol/l gastrin is reached in the serum (14), which is in a range similar to the concentrations we used in culture experiments. Experiments in vitro showed that gastrin exerts a strong mitogenic effect on ductlike cells, obtained by culturing acinar cells, as described previously (8). However, in these monolayers, we could not detect an increase in the number of insulin-expressing cells (unpublished observations). These preliminary in vitro observations may indicate that additional factors, perhaps extracellular matrix components, are required to reproduce the in vivo effects of gastrin.

The potential islet precursor cells may reside in apparently terminally differentiated exocrine acinar and duct tissue instead of in a small, yet undefined, population of undifferentiated stem cells.

The combined result of growth and differentiation of the presumed precursor cells that is stimulated by administration of gastrin may be promising for applications in the treatment of diabetes. Stimulation of neogenesis might find application in islet cell transplantation to increase the number of donor cells or in regeneration of the endogenous β-cells in diabetic patients.

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