Cultured pancreatic ductal cells undergo cell cycle re-distribution and β -cell-like differentiation in response to glucagon-like peptide-1

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Abstract

The intestinal hormone glucagon-like peptide-1 (GLP-1) has been shown to promote an increase in pancreatic β -cell mass via proliferation of islet cells and differentiation of non-insulin-secreting cells. In this study, we have characterized some of the events that lead to the differentiation of pancreatic ductal cells in response to treatment with human GLP-1.

Rat pancreatic ductal (ARIP) cells were cultured in the presence of GLP-1 and analyzed for cell counting, cell cycle distribution, expression of cyclin-dependent-kinase (Cdk) inhibitors, transcription of β -cell-specific genes, loss of ductal-like phenotype and acquisition of β -cell-like gene expression profile.

Exposure of ARIP cells to 10 nM GLP-1 induced a significant reduction in the cell replication rate and a significant decrease in the percentage of cells in S phase of the cell cycle. This was associated with an increase in the number of cells in G0-G1 phase and a reduction of cells in G2-M phase. Western blot analysis for the Cdk inhibitors, kinase inhibitor protein 1 (p27^{Kip1}) and Cdk-interacting protein 1 (p21^{Cip1}), demonstrated a significant increase in p27^{Kip1} and p21^{Cip1} levels within the first 24 h from the beginning of GLP-1 treatment. As cells slowed down their proliferation rate, GLP-1 also induced a time-dependent expression of various β -cell-specific mRNAs. The glucose transporter GLUT-2 was the first of those factors to be expressed (24 h treatment), followed by insulin (44 h) and finally by the enzyme glucokinase (56 h). In addition, immunocytochemistry analysis showed that GLP-1 induced a time-dependent down-regulation of the ductal marker cytokeratin-20 (CK-20) and a time-dependent induction of insulin expression. Finally, GLP-1 promoted a glucose-dependent secretion of insulin, as demonstrated by HPLC and RIA analyses of the cell culture medium.

The present study has demonstrated that GLP-1 induces a cell cycle re-distribution with a decrease in cell proliferation rate prior to promoting the differentiation of cells towards an endocrine-like phenotype.

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Introduction

Growth and differentiation of islet β -cells is not limited to the embryological state (Hellestrom *et al.* 1996, Bonner-Weir 2000a). A constant remodeling of size and function of the islets of Langerhans occurs during the entire life of individuals and is likely to play an essential role in the prevention of diabetes (Bonner-Weir 2001). The mass of β -cells is dynamically regulated to maintain euglycemia and

its expansion has been proposed to be a compensatory mechanism for glucose homeostasis (Bonner-Weir 1994). An increase in the overall islet mass has been shown in animals subjected to prolonged glucose infusion (Bonner-Weir *et al.* 1988), as well as in animal models of insulin resistance (Pick *et al.* 1998, Finegood *et al.* 2001) or in human subjects with obesity (Klöppel *et al.* 1985), and finally during pregnancy (Sorenson & Brelje 1997). Failure to increase β -cell mass to compensate for an increased

demand for insulin secretion leads inevitably to the onset of diabetes. The identification of cells capable of undergoing differentiation into insulin-producing cells, as well as the characterization of stimuli capable of promoting the acquisition of such a phenotype, may provide important insights in the designing of novel therapeutic agents capable of enhancing the proliferation and/or differentiation of β -cells so as to restore normal glycemia in subjects with diabetes.

It has been proposed that in adulthood two independent pathways are utilized for the proliferation of pancreatic endocrine cells: with the first one, new endocrine cells arise from the division and differentiation of cells within the islets; while with the second pathway newly formed islet cells originate from precursor cells located in the pancreatic ductal epithelium (Brockenbrough *et al.* 1988, Bonner-Weir *et al.* 1993, Gu & Sarvetnick 1993, Wang *et al.* 1995, Fernandes *et al.* 1997, Waguri *et al.* 1997, Anastasi *et al.* 1999, Perfetti *et al.* 2000, Hui *et al.* 2001). In adult animals, the onset of hyperglycemia that follows subtotal pancreatectomy (Sharma *et al.* 1999) or treatment with the toxic agent streptozotocin (Wang *et al.* 1996) are characterized not only by a reduction of the pre-existing islet mass, but also by a compensatory attempt to replace the normal population of insulin-secreting cells. New β -cells are formed from existing islets and from ductal epithelial cells (Bonner-Weir *et al.* 1993). The latter source has greater intrinsic biological relevance. Indeed, the possibility of differentiating insulin-secreting cells from non-endocrine cells supports the hypothesis that the biological source (pancreatic ductal epithelium) for this compensatory mechanism may be present even in the setting of a generalized destruction of the entire population of islet β -cells. This is strongly supported by recent studies demonstrating that primary cultures of epithelial ductal cells (from human and mouse pancreas) are susceptible to undergo differentiation into endocrine cells (Bonner-Weir *et al.* 2000b, Ramiya *et al.* 2000).

It is likely that a co-ordinated activation of multiple differentiation factors, in a fashion similar to the sequence of events occurring during fetal development, may be required for the cellular growth of the endocrine pancreas in adults. The mechanism(s) for the activation of such a complex regulatory network in adulthood is not known. We have previously demonstrated that treatment of

glucose-intolerant aging Wistar rats with the incretine hormone glucagon-like peptide-1 (GLP-1) restored normal glucose tolerance and induced islet cell proliferation (Wang *et al.* 1997, Perfetti *et al.* 2000). Similarly, Xu *et al.* (1999) demonstrated that an analogue of GLP-1, termed exendin-4, was able to increase islet mass in adult animals previously subjected to subtotal pancreatectomy. These studies suggest that exogenously administered stimuli are able, *in vivo*, to increase the mass of insulin-secreting cells and ameliorate glucose tolerance by inducing neogenesis of islet cells. Our previous studies have indicated that, by culturing pancreatic ductal cells with GLP-1, we could induce their differentiation into insulin-producing and -secreting cells (Hui *et al.* 2001). To gain additional insight into the mechanism(s) by which GLP-1 produces differentiation of insulin-producing cells, in the present study we analyzed the effect of GLP-1 on cell cycle distribution and G1 phase cyclin-dependent kinase (Cdk) inhibitor expression, as well as on the loss of the ductal-like phenotype, leading finally to the acquisition of a β -cell-like gene expression profile.

Materials and Methods

Cell culture

Rat pancreatic ductal (ARIP) cells were cultured in F12 medium (Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL), 50 μ g/ml streptomycin and 100 μ g/ml penicillin at 37 °C under a humidified condition of 95% air and 5% CO₂. Cultures used for cell cycle analysis and immunocytochemistry were grown to approximately 70% confluence in serum-containing medium and cells were deprived of serum for 6 h before the addition of GLP-1 (10 nM; Bachem, Torrance, CA, USA).

To determine the effect of GLP-1 on p27 or kinase inhibitor protein 1 (p27^{Kip1}) and p21 or Cdk-interacting protein 1 (p21^{Cip1}) expression, ARIP cells were cultured to 50% confluence ($\sim 2 \times 10^6$ cells) in serum-containing medium and then serum starved for 24 h in serum-free F12 (time 0). The cells were re-exposed to serum-containing medium for 24 h before being cultured for increasing lengths of time with or without GLP-1 (10 nM). The glucose concentration in the culture medium was 12 mmol/l.

Fluorescent bead-based flow cytometric counting of synchronized cells

Exponentially growing ARIP cells were exposed to thymidine (final concentration 2 mM) for 12 h, then washed twice, and resuspended in serum-containing medium. After 12 h their growth was arrested by incubating the cells in the presence of thymidine (final concentration 2 mM) for an additional 12 h. After two washings with fresh serum-containing medium, the cells were exposed to normal growth medium, in the presence or absence of GLP-1 (10 nM). GLP-1 was added every 8 h for different lengths of time (6, 10, 14, 18, 24, 36, 54 h). At the end of the treatment the cells were trypsinized, washed in 5 ml serum-containing medium and resuspended in 1% paraformaldehyde. Ten microliters of fluorescent bead solution (SpheroTM rainbow fluorescent particles; PharMingen, San Diego, CA, USA) were added to each sample immediately before analysis. Samples were analyzed by FACScan (Becton Dickinson, San Jose, CA, USA) with Cell Quest 3.1 acquisition software (Becton Dickinson). Fluorescent beads were used as a counting reference for the quantitative analysis of cells and then, based on the simultaneous analysis of a fixed number of beads and an unknown number of cells, the following formula was used to calculate the number of cells present in the samples: number of cells = number of counted cells / (number of counted beads / number of beads added) $\times$ volume of the sample.

Cell cycle analysis

Cell cycle distribution was analyzed by fluorescence-activated cell sorting (FACS) analysis of DNA content. The cells were incubated in serum-free medium with or without GLP-1 (10 nM) for increasing lengths of time, trypsinized, washed in 5 ml serum-containing medium, resuspended in 1 ml cold phosphate-buffered saline (PBS) and fixed in cold ethanol (100%). Fixed cells were washed with PBS and incubated with propidium iodide (1 μ g/ml in PBS) for 1 h at room temperature in the dark. Analysis of DNA content in propidium iodide-stained cells was performed by FACScan. Cells were excited at 480 nm (15 mW) using an argon ion laser, and red fluorescence (as a measure of the total DNA content) was recorded at 575/26 nm with a band-pass filter. The percentages of cells in G1, S and G2-M phases of the cell

cycle were determined using ModFit LT (Verity Software House, Inc, Topsham, Maine).

Cell viability was evaluated by the Trypan blue dye (Gibco-BRL) exclusion technique.

Immunoprecipitation and immunoblotting

Cells cultured in the presence or absence of GLP-1 were collected and washed twice with cold PBS and lysed in 500 μ l M-PER lysis buffer (Pierce, Rockford, IL, USA) containing proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 8.3 μ M aprotinin, 50 μ M leupeptin and 30 mM sodium orthovanadate) (Sigma Chemicals, St Louis, MO, USA) and spun at 90 000 *g* for 5 min at room temperature. The lysate was collected and centrifuged at 13 000 *g* for 5 min at 4 °C. One microgram of control IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) together with 20 μ l Protein-G-agarose (Santa Cruz) were added to the whole cell lysate at 4 °C for 30 min. The protein concentration was then measured in the supernatant at least twice using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Soluble proteins (200–500 μ g) were incubated overnight at 4 °C on a rotating device with 1 μ g anti-p27^{Kip1} (Transduction Laboratories, Lexington, KY, USA) or anti-p21^{Cip1} (PharMingen, San Diego, CA, USA) and 50 μ l agarose-conjugated Protein-G. The beads were washed with 10% M-PER lysis buffer in PBS, and the pellet was resuspended in 30 μ l electrophoresis sample buffer for immunoblotting.

For immunoblotting, precipitated proteins were separated on 12% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were then soaked in Poinceau-S solution (Sigma) to verify that an equal amount of proteins was loaded on the gel and the homogeneity of the transfer. After incubation of the membrane with 3% bovine serum albumin (Sigma) in PBS–Tween 20 (Sigma) for 1 h at room temperature to inhibit non-specific binding, the membranes were immunoblotted with anti-p27^{Kip1} (1:1000) or anti-p21^{Cip1} (1:500) for 1 h at room temperature. After three washes in PBS–Tween 20 the membranes were incubated with a secondary antibody conjugate with horseradish peroxidase for 45 min at room temperature. Immunoreactive proteins were visualized by the ECL immunodetection system (Amersham Pharmacia Biotech, Amersham, Bucks, UK).

Table 1 PCR primers

	Primer sequence	Size of PCR product (bp)	Gene Bank accession number
Target gene			
Insulin	CCTGCCCAGGCTTTTGTCAA(+) CTCCAGTGCCAAGTCTGAA (-)	187	J00747
GLUT2	TTAGCAACTGGGTCTGCAAT(+) GGTGTAGTCCTACACTCATG(-)	343	J03145
Glucokinase	AAGGGAACCAACATCGTAGGA(+) CATTGGCGGTCTTCATAGTA(-)	136	AH002177
β -Actin	CGTAAAGACCTCTATGCCAA(+) AGCCATGCCAAATGTCTCAT(-)	349	V01217

RT-PCR of synchronized cells

ARIP cells were synchronized as described for cell counting and then exposed to F12 medium, in the presence of GLP-1 (10 nM/l). GLP-1 was added every 8 h, and cells were harvested after 12, 24, 30, 36, 44, 50, 56, 68 or 74 h. At the end of the treatment the culture medium was removed and the cells were washed twice with serum-free medium. Total RNA was extracted using the TRIazol-method (Gibco-BRL), and treated with DNase (amplification grade; Gibco-BRL) in 20 mmol/l Tris-HCl (pH 8.4), 2 mmol/l MgCl₂ and 50 mmol/l KCl to remove any trace of contaminating genomic DNA. RNA (2.5 µg) was then subjected to reverse transcription (RT reagents; Promega, Madison, WI, USA). RT-PCR was undertaken in a volume of 50 µl buffer containing 50 mmol/l KCl, 10 mmol/l Tris-HCl, 3.5 mmol/l MgCl₂, 200 µmol/l each dNTPs (Gibco-BRL) and 0.4 µmol/l each of sense and antisense primers to rat insulin. Amplification was performed for 30 cycles at the denaturing temperature of 94 °C for 1 min, annealing temperature of 60 °C for 45 s and an extension temperature of 72 °C for 1 min. For the amplification of the glucose transporter (GLUT)-2 and glucokinase mRNAs, we used the same PCR conditions as described above, in the presence of gene-specific primers. For β -actin, the annealing temperature was raised to 64 °C for 1 min and gene-specific primers were used. All other experimental conditions to amplify GLUT-2 and glucokinase mRNAs were identical to those described for the amplification of insulin mRNA. Oligonucleotide primers for insulin, GLUT-2, glucokinase and β -actin were derived from the

published gene sequences (Ullrich *et al.* 1977, Nudel *et al.* 1983, Thorens *et al.* 1988, Magnuson *et al.* 1989) and are presented in Table 1.

Immunofluorescence microscopy

ARIP cells were grown in monocoated chamber slides (Nange Nunc International, Naperville, IL, USA) in the presence or absence of GLP-1 (10 nM) for 0, 12, 24 or 48. The cells were then washed in PBS and fixed with paraformaldehyde 4% (Sigma) for 30 min at 37 °C in PBS. After three washings in PBS, they were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min. The cells were then incubated sequentially with 10% normal blocking serum in PBS (Santa Cruz) for 20 min, followed by overnight incubation with guinea pig anti-porcine insulin antibody (Dako, Carpinteria, CA, USA) at a dilution of 1:125, or with mouse anti-human cytokeratin 20 (CK-20; Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:50, at 4 °C, in a humid chamber. After three washes in PBS, the cells were incubated with Texas Red-conjugated rabbit anti-guinea pig IgG (Vector Laboratories, Inc., Burlingame, CA, USA) (1:200) for insulin detection and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA, USA) (1:50) for CK-20 detection. The secondary antibody incubations were 1 h at room temperature in a humid chamber. Slides were mounted with a medium containing 4',6 diamidino-2-phenylindole (Vectashield; Vector Laboratories) and examined using a fluorescent microscope (Olympus AX-70, Olympus America Inc., Melville, NY, USA). Images were captured by Apogee Digital Camera (Olympus America Inc) and processed by the

Figure 1 Cell counting by fluorescent bead-based flow cytometry. Exponentially growing ARIP cells were double synchronized with thymidine and exposed to serum containing F12 medium in the presence or absence of GLP-1 (10 nM) for different lengths of time. Fluorescent beads were used as a counting reference for the quantitative analysis of cells by FACS analysis. Statistical differences between GLP-1-treated and control cultures were evaluated by ANOVA. The *P* value on the Figure refers to the difference between the two curves.

Image-Pro Computer software (Mediacybernetics, Carlsbad, CA, USA).

Staining for insulin and CK-20 experiments was repeated at least three times, using independent cultures.

Control staining for insulin (islets of Langerhans) and CK-20 (pancreatic ductal epithelium) was performed using normal rat pancreas.

HPLC analysis

The culture media of ARIP cells grown in the presence of GLP-1 (or vehicle) for 48 h was utilized for HPLC for insulin, following the procedure described by Khaksa *et al.* (1998). In brief, 1 ml of the cell culture medium was removed and treated with 1 ml dichloromethane by rotating for 5 min. The supernatant layer was decanted and the organic layer transferred to an HPLC sample tube and a 20 ml aliquot injected into the C18 reverse phase HPLC column (Nova-Pak; Waters Corporation, Milford, MA, USA) for chromatographic separation. The mobile phase consisted of 90% methanol/water (50% v/v) and 10% glacial acetic acid. A Finnegan LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) connected to the HPLC column was used to analyze mass distribution of alleles of rat insulin

secreted in response to GLP-1 treatment. Insulin was ionized using electro-spray ionization and analyzed in normal mass range (m/z 200–2000) operation mode to recover the multiple charged peptide alleles. Control rat insulin was used as standards.

Regulation of insulin secretion by glucose

Cells were plated at a density of 10^6 cells/well in a 6-well plate. Once they reached 80% of confluence they were washed with serum-free medium containing 12 mM glucose and then exposed to medium containing increasing concentrations of glucose. Glucose-dependent insulin secretion was evaluated in the presence of a determined concentration of GLP-1 (10 nM for 48 h) with increasing concentrations of glucose in the culture medium (0, 1, 6, 10 and 20 nM). Insulin released into the medium was measured by radioimmunoassay (RIA) (Linco Research Inc., St Charles, MA, USA). Total insulin accumulation in the culture medium was then normalized for total cellular protein content for each individual culture.

Protein assay

Total cellular protein content was measured by utilizing the Bradford method (Bio-Rad). The

Figure 2 Flow cytometry for cell cycle analysis. ARIP cells were cultured in serum-free medium and then treated with GLP-1 (10 nM) for increasing lengths of time. Cells cultured in serum-free medium or in medium containing serum were used as controls. The Figure depicts a representative FACscan experiment. In each graph the cell number is plotted on the vertical axis and the channel number (i.e. channel of fluorescence emission) on the horizontal axis. Since the intensity of fluorescence is directly proportional to the amount of DNA per cell, the trace gives a distribution analysis of the cell population by DNA content. The percentages of cells in G1, S and G2-M phases of the cell cycle, as determined by Lysis II software analysis, are shown in each individual graph.

amount of protein measured was used as a correction factor for determining the relative amount of medium to be assayed for each individual RIA for insulin.

Statistical analysis

The data are expressed as means $\pm$ S.E.M. Significance was evaluated by unpaired Student's *t*-test. One-way analysis of variance (ANOVA) was used to evaluate statistical significance when more than two data points were analyzed. Statistical significance by either unpaired Student's *t*-test or

ANOVA is explicitly identified in the text or in the Figure legends.

Results

Fluorescent bead-based flow cytometry for cell counting

Cell counting demonstrated that there were fewer cells in cultures grown in the presence of GLP-1, compared with controls (Fig. 1). After 6 h from the beginning of the treatment with GLP-1 the number of cells was 17% lower in the GLP-1-treated

culture in comparison with cultures where only the vehicle was added. The difference between the number of cells grown in the presence and those grown in the absence of GLP-1 reached 25% after 10 h of exposure ($P<0.05$; by Student's *t*-test comparing cell number of GLP-1-treated vs control cultures at 10 h) and was maintained over time up to 54 h from the earliest exposure to GLP-1 ($P<0.01$; by ANOVA comparing curves derived from GLP-1-treated vs untreated cultures).

Cell cycle distribution

The effect of GLP-1 on cell cycle progression of ARIP cells was analyzed by FACS. Exposure to GLP-1 lowered the proportion of cells in S phase compared with control cultures (Fig. 2). After 12 h from the beginning of the treatment with GLP-1, 9% of the cell population was in S phase of the cell cycle, and reached 11% after 24 h. This was lower than the proportion of cells that were in S phase in the cultures grown with vehicle alone in the absence of FBS (16% and 13% at 12 and 24 h respectively; $P<0.05$ at 12 h, not significant at 24 h), or in the presence of 10% FBS (37% and 25% at 12 and 24 h respectively; $P<0.01$ for both 12- and 24-h time-points). An average of three independent experiments revealed a 43% reduction of cells in S phase at 12 h and a 15% reduction of cells in S phase after 24 h of treatment with GLP-1 compared with control cultures grown in the absence of FBS. The mean reduction of GLP-1-treated cells that were in S phase in comparison with cells grown in FBS alone was about 75% at 12 h and 56% at 24 h ($P<0.001$ and $P<0.01$ respectively). We also observed a parallel increase of the percentage of GLP-1-treated cells in G1 phase (84% at 12 h and 77% at 24 h) compared with cells grown in serum-free medium (68% and 74% at 12 and 24 h respectively) and with cells grown in FBS (54% and 55% at 12 and 24 h respectively) (Figs 2 and 3; $P<0.01$ for GLP-1-treated vs serum-free medium at 12 h; $P<0.05$ for GLP-1-treated vs FBS at 12 h; $P<0.05$ for GLP-1-treated vs FBS at 24 h; not significant GLP-1-treated vs serum-free medium at 24 h). An effect of GLP-1 treatment on the proportion of cells in G2-M phase was also observed (Figs 2 and 3). Indeed, 6.5% of the GLP-1-treated cells were in G2-M phase at 12 h and 11% at 24 h, compared with 15.5% and 12.5% of the cells grown in the

Figure 3 Percentage of cells in the different phases of the cell cycle at each time-point for each experimental condition, as derived by FACS analysis. The graphs represent the average of three independent experiments. Statistical significance of the data was evaluated by unpaired Student's *t*-test. Asterisks indicate individual time-points at which GLP-1-treated cells showed a significant cell cycle distribution compared with cells cultured in the absence of serum. Cells cultured in the presence of FBS are shown as controls for actively growing cells.

presence of the vehicle alone, 8% and 19% of the cells grown in medium containing FBS ($P<0.01$ for GLP-1-treated vs serum-free medium at 12 h; not significant for GLP-1-treated vs serum-free medium at 24 h; $P<0.05$ for GLP-1-treated vs FBS at 12 h; $P<0.01$ for GLP-1-treated vs FBS at 24 h;

Figure 4 Western blot analysis of p27^{Kip1}. ARIP cells were serum starved for 24 h and then re-exposed to serum-containing medium for 24 h before incubation with GLP-1 (10 nM) for 24 h. Total cell lysates were immunoprecipitated with anti-p27^{Kip1} antibody and resolved on 12% SDS-polyacrylamide gel. This experiment was repeated four times providing results very similar to the one depicted in this blot.

not significant for GLP-1-treated vs serum-free medium at 24 h). The difference in cell cycle distribution between GLP-1-treated and control cultures progressively declined over time, losing statistical significance after 48 h.

Effects of GLP-1 on the expression of G1 Cdk inhibitors, p27^{Kip1} and p21^{Cip1}

After the induction of growth arrest by a 24-h serum starvation (time 0), cells were re-exposed to serum-containing medium for 24 h and

subsequently grown for another 24 h in the presence or absence of GLP-1 (10 nM). As shown in Fig. 4, p27^{Kip1}, abundantly expressed in growth-arrested cells, was down-regulated by a 24-h exposure to serum-containing medium. The addition of GLP-1 resulted in an up-regulation of p27^{Kip1}, occurring within 24 h of GLP-1 treatment. By contrast, in the control cells, grown in the absence of GLP-1, the p27^{Kip1} protein remained at a low level over time.

The expression of another member of the Kip/Cip family of Cdk inhibitors, p21^{Cip}, was also

Figure 5 Western blot analysis of p21^{Cip1}. ARIP cells were serum starved for 24 h and then re-exposed to serum-containing medium for 24 h before incubation with GLP-1 (10 nM) for 24 h. Total cell lysates were immunoprecipitated with anti-p21^{Cip1} antibody and resolved on 12% SDS-polyacrylamide gel. This experiment was repeated four times providing results very similar to the one depicted in this blot.

Figure 6 RT-PCR for β -cell-specific transcripts. ARIP cells growing exponentially were double synchronized using thymidine and exposed to GLP-1 (10 nM) every 8 h for different lengths of time. Total RNA was extracted from each culture at different time-points and analyzed by RT-PCR for the expression of GLUT-2, insulin and glucokinase (GK) mRNA. Each experiment was repeated three times, using RNA samples from independent cultures.

examined. As shown in Fig. 5, $p21^{Cip}$ was expressed in growth-arrested cells and was down-regulated by a 24-h exposure to serum-containing medium. As for $p27^{Kip1}$ protein, the addition of GLP-1 resulted in an up-regulation of $p21^{Cip}$ protein expression at 24 h from the beginning of GLP-1 treatment. In contrast to GLP-1-treated cells, control cells maintained a drastic reduction of $p21^{Cip}$ level, likely due to the re-exposure to serum-containing medium.

GLP-1 promoted the expression of β -cell-specific genes in synchronized cells

RT-PCR analysis revealed the earliest detection of insulin mRNA after 44 h from the beginning of GLP-1 treatment of growth-arrested cells (Fig. 6). Insulin mRNA level remained constant up to 74 h after the first GLP-1 exposure. RT-PCR for GLUT-2 revealed the presence of this transcript after only 24 h of treatment with GLP-1, preceding the earliest detection of insulin mRNA by approximately 1 day. After the initial expression at

24 h, GLUT-2 mRNA remained constant over time. Glucokinase mRNA was first detectable at 56 h, 12 h after the detection of insulin, its levels also remained unchanged after the first detection. No RT-PCR products for insulin, GLUT-2 or glucokinase were detectable in non-GLP-1-treated cells. RT-PCR for β -actin was used as a control for cDNA loading.

Immunocytochemistry and immunofluorescence microscopy

Treatment with GLP-1 induced the differentiation of ductal epithelial cells into insulin-expressing cells. As shown in Fig. 7, no insulin immunoreactivity was observed prior to treating ARIP cells with GLP-1. Insulin was first detected in a few cells after 12 h of GLP-1 treatment. At 24 h from the early exposure to GLP-1, there was a greater number of cells that contained insulin. We also observed a time-dependent increase in insulin immunoreactivity per individual cell, suggesting an accumulation of insulin over time.

Figure 7 Immunocytochemistry for insulin. ARIP cells were cultured with GLP-1 (10 nM) for 48 h. ARIP cells treated with GLP-1 (10 nM for 48 h) and stained solely with secondary antibody were used as negative control (blank). Rat pancreas with characteristic islets of Langerhans was used as positive control. This experiment was repeated three times providing results very similar to the one depicted here. Pictures were taken at 20 $\times$ magnification. The boxed area in the 48 h panel shows a greater magnification (40 $\times$) of two individual cells.

As cells acquired a β -cell-like phenotype, with the capability of producing insulin, we observed a progressive reduction of the expression of CK-20, a protein marker of ductal epithelial cells. As shown in Fig. 8, a positive staining for CK-20 was detected in the entire population of ARIP cells prior to the exposure to GLP-1; however, the expression of CK-20 was strongly reduced after 24 h of treatment with GLP-1, with few cells being still immunoreactive with the CK-20 antibody at later time-points.

HPLC analysis for insulin

HPLC analysis of culture medium revealed the presence of the two non-allelic rat insulin proteins (insulin-I and insulin-II) secreted by ARIP cells

when grown in the presence of GLP-1 (Fig. 9A and B). Control cultures, treated with vehicle only, did not produce chromatographic rat insulin peaks indicating that no insulin synthesis was induced in untreated cultures.

Glucose-dependent secretion of insulin

Cells cultured in the presence of increasing concentrations of glucose were able to release insulin in a glucose-dependent manner when exposed to GLP-1 (Fig. 9C). No insulin secretory response was observed when cells were cultured with vehicle alone. The lowest concentration of glucose required to induce the secretion of insulin was 3 mM, this was followed by a progressive increase in insulin accumulation into

Figure 8 Immunocytochemistry for CK-20. ARIP cells cultured with GLP-1 (10 nM) for 48 h. ARIP cells treated with GLP-1 (10 nM for 48 h) and stained solely with secondary antibody were used as negative control (blank). Rat pancreas with characteristic ductal epithelial cells was used as positive control. This experiment was repeated three times providing results very similar to the one depicted here. Pictures taken at 20 $\times$ magnification. The boxed area in the 12 h panel shows a greater magnification (40 $\times$) of two individual cells.

the culture, with a plateau detected with glucose concentrations between 10 and 20 mM.

Discussion

In this study we have demonstrated that the exposure of pancreatic ductal cells to GLP-1 induced a cell cycle re-distribution leading to their differentiation into insulin-producing cells. Treatment with GLP-1 was associated with an up-regulation of the G1 phase cyclin-Cdk inhibitors, p27^{Kip1} and p21^{Cip1}. As cells slowed down their proliferation rate, a time-dependent transition from a ductal-like phenotype to the acquisition of β -cell-like characteristics was also demonstrated.

The intestinal hormone GLP-1 has been shown to play an important role in the expansion of islet cell mass in animal models of glucose intolerance and diabetes (Wang *et al.* 1997, Pederson *et al.* 1998, Xu *et al.* 1999, Perfetti *et al.* 2000, Stoffers *et al.* 2000). The improvement of glucose tolerance that followed the administration of GLP-1 was shown to result from the neo-differentiation of pancreatic ductal cells into insulin-expressing cells (Xu *et al.* 1999, Stoffers *et al.* 2000). In the present study, we elected to use a rat pancreatic ductal cell line, termed ARIP, as a biological model to characterize some of the events leading to the trans-differentiation of ductal cell into insulin-producing cells.

Differentiation of mammalian cells characteristically follows a slow-down of the cell proliferation rate and exit from the cell cycle (Coffman & Studzinski 1999). Initial experiments were therefore performed to investigate the effect of GLP-1 on cell proliferation and to characterize its effect on the distribution of cells in different phases of the cell cycle. The number of cells present in the GLP-1-treated cultures was significantly lower than in controls. Furthermore, the population of cells entering in S phase was substantially reduced by GLP-1, while an increase in the percentage of resting cells in G0-G1 phase and a reduction in mitotic cells in G2 phase occurred.

The potential control by cell cycle modulators (i.e. hormones, growth factors, etc.) takes place at different and defined check points of the cell cycle (Pestell *et al.* 1999). The transition between G1 and S phase is one of the most important regulated steps in this process (Reed 1996). Entering S phase is strictly regulated in the early and late G1 phase by the balance between cell cycle activators (Cdks) and cell cycle inhibitors (Sherr 1994, Sherr & Roberts 1995). Progression through G1 phase requires the activity of the cyclin D-dependent kinases, Cdk4 and/or Cdk6, early in G1 and the cyclin E-dependent kinase, Cdk2, in the later G1 phase. Cdk inhibitors can prevent the activation of Cdks by binding the cyclin-Cdk complexes and thus preventing cells from entering S phase. Among the Cdk inhibitors, p27^{Kip1} and p21^{Cip1} have been shown to inhibit a wide variety of cyclin-Cdk complexes *in vitro*. We therefore investigated whether the pro-differentiation property of GLP-1 required a GLP-1-dependent regulation of the growth-arrest signaling proteins p27^{Kip1} and p21^{Cip1} (Polyak *et al.* 1994a,b, Toyoshima & Hunter 1994, Harper *et al.* 1995). Our data demonstrated that the levels of the two proteins were both increased by GLP-1, thus showing that the up-regulation of these Cdk inhibitors is one of the

molecular mechanisms underlying the cell cycle modulation by GLP-1.

It is likely that the transition from the phenotype of ductal cells into that of insulin-producing cells requires that a complex network of genes and their products is activated/deactivated in a finely co-ordinated fashion in order to ensure that a proper differentiation process takes place. We have demonstrated that GLP-1 induced a time-dependent reduction of the duct cell specific marker, CK-20, and this was associated with a progressive increase in the synthesis of insulin. In addition to insulin, GLP-1 induced a time-dependent expression of two other main regulators of the glucose-sensing machine of normal β -cells, the glucose transporter, GLUT-2, and the glucose-phosphorylating enzyme, glucokinase. Finally the acquisition of a β -cell-like phenotype was associated with the capability of a glucose-dependent secretion of insulin into the culture medium.

In summary, the present study elucidates some of the early events that allow GLP-1 to slow down cell growth in order to promote their differentiation into insulin-secreting cells.

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Figure 9 HPLC and RIA for insulin. ARIP cells cultured in the presence of 10 mM glucose with GLP-1 (10 nM) or vehicle for 48 h were subjected to HPLC analysis. Cells cultured in the presence of GLP-1 revealed the presence (retention time 7.53 min) of the two known non-allelic rat insulin proteins (insulin-I and insulin-II) (A), while no insulin was detected in control cultures (B). Repetition of HPLC analysis in the independent cultures of GLP-1-treated and controls showed very similar results. To study the cell responsiveness to glucose, ARIP cells were cultured in serum-free medium with GLP-1 (10 nM for 48 h) or vehicle in the presence of various concentrations of glucose (0, 1, 6, 10 and 20 mM) (C). Each experiment was repeated three times and the data represent the means $\pm$ s.d. Insulin levels were normalized for total protein content in each individual sample of culture medium. * $P < 0.001$ for GLP-1-treated cultures vs controls for individual glucose concentrations (Student's *t*-test).

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