The acquisition of an insulin-secreting phenotype by HGF-treated rat pancreatic ductal cells (ARIP) is associated with the development of susceptibility to cytokine-induced apoptosis

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Abstract

The elucidation of mechanisms regulating the regeneration and survival of pancreatic beta cells has fundamental implications in the cell therapy of type 1 diabetes. The present study had the following three aims: 1. to investigate whether pancreatic ductal epithelial cells can be induced to differentiate into insulin-producing cells by exposing them to hepatocyte growth factor (HGF); 2. to characterize some of the molecular events leading to their differentiation toward a beta-cell-like phenotype; 3. to evaluate the susceptibility of newly differentiated insulin-secreting cells to cytokine-induced apoptosis, a mechanism of beta-cell destruction occurring in type 1 diabetes. We demonstrated that HGF-treated rat pancreatic ductal cell line (ARIP) cells acquired the capability to transcribe the insulin gene and translate its counterpart protein. HGF-treated cells also exhibited a glucose-dependent capability to secrete insulin into the cultured medium. Expression analysis of some of the genes regulating pancreatic beta-cell differentiation revealed a time-dependent transcription of neurogenin-3 and Neuro-D in response to HGF. Finally, we determined the susceptibility to proinflammatory cytokine (PTh1)-induced apoptosis by incubating HGF-treated and untreated ARIP cells with a cocktail of interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ). Such treatment induced apoptotic death, as determined by the TUNEL technique, in about 40% of HGF-treated, insulin-secreting ARIP cells, while untreated ARIP cells were resistant to PTh1-induced apoptosis. In conclusion, we showed that HGF promotes the differentiation of ARIP cells into pancreatic beta-cell-like cells, and that the differentiation toward an insulin-secreting phenotype is associated with the appearance of susceptibility to cytokine-induced apoptosis.

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Introduction

Type 1 diabetes is an autoimmune disease characterized by the progressive and selective destruction of pancreatic beta cells (Wucherpfennig & Eisenbarth 2001, Yoon & Jun 2001). Factors other than autoimmunity may play a crucial role in the progression of the disease, such as an impaired balance between the rate of immune-mediated apoptotic phenomena and the capability of the pancreas to regenerate and expand the beta cell mass after damage (Risbud & Bhonde 2002). The process of islet cell regeneration leads, after an initial phase of cell replication, to the acquisition of a number of phenotypic features that are characteristic of differentiated insulin-producing cells (Hardikar 2004). However, whether such a phenomenon of islet cell neogenesis and tissue repair is

coupled to the acquisition of additional phenotypic characteristics, such as the ability to start and execute the apoptotic program in response to environmental stimuli, is presently unknown. A number of growth factors have been shown to be involved in the neogenesis of endocrine cells. In particular, a role for hepatocyte growth factor (HGF) and its receptor, in pancreas embryonal development (Sonnenberg *et al.* 1993, Calvo *et al.* 1996), as well as in beta-cell regeneration in autoimmune diabetes (Bulotta *et al.* 2001), has been demonstrated. The *in vivo* beta-cell neogenetic process can be recapitulated *in vitro*, as beta-cell precursors and islet beta cells have been shown to proliferate and to differentiate toward an endocrine phenotype when stimulated with various growth factors (Hui *et al.* 2001, Nielsen *et al.* 2001, Brand *et al.* 2002, Egan *et al.* 2003).

During embryonic life, one of the recently described major regulatory networks in the control of beta-cell development is represented by the Notch receptor/ligand pathway (Apelqvist *et al.* 1999). Notch proteins belong to a conserved family of transmembrane receptors, including Notch 1 to 4. Via interaction with a number of specific ligands (Delta-like 1 to 3 and Serrate 1 and 2), Notch receptors play a critical role in cell fate specification and in the control of cell growth and differentiation in a variety of cell lineages in vertebrates and invertebrates (Lewis 1998, Mumm & Kopan 2000, Baron *et al.* 2002, Guidos 2002). A number of recent reports assessing knockout and transgenic mice for components of the Notch pathway suggest that Notch 1, via RBP-Jk- and HES-1-dependent downregulation of neurogenin-3 (*ngn-3*) and Neuro-D, either keeps enteroendocrine precursor cells in an undifferentiated state or drives them toward an exocrine fate (Apelqvist *et al.* 1999, Gradwohl *et al.* 2000, Schwitzgebel *et al.* 2000). Conversely, Notch 3 acts as an antagonist of Notch 1, thus promoting the beta-cell differentiation program (Apelqvist *et al.* 1999). Although the apoptotic program is linked to proliferation and differentiation processes in a number of tissues (reviewed in Medh & Thompson 2000, Wagers *et al.* 2002), the acquisition of the apoptotic regulatory and execution machinery during beta-cell development remains to be investigated. Apoptosis is a mechanism of beta-cell death during immune-mediated damage, triggered by T cells and proinflammatory cytokines. Specifically, the proinflammatory cytokines interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) play an important role in immune-induced damage of islet beta cells (Argiles *et al.* 1992, Corbett & McDaniel 1992, Sandler *et al.* 1994, Rabinovitch & Suarez-Pinzon 1998). We have previously demonstrated that Th1 and proinflammatory cytokines (PTh1) determine the apoptotic death of beta cells associated with downregulation of bcl-2 expression, and that the extent of apoptosis can be significantly reduced by the incubation of beta cells with Th2 cytokines (IL-10 and IL-4), thus indicating that cytokine signaling plays a major role in determining the death or survival of islet cells during inflammation (Marselli *et al.* 2001).

In order to identify a possible link between beta-cell neogenesis and susceptibility to apoptosis, we described here the ability of HGF to differentiate rat pancreatic ductal epithelial cells into insulin-producing cells. We demonstrated that the acquisition of beta-cell-like phenotype is associated with the expression of *ngn-3* and Neuro-D, the two chief transcription factors involved in the regulation of beta-cell differentiation during embryogenesis in man and other vertebrates (Apelqvist *et al.* 1999, Gradwohl *et al.* 2000, Schwitzgebel *et al.* 2000). Such an HGF-induced differentiation is associated with a newly acquired susceptibility of cells to undergo

apoptosis when exposed to cytokines. The elucidation of the molecular events responsible for the acquisition of susceptibility to apoptosis during beta-cell neogenesis might lead to the identification of intervention strategies to uncouple the two processes, thereby re-establishing a favorable balance between the neogenetic beta-cell expansion and the autoimmune cell damage by inhibiting the apoptotic response.

Materials and methods

Cell culture

The rat pancreatic ductal cell line (ARIP) and Hep-G2 hepatoma cell line were gifts from the Diabetes Research Laboratories at Cedars-Sinai Medical Center, Los Angeles, CA, USA, respectively. ARIP cells were cultured in F12 medium (Gibco, BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco), 50 μ g/ml streptomycin, 100 μ g/ml penicillin at 37 °C under a humidified condition of 95% air, and 5% CO₂. Hep-G2 cells were cultured in Minimum Alpha Essential Medium (Gibco, BRL) supplemented with FBS and antibiotics, as indicated for F12 medium. The glucose concentration in the culture medium was 12 mmol/l. Treatment of ARIP cells with human recombinant HGF (R & D Systems, Minneapolis, MN, USA) was carried out with cells grown in serum containing medium to 80% confluence, after a 'wash-out' incubation for 6 h with serum-free medium. To evaluate the time course of response, ARIP were exposed to HGF (50 ng/ml) or vehicle alone for 0, 12, 24, 36, 48 or 72 h. To detect the presence of *c-met* receptor, ARIP cells were cultured in the absence of HGF. Susceptibility to cytokine-induced apoptosis was analyzed by incubating untreated and HGF-treated ARIP cells (50 ng/ml) for 72 h in the presence or absence of human recombinant IL-1 β (50 U/ml), TNF- α (1000 U/ml) and IFN- γ (1000 U/ml) (Roche Diagnostics, Mannheim, Germany).

Immunofluorescence microscopy

ARIP cells were grown in monocoated chamber slides (Nange Nunc International, Naperville, IL, USA) in the presence or absence of HGF (50 ng/ml) for 0, 12, 24, 36, 48, and 72 h. For the detection of *c-met*, ARIP cells, as well as HEP-G2 cells, were grown in serum containing medium to approximately 80% confluence in the absence of HGF. Cells were washed in PBS and fixed with paraformaldehyde 4% (Sigma) for 30 min at 37 °C in PBS. After three washings in PBS, the cells were permeabilized with 0.1% (v/v) of Triton X-100 in PBS for 10 min. Cells were then incubated sequentially with 10% normal blocking serum in PBS (rabbit or goat normal serum; Santa Cruz Biotechnology, San Diego, CA, USA) for 20 min, followed by overnight incubation

with guinea pig anti-porcine insulin antibody (Dako, Glostrup, Denmark) at a dilution of 1:100, or with rabbit polyclonal antihuman *c-met* (Met H-190, sc-8307; Santa Cruz Biotechnology) (1:25) at 4 °C in a humid chamber. After three washes in PBS, the cells were incubated with FITC-conjugated rabbit anti-guinea pig immunoglobulin (Ig) G (Dako) (1:40) for insulin detection, and goat antirabbit IgG (Chemicon, Temecula, CA, USA) (1:50) for *c-met* detection. The secondary antibody incubations were 1 h at room temperature in a humid chamber. Slides were mounted in DAPI fluorescent mounting medium (Vector Laboratory, Peterborough, UK) and examined under a fluorescent microscope (Olympus AX-70). Images were captured by Apogee Digital Camera and processed by Image-Pro computer software (Media Cybernetics, San Diego, CA, USA). Staining for insulin and for *c-met* was repeated at least three times, using independent cell cultures.

Immunoprecipitation and immunoblotting

Cells were washed twice with cold PBS and lysed in 500 µl M-PER lysis buffer (Pierce, Ruckford, IL, USA) containing protein inhibitors (1 mM phenylmethylsulfonyl fluoride, 8.3 µM aprotinin, 50 µM leupeptin and 30 mM sodium orthovanadate) (Sigma), and spun at 90 000 *g* for 5 min at room temperature (RT). The lysate was collected and centrifuged at 13 000 *g* for 5 min at 4 °C. The whole-cell lysate was precleared by adding 1 µg control IgG (Santa Cruz Biotechnology) together with 20 µl resuspended volume of protein G agarose conjugate (Santa Cruz Biotechnology) at 4 °C for 30 min. The protein concentration was then measured in the supernatant at least twice with protein assay reagent (Bio-Rad). Soluble proteins (200–500 µg) were incubated overnight at 4 °C on a rotating device with 1 µg antihuman *met* (Met H-190, sc-8307; Santa Cruz Biotechnology) and 50 µl protein G agarose conjugate. The beads were washed with 10% M-Per in PBS, and the pellet was resuspended in 30 µl electrophoresis sample buffer for immunoblotting. For immunoblotting, precipitated proteins were separated on 7.5% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad). The transferred membranes were soaked in Poinceau-S solution (Sigma) to check that comparable amounts of proteins were loaded on the gel and to check the homogeneity of the transfer. After blocking from nonspecific binding with 3% bovine serum albumin in PBS-Tween 20 (Sigma) for 1 h at RT, the membranes were immunoblotted with antihuman *c-met* (Met H-190, sc-8307; Santa Cruz Biotechnology) (1:500) overnight at 4 °C on a rotating device. After three washes in PBS-Tween 20, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase for 1 h at RT. Immunoreactive proteins were visualized by the

ECL immunodetection system (Amersham, Arlington Heights, IL, USA).

Measurement of insulin release

ARIP cells were plated at a density of 10⁶ cells/well in a six-well plate. Glucose-dependent secretion of insulin was evaluated in ARIP cells cultured in the presence or absence of HGF (50 ng/ml added every 8 h) for 60 h. Cells were kept in 5.5 mM glucose until the day of the experiment and then either challenged with 20 mM glucose or re-exposed to 5.5 mM glucose, for 60 min. The level of insulin in the culture medium was measured by RIA (Linco Research, St. Charles, MO, USA) and normalized for the total cellular protein content detected in the pellet of each individual culture, as measured by the Bradford method (Bio-Rad).

RT-PCR

The expression of mRNA encoding for Notch, *ngn-3* and *Neuro-D* was evaluated by RT-PCR in four separate experiments. RT-PCR was performed by a first step of reverse transcription from DNase I-treated total RNA isolated from ARIP cells, followed by PCR, essentially as previously described (Felli *et al.* 1999) with a Gene-Amp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT, USA). Samples of PCR reactions were taken at different cycles throughout the amplification, allowing accurate quantitation of the product during the exponential phase of DNA amplification. Negative controls included RT-PCR without reverse transcriptase or without RNA. Amplification of beta-actin with 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGA TTTC-3' as 5' and 3' primers was used as an internal control for both reverse transcription and PCR, and as a measure of the relative amount of RNA. Amplifications of Notch 3 cDNAs were performed at an annealing temperature of 55 °C with the following primers: 5'-CAGGACAGCAAGGAGGA GAC-3', forward; 5'-GTG ATCTGTGATCTC CC GGT-3', reverse (rat Notch 3 sequence). Amplification of *ngn-3* cDNA was performed with the 5'-CAG CAACCCCTTTCCCGGAG-3' forward and 5'-TCAGT GCCAACTCGCTCTTG-3' reverse primers (rat *ngn-3* sequence). Amplification of *Neuro-D* cDNA was performed with the 5'-CTTGGCCAAGAAGTATAT CTGG-3' forward and 5'-GGAGTAGGGATGCACC GGGAA-3' reverse primers (rat *Neuro-D*). The hybridizations were performed with a specific oligomer internal to the amplified sequences. Semiquantitative analysis was performed by densitometric gel scanning with the Gel Doc 2000 video image system (Bio-Rad). The expression of insulin (INS), GLUT2, glucokinase (GK), IL-1β, TNF-α and IFN-γ receptors was evaluated by RT-PCR with the following primers: (INS) forward

5'-CCT GCC CAG GCT TTT GTC AA-3' and reverse 5'-CTC CAG TGC CAA GGT CTG AA-3'; (GLUT2) forward 5'-CTG TAC TGT GGG CTA ATT TCA GG-3' and reverse 5'-GGT GAA CTT ATC CAG CAA CAC C-3'; (GK) forward 5'-GCC GAG ATG CTC TTT GAC TAC-3', reverse 5'-GCC GAG ATG CTC TTT GAC TAC-3' and reverse 5'-CAG TGC CCA CAA TCA TGC-3'; (IL-1R) forward 5'-GCT GGA GAG TAC TCT TCA TGC TG 3' and reverse 5'-GAT GAA TCC TGG AGT CCT TGT CC-3'; (TNF-RI) forward 5'-ACC AAG TGC CAC AAA GGA ACC-3' and reverse 5'-TAC ACA CGG TGT TCT GTT TCT CC-3'; (IFN- γ -R) forward 5'-CCT AAG GTC AAT GTG AGT CAG G-3' and reverse 5'-GAA GAG AAG AGG AGC AAC AAG C-3'. All PCR products were electrophoresed on agarose gel and the bands visualized by ethidium bromide staining.

Evaluation of apoptosis

Untreated and HGF-treated ARIP cells were grown in monocoated chamber slides and treated with cytokines as described above. The presence of apoptotic cells was determined by propidium iodide (PI) staining and TUNEL assay (*In Situ* Cell Death Detection System; Roche Diagnostics), according to the manufacturer's instructions. Briefly, cells were fixed with 4% formaldehyde/PBS for 25 min at 4 °C, washed in PBS and permeabilized with prechilled 0.2% Triton X-100/PBS for 5 min on ice. After two washes with PBS, cells were covered with terminal deoxynucleotidyl transferase (TdT) buffer containing TdT enzyme and fluorescein-dUTP, and the slides were incubated in a humidified chamber at 37 °C for 60 min. A TdT-minus negative control was performed by replacing the TdT enzyme with deionized H₂O. The reaction was terminated by immersing slides in 2 SSC for 15 min at RT. The slides were then washed twice in PBS and the cells stained with 10 ng/ml propidium iodide (PI)/PBS at room temperature for 10 min. After two washes in deionized H₂O, slides were mounted in DAPI fluorescent mounting medium (Vector Laboratory) and examined under a fluorescent microscope (Olympus AX-70). Results were expressed as means $\pm$ S.D. Statistical analysis was performed by Student's *t*-test.

Results

The *c-met* ligand, HGF, induces the pancreatic ductal ARIP cell line to differentiate into insulin-producing beta-like cells

Recent studies indicated that treatment of ARIP cells with GLP-1 resulted in their differentiation into insulin-producing and -secreting cells (Hui *et al.* 2001).

These cells could thus represent a valuable *in vitro* model to study pancreatic islet neogenesis. When analyzed by immunofluorescence, cultured ARIP cells were shown to express a significant amount of *c-met* receptor protein (Fig. 1A). In contrast, no *c-met* positive staining was detected when the primary antibody was omitted and the cells were incubated solely with the secondary antibody. The expression of *c-met* protein was also demonstrated by immunoprecipitation and Western blotting of ARIP cell lysates (Fig. 1B). As shown in Fig. 1B, both the 170 kDa single-chain precursor and the 145 kDa beta subunit of the receptor were detected. Treatment with HGF (50 ng/ml) induced the differentiation of ARIP ductal epithelial cells into insulin-expressing cells. As shown in Fig. 2, no insulin immunoreactivity was observed prior to the exposure of cells to HGF (time 0) or in cells cultured in the presence of vehicle alone (control 72 h). Insulin was first detected after 12 h of HGF treatment. By day 3 of HGF treatment, the number of insulin-expressing cells was sevenfold above basal condition (68.3 ± 7.0 , mean $\pm$ S.E. for three individual experiments). HGF-differentiated insulin-producing cells express GLUT2 and glucokinase (GK) (Fig. 2, lower panel) and can also secrete insulin in a glucose-dependent fashion (Fig. 3). Indeed, cells cultured in the presence of HGF and challenged to high glucose concentration (20 mM) secrete higher amounts of insulin than cells exposed to low glucose medium (5.5 mM).

ngn-3 and Neuro-D are upregulated during HGF-induced beta-cell differentiation

As Notch-3 receptor and its downstream effectors ngn-3 and Neuro-D have been found to be critical for endocrine pancreas differentiation, we investigated their expression during HGF-induced differentiation of pancreatic ductal cells into insulin-producing cells. Notch 3 was present in untreated ARIP cells, and its level did not vary during the process of cell differentiation. The expression of ngn-3 and Neuro-D was instead modulated during differentiation. ngn-3 became detectable 6 h after the beginning of HGF treatment, reaching a maximum after 10 h and then diminishing to become undetectable after 24 h. The effect of HGF on the expression of Neuro-D was delayed when compared with ngn-3, becoming detectable 12 h after the beginning of treatment, with a peak after 24 h followed by a slow decline (although still detectable) after 72 h (Fig. 4).

HGF-differentiated ductal cells undergo apoptosis in response to proinflammatory cytokines

TUNEL staining revealed that untreated ARIP cells were resistant to cytokine-induced damage, apoptotic phenomena affecting very few cells, whereas cell death

Figure 1 (A) Immunocytochemistry for *c-met*. ARIP cells were cultured for 24 h in F12 medium containing serum and then incubated for 6 h with serum-free medium (left panel). The HepG2 (hepatoma) cell line was used as positive control (right panel). Both ARIP and HepG2 cells stained solely with secondary antibody were used as negative control (Magnification: $\times 40$). (B) Western blot analysis of *c-met*. ARIP cells were cultured for 24 h in F12 medium containing serum and then incubated for 6 h with serum-free medium. Total cell lysates were immunoprecipitated with antihuman *c-met* antibody and resolved on 7.5% polyacrylamide gel. Total cell lysates of HepG2 cell line (hepatoma) immunoprecipitated with the same antihuman *c-met* antibody were used as positive control. Arrows indicate the 170 kDa single-chain precursors and the 145 kDa the beta subunit of the receptor.

was dramatically induced in HGF-treated insulin-secreting cells cultured with a cocktail of proinflammatory cytokines, including IL-1 β (50 U/ml), TNF- α (1000 U/ml) and IFN- γ (1000 U/ml). By this technique, apoptotic cells were identified by yellow, highly condensed and/or fragmented nuclei; the yellow emission of fluorescence was due to the overlay of TUNEL-positive (green) and PI-positive (red) staining (Fig. 5b). The percentage of apoptotic cells (calculated as number of apoptotic cells/apoptotic + non-apoptotic cells $\times 100$) was $6.3 \pm 1.8\%$ in undifferentiated ARIP cells treated with proinflammatory cytokines, and increased by up to $40.3 \pm 7.9\%$ in cytokine-exposed HGF-treated cells ($P < 0.001$, Student's *t*-test).

Discussion

The differentiation of pancreatic beta cells requires the coordinated action of multiple signaling molecules. We

used ductal derived ARIP cells as an *in vitro* model to investigate the role of extracellular and intracellular factors regulating the neogenesis and survival of newly differentiated beta cells. In the present study, we demonstrated that treatment of pancreatic ductal cells with HGF promoted their differentiation into insulin-secreting cells. Such an ability of HGF to promote beta-cell terminal differentiation extends the spectrum of activities of this growth factor, previously shown to induce *in vivo* and *in vitro* differentiation of beta cells (Otonkoski *et al.* 1994a,b, 1996, García-Ocaña *et al.* 2000, Gahr *et al.* 2002). HGF has also been shown to increase *in vitro* the expression of Reg, a protein implicated in pancreatic regeneration (Anastasi *et al.* 1999, Kobayashi *et al.* 2000), and to convert, when administered alone or in combination with activin A, pancreatic acinar AR42J cells into insulin-producing cells (Mashima *et al.* 1996). Additionally, the capacity of HGF to induce the differentiation of a ductal cell line

Figure 2 (Upper panel) Immunocytochemistry for insulin of ARIP cells cultured with HGF (50 ng/ml), or vehicle alone, for 0–72 h. This experiment was repeated three times, providing results very similar to the one depicted here. Pictures were taken at $\times 20$ magnification. Panel e shows a greater magnification. (Lower panel) Expression of insulin, GLUT-2 and glucokinase (GK) and the endogenous control GAPDH evaluated by RT-PCR, using specific primers, in untreated and in cells treated with HGF (50 ng/ml) for 72 h.

(ARIP) into insulin-producing cells suggests that this growth factor may be able to recapitulate, at least in part, the neogenetic process that leads from the cell expansion of precursor cells to the terminal differentiation of insulin-producing cells. Indeed, pancreatic ductal cells are known to give rise to pancreatic endocrine cells both during pancreatic organogenesis and islet cell neogenesis after pancreatic injury in adult animals (Teitelman & Lee 1987, Bonner-Weir *et al.* 1993, Bouwens 1998). During pancreas organogenesis, the Notch pathway and downstream signals act as crucial determinants of cell growth and differentiation, directing the cell fate toward either a differentiated endocrine phenotype or a progenitor-like characteristic

(Edlund 2002). Among the members of Notch protein family, Notch 3 has been shown to promote the beta-cell differentiation program (Apelqvist *et al.* 1999) through the modulation of *ngn-3* and *Neuro-D*, which in turn promote beta-cell terminal differentiation. We demonstrated that *ngn-3* and *Neuro-D* genes were expressed in epithelial ductal cells in response to HGF exposure. It is worth noting that a time-dependent course of the expression of both genes could be demonstrated during differentiation. In fact, *ngn-3* was the first protein to be measurable after the beginning of HGF treatment, followed by a plateau and then a progressive decline until it became undetectable at 24 h. The effect of HGF on the expression of *Neuro-D* was slightly delayed when

Figure 3 Glucose-dependent secretion of insulin evaluated in ARIP cells cultured in the presence or absence of HGF (50 ng/ml) for 60 h. Cells were kept in 5.5 mM glucose until the day of the experiment and then challenged with either 20 mM glucose or re-exposed to 5.5 mM for 1 h. Each experiment was repeated at least three times, data are means $\pm$ S.D. * $P < 0.01$ (Student's *t*-test).

Figure 4 Time course of Notch 3, ngn-3 and Neuro-D gene expression in ARIP cells cultured with HGF (50 ng/ml) from 0 to 72 h. The expression of Notch 3, ngn-3 and Neuro-D was evaluated by RT-PCR of total RNAs. Notch 3, ngn-3 and Neuro-D amplified products are represented as the densitometric analysis of autoradiographic bands normalized to beta-actin amplified products evaluated at the same times. Results shown represent one point of the multiple samples taken during the exponential phase of DNA amplification for appropriate quantitation, as described in Materials and methods. Results depicted represent three similar experiments (differing less than 20% from each other).

compared with the expression of ngn-3, reaching a peak after 24 h, and being still detectable 72 h after the beginning of HGF treatment.

The expression profile of the those two transcripts, as detected in the experimental model described in the present report, was very similar to what has been shown to happen during pancreatic development. Indeed, during pancreatic organogenesis, the expression of ngn-3, after reaching a peak during the major wave of endocrine cell genesis, greatly diminishes until it becomes virtually absent in the adult pancreas. In the same process, Neuro-D is expressed slightly later than ngn-3, and, unlike ngn-3, its expression persists in adult islet cells, where it modulates the expression of various genes, including insulin (Wilson *et al.* 2003, Chakrabarti & Mirmira 2003). Our observation that rat ductal cells constitutively express Notch 3 receptor agrees with previous results obtained in human ductal cells (Heremans *et al.* 2002). However, whereas the HGF-induced beta-cell differentiation process is associated with an upregulation of ngn-3 and Neuro-D gene expression, such a process does not result in any change in the expression level of the Notch 3 receptor.

Beta-cell mass is physiologically kept in balance between neogenesis and apoptosis, and this balance is impaired in type 1 diabetes. We here show that the acquisition of susceptibility to apoptosis after exposure to IL-1 β , TNF- α and IFN- γ is part of the HGF-induced beta-like terminal differentiation program. In type 1 diabetes, the impaired function and destruction of beta cells results from the direct contact with islet-infiltrating macrophages and T cells and/or exposure to inflammatory products of the islet-infiltrating cells, such as free radicals and cytokines. Specifically, *in vitro* studies have shown that inflammatory cytokines are capable of inducing apoptosis in purified primary human, rat and mouse beta cells (Delaney *et al.* 1997, Stassi *et al.* 1997, Rabinovitch & Suarez-Pinzon 1998). In this study, we demonstrate that HGF treatment confers susceptibility to Th1 cytokine-induced apoptosis in ARIP cells, a cell line resistant to cytokine apoptotic stimuli in the basal condition. It is worth mentioning that, when characterized by RT-PCR for the presence of inflammatory cytokine receptors, ARIP cells were shown to express (in the basal condition, before being differentiated in insulin-producing cells by HGF) high levels of IL-1 β , TNF- α and IFN- γ cytokine receptors, thus demonstrating the constitutive expression of those receptors independently of HGF exposure. We therefore suggest that the susceptibility to cytokine-induced apoptosis is strictly related to the differentiation process that modulates pathways or genes that would modify the cellular response to environmental stimuli.

Our results are consistent with studies performed with primary cultures of rat oligodendrocyte progenitor cells showing that, as cells differentiated, they become progressively more susceptible to cytokine-induced apoptosis. The authors have hypothesized that differential susceptibility is due to developmental changes in

Figure 5 (Left panel) TUNEL detection of apoptotic ARIP cells. Untreated control (c and d) and HGF-treated (a and b) ARIP cells were cultured with or without a combination of IL-1 β (50 U/ml), TNF- α (1000 U/ml) and IFN- γ (1000 U/ml) proinflammatory cytokines (PTh1) for 72 h. Apoptotic cells (b) are identified by highly condensed and/or fragmented nuclei. Similar results were obtained in three independent experiments ($P < 0.001$ vs control ARIP cells incubated with proinflammatory cytokines). In the insets are represented a particular of the insulin-positive cells stained with DAPI (magnification: $\times 20$). (Right panel) Expression of IL-1 β , TNF- α and IFN- γ receptors, and the endogenous control GAPDH was evaluated by RT-PCR, in untreated and in HGF-treated cells (50 ng/ml) for 72 h.

molecules involved in apoptotic signaling, having observed a shift of balance toward proapoptotic molecules (Osterhout *et al.* 2002). All these findings, taken together, suggest that death susceptibility is intrinsic to the differentiation state of the cell, and that this may render the mature cells very sensitive to apoptotic signals generated after injury or illness.

In summary, our data indicate that HGF promotes the differentiation of ARIP cells into a pancreatic beta-cell-like cell, as in the molecular events occurring in endocrine pancreas embryogenesis, and that the differentiation toward an insulin-secreting phenotype is associated with the appearance of susceptibility to cytokine-induced apoptosis. Our observations indicate that ARIP ductal cell lines differentiated by HGF may be a good model for the identification of those target genes that confer the susceptibility of beta-cell-like cells to cytokine-induced apoptosis. Moreover, these genes could counteract the benefits of beta-cell regeneration and, consequently, the success of transplantation strategies based on the use of stem and/or not fully differentiated endocrine cells. The recognition and characterization of these genes may direct

future research to identify pharmacologic agents able selectively to suppress the expression of unwanted mRNAs.

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