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Isolation and Culture of β-Like Cells From Porcine Wirsung Duct

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ABSTRACT

We sought to develop a protocol to isolate and culture porcine Wirsung duct cells in order to determine their potency to differentiate into insulin-expressing β-like cells. The porcine Wirsung duct isolated by a surgical microdissection was digested with collagenase P and trypsin to dissociate ductal cells. These elements were cultured in serum-free supplemented media: for 2 weeks. Thereafter the cells were exposed to varying concentrations of glucose (0, 5.6, 17.8, and 25 mmol/L) to induce a β-like phenotype, as identified by immunohistochemical staining. Cell growth proceeded slowly for the first 2 weeks of culture. After glucose induction for 2 weeks, they formed pancreatic islet-like structures. These cells were stained for the pancreatic ductal cell marker cytokeratin-19 (CK-19) and the pancreatic endocrine markers insulin and glucagon. After the second week, 90% of cells were positive for CK-19. Up to 20.1% of the cells in pancreatic 3-dimensional structures induced by 17.8 mmol/L glucose were positive for insulin, and <3.2%, for glucagon. The positive ratio of immunoreactive staining was dependent on the glucose concentration; 17.8 mmol/L glucose effectively stimulated insulin- and glucagon-secreting cells. We concluded that porcine Wirsung duct cells were capable of proliferation with the potential to differentiate toward β cells upon glucose induction in vitro.

DIABETES CONSTITUTES a major health care problem.[1–3] Although insulin therapy is successful,[4] diabetic patients still suffer complications, especially nephropathy, retinopathy, and cardiovascular diseases.[5–7] Many efforts have therefore been directed toward methods to replace β cells. Transplantation therapies for diabetes have been developed, but islet cell transplants still involve problems of immune rejection and donor supply.[8,9] One alternative is to generate β cells in vitro using islet progenitor cells—adult or embryonic stem cells. No direct progenitor cells have been identified from bone marrow, peripheral blood, or umbilical cord blood.[10] The pancreas is composed of exocrine (duct and acinar) and endocrine compartments, but embryonically all the cells differentiate from a common ductal epithelium.[11] During pre- and postnatal development, β-cell expansion with an increase in islet size results from both addition of new islet cells and increase in individual cell volume.

The aim of the present study was to induce differentiation of ductal cells[12,13] from which β cells arise during normal development in the mouse and in humans.[11] We sought to investigate the capacity of cells from a different mammalian species to differentiate into insulin-producing cells. We evaluated the culture conditions, suitable extracellular matrices, and soluble factors that push differentiation of ductal cells to β cells. We then assayed insulin secretion under various culture conditions. One particular difference in the present investigation was our method of isolation of ductal cells from the porcine Wirsung duct through microsurgical dissection compared with previous techniques, which allowed us to obtain these cells without using the whole pancreas.

MATERIALS AND METHODS

For our study, we used whole pancreata of 12- to 18-month-old pigs of average weight (120 ± 40 kg). Pancreata were collected by a non–heart-beating technique after complete exsanguination of the animal following the guidelines approved by our department.

On the operative table we identified 3 sites of the pancreas region and avoided direct traction on the organ itself: the pylorus, Treitz angle at the end of the duodenum and the beginning of the small intestine, and the spleen, which in the pig has a "dog's

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tongue" shape in the pancreatic tail. The whole duodenal tract until the Treitz ligament was isolated and bound with 2 strong sutures and then cut. In the porcine pancreas there are some embryonic residues. The pancreas head is not compacted but is more like a soft tissue spreading on the region from the pylorus to the Treitz angle which is the reason to include it amid the collected tissues. Opening the gastrosplenic ligaments with a section of short vascular structures allowed us to access the lesser sac and the anterior dislocation of the spleen, which we used as a handle to separate the posterior pancreas face from connective tissue by delicate dissection with a blunt forceps. The duodenal C loop was mobilized from the peritoneal ligament by a Kocher manuever that allowed us to dislocate the pancreas head toward the midline. The transverse colon was retracted, putting tension on the ligament between the colon and spleen, and then divided. The gastrohepatic ligament, common bile duct, portal vein next to the hepatic vessels, an aortic patch, including the origin of celiac trunk, the superior mesenteric artery, and the mesenteric vessels along the inferior pancreas side were then serially sectioned. To reduce contamination the duodenum was washed with saline-buffered solution containing antibiotics and antifungals by performing a "round block" purse-string suture on its pyloric side through which we introduced a 3-way Foley catheter with an inflated retaining balloon. After washing we started to separate the duodenum and fatty tissue from the pancreas.

We localized the Wirsung duct with a microsurgical technique (loops 2.5×). It was sectioned to introduce an 18-gauge catheter cannula (Fig 1), via which we perfused about 50 mL of 4°C Hanks' solution (Sigma-Aldrich) containing antibiotics (200 U/mL penicillin, 200 μg/mL streptomycin) and antifungal drugs with the purpose to wash out residual exocrine ducts, cool the parenchyma, and sterilize the ductal cavity. We dissected the Wirsung duct and its major ramifications using the same catheter as a support for the delicate manipulation.

Specimen

The ducts were stored in Hanks' solution at 4°C with 200 U/mL penicillin, 200 μg/mL streptomycin, and 2% fungizone. After Hanks' solution was removed, the ducts were washed with 50 mL of medium A: CMRL 1066, 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 0.1 IU/mL human insulin, 200 IU/mL penicillin, 200 μg/mL streptomycin, 2% fungizone, and 2 μg/mL dexamethasone. After incubation at 37°C 5% CO_2 for 2 to 3 days to permit ductal expansion, the supernate was removed and ducts were washed 3 times with medium B: 15 mmol/L DMEM/F12-HEPES, 0.1 mg/mL trypsin inhibitor, 30 mg/mL bovine serum albumin (BSA), 2 mmol/L L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, 2% fungizone, pH 7.4.

Cell Culture

Tissue was transferred to digestion medium C: 15 mmol/L DMEM/F12-HEPES, 0.1 mg/mL trypsin inhibitor, 30 mg/mL BSA, 2 mmol/L L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, 2% fungizone, 1 mg/mL collagenase P (Roche), pH 7.4. After mincing into small fragments and incubation for 40 minutes at 37°C 5% CO_2, the supernate of centrifugation at 1000 rpm for 10 minutes was discard; the remaining tissue-digested in the same medium with 2 mg/mL trypsin for 30 minutes at 37°C followed by centrifugation for 10 minutes at 1000 rpm. The supernate was discarded; the pellet was washed 3 times with medium B followed by centrifugations for 3 minutes at 2000 rpm to obtain a second pellet. The pellets were suspension in medium D: Ca^{2+}/Mg^{2+}-free Krebs' Ringer buffer, 15 mmol/L HEPES, 145 mmol/L NaCl, 4.5 mmol/L KCl, 2 mmol/L EGTA, 5.6 mmol/L glucose, 200 U/mL penicillin, 200 μg/mL streptomycin, 2% fungizone, pH 7.4. After incubation for 10 minutes at 37°C 5% CO_2, the cells were distributed into nontreated T-75 flasks (Becton Dickinson) for incubation for 4 days at 37°C 5% CO_2 in medium E: CMRL 1066, 10% FCS, 2 mmol/L L-glutamine, 0.1 Iu/L human insulin, 2 μg/mL dexamethasone, 200 U/mL penicillin, 200 μg/mL streptomycin, 2% fungizone, 5.6 mmol/L glucose, pH 7.4.

Cell Induction and Differentiation

After 4 days we removed suspended cells and harvested the adherent cells to layer in Matrigel (Collaborative Research-Becton Dickinson) with the glucose-free medium F: 15 mmol/L DMEM/F12-HEPES, 2 mmol/L L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, 2% fungizone, 1 g/L insulin-transferrin-sodium selenite (ITS; Sigma-Aldrich), 2 g/mL BSA, 10 mmol/L and 20 ng/mL keratinocyte growth factor (KGF; Roche Diagnostics GmbH). After another 7-day incubation to promote the association of ductal cells in 3-dimensional structures, the flasks were washed 3 times in medium G: RPMI, 5 mmol/L glucose, 10 mmol/L HEPES, 200 U/mL penicillin, 200 μg/mL streptomycin, 10% FBS. After 15 aliquots from each flask were incubated in the same media in 15-well plates for 4 hours at 37°C, the media were removed to measure preincubation insulin levels, and fresh media added for another 24-hour incubation. The next day the media were removed and measured for basal insulin secretion. Three-dimensional structures were subdivided into 4 groups to be exposed to varying glucose concentrations: glucose-free (control), 5.6, 17.8, and 25 mmol/L for a 2-week incubation. Measurements of insulin and glucagon in the media were performed using a radioimmunoassay kit.

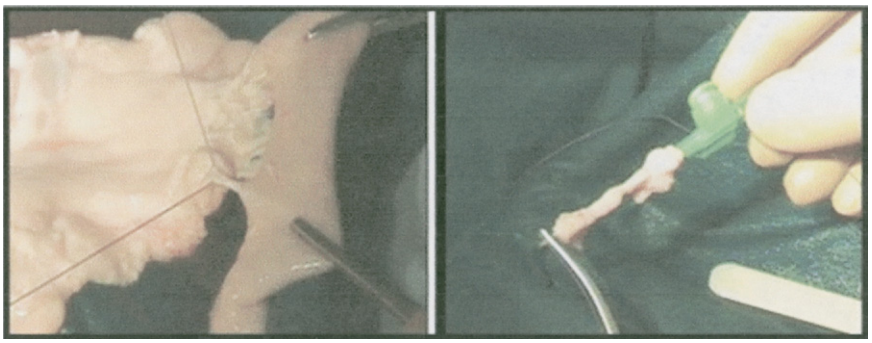

Fig 1. Isolation of porcine Wirsung duct.

Tissue Fixation and Immunochemistry

Monolayer cells were fixed for 30 minutes in 40 g/L polyformaldehyde (PFA) in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.2). After incubation with 0.3% H_2O_2 in pure methanol for 30 minutes at room temperature and retrieval 3 times (1 minute each time) in 0.1 mol/L pH 6.0 citrate buffer in a microwave (750 W) the specimens were incubated with 10 g/L BSA plus 4 g/L Triton X-100 at 37°C for 30 minutes.

Immunocytochemical staining used primary antibodies from different species: mouse monoclonal antihuman insulin antibodies (1:200); rabbit polyclonal antihuman glucagon antibodies (1:100); or mouse monoclonal anticytokeratin-19 (anti-CK-19) antibodies (1:50). The secondary antibodies used for immunofluorescence were: donkey antimouse IgG Texas red (1:200); goat antirabbit IgG fluorescein isothiocyanate (FITC; 1:200); or goat antimouse IgG FITC (1:250). All the antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif, United States). The specimens were mounted cell-side down on glass slides with a drop of DPX mounting medium (Merck Chemicals-Italy) for examination using a Zeiss light microscope.

RESULTS

The cells grew slowly during the first 2 weeks; 90% (885/984) of them were positive for CK-19 staining. With the additional time of 3 to 4 weeks, the cells grew from adherent clumps to form a monolayer with clear epithelial morphology. After 2 weeks of induction culture in media without glucose (Fig 2), the total counting of cells for CK-19+ was 885/984 (90%), while 80/1114 (7.2%) were positive for insulin and 11/995 (1.1%) were positive for glucagon. Using a glucose concentration of 5.6 mmol/L, CK-19-positive cells were 765/1021 (75%) with 138/1109 (12.5%) insulin positive and 14/1092 (1.4%) glucagon positive. In medium containing 17.8 mmol/L glucose, 526/1013 (52%) were CK-19-positive, 200/996 (20.1%) insulin positive, and 31/987 (3.2%) glucagon positive. When the concentration of glucose was 25 mmol/L, the CK-19-positive cells were 243/1008 (24%); insulin-positive cells, 227/1058 (21.5%); and glucagon-positive cells, 28/1038 (2.7%). The insulin content of samples at 2 weeks with 0 mmol/L was not detectable; in contrast; with 5.6 mmol/L glucose, it was 1.9 ± 0.1 ng/mL; 2.8 ± 0.3 ng/mL with 17.8 mmol/L glucose; and 4.2 ± 0.5 ng/mL with 25 mmol/L glucose (Fig 3).

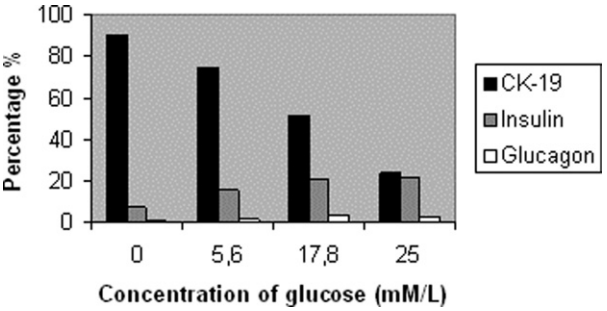

Fig 3. Percentages of immunopositive cells induced by varying concentrations of glucose.

DISCUSSION

Although pancreas transplantation represents a valid replacement for insulin therapy in type 1 diabetes, clinical results show difficulty in patient management. The main problems are the scarce organ availability, the low quantity of islet tissue recovered, and the selection of optimal immunosuppressive therapy. In the past the scarce availability of pancreas induced scientists to choose the porcine model to obtain insulin-producing tissue which showed biochemical affinities with human insulin. When ductal cells are placed on an extracellular matrix substrate, they differentiate into 3-dimensional islet-like structures. These data confirmed the hypothesis that pancreatic ductal cells have the potential to lose their specific ductal epithelial phenotype in vitro, reverting to multipotent cells able to differentiate into insulin-producing cells under the influence of appropriate exogenous factors. We have been able to culture porcine Wirsung duct cells and induce them toward insulin- and glucagon-producing cells. The capacity to cul-

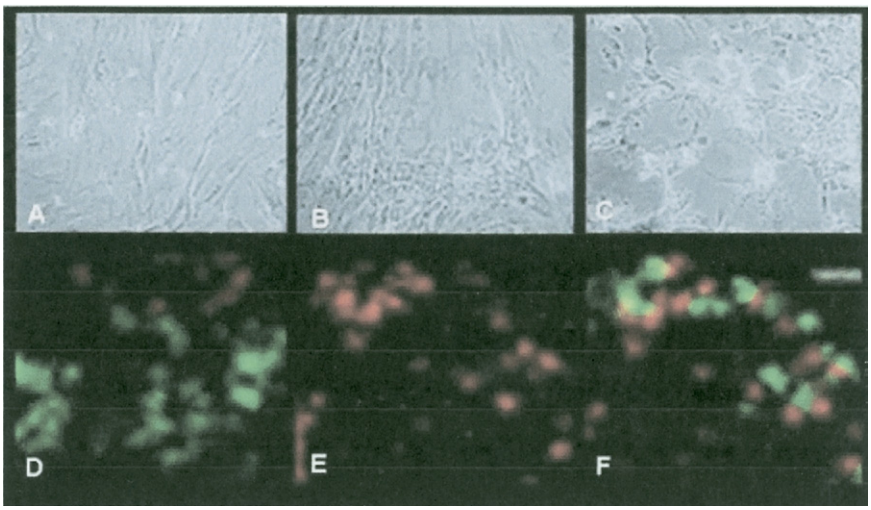

Fig 2. Differentiation of pancreatic ductal stem cells into insulin-producing cells: **(A)** free ductal cells after 2 weeks of culture; **(B)** cell association in adherent monolayer at 4 weeks; **(C)** 3-dimensional structure differentiation after being cultured in media containing 17.8 mmol/L glucose for 1 week. Immunostaining; **(D)** CK-19-positive ductal cells (FITC green) after 2 weeks; **(E)** insulin-positive ductal cells (Texas red) after 4 weeks. Double immunostaining: **(F)** glucagon (FITC green)-positive ductal cells and insulin-positive cells (Texas red) after being cultured in media containing 17.8 mmol/L glucose for 1 week.

tivate islets from digested porcine pancreatic ductal cells opens a new approach for β-cell therapy in animal experimentation.

We sought to obtain insulin-producing cells from elements isolated by microsurgical dissection of the porcine Wirsung duct without having to use the whole pancreas. In fact, obtaining ductal cells from the whole pancreas would involve the use of separation gradients with a high probability of the presence of both exocrine cells and endocrine cells in the digest. The mixture would interfere with separating individual epithelial cells. We have been able to repeatedly effect the protocol, due to the plentiful supply of pancreata from a near by slaughterhouse. Nevertheless, there has been a notable difficulty to get samples under sterile conditions requiring the use of abundant amounts of antiseptic and antifungal drugs. In our study, having isolated and selected cells in the presence of Matrigel in serum- and glucose-free conditions using keratinocyte growth factor and nicotinamide, we obtained 3-dimensional structures. We have shown that increasing glucose concentrations in the culture media (to 17.8 mmol/L) lead to a decreased number of CK-19-positive cells, but an increased number of insulin- and glucagon-producing cells, as previously reported.[14] Although limited, our results nevertheless provide a basis for expansion and differentiation of these elements, but doubts still remain concerning the resistance of such cells. We need to better characterize these cells to discover more about the bases of their transdifferentiation.

Despite the limitations of this initial phase, these findings raise the tantalizing possibility that this approach, once optimized, could produce meaningful amounts of insulin-producing cells from porcine Wirsung duct cells for in vivo use as transplants through the portal system to new onset diabetic mice. This approach could be the first step toward a protocol for the use of Wirsung duct bioptic fragments of the same diabetic patient withdrawn via endoscopic or video-laparoscopic techniques. Such cells, opportunely transdifferentiated and expanded in vitro, would possibly represent an inexhaustible insulin-producing cell source. Due to their autologous origin, they would resolve the inevitable complications tied to immunosuppressive therapy.

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