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Abstract

In diabetes, β-cell mass is not static but in a constant process of cell death and renewal. Inactivating mutations in transcription factor 1 (tcf-1)/hepatocyte nuclear factor1a (hnf1a) result in decreased β-cell mass and HNF1A-maturity onset diabetes of the young (HNF1A-MODY). Here, we investigated the effect of a dominant-negative HNF1A mutant (DN-HNF1A) induced apoptosis on the regenerative capacity of INS-1 cells.

Reference


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INS-1 Cells Undergoing Caspase-Dependent Apoptosis Enhance the Regenerative Capacity of Neighboring Cells

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OBJECTIVE—In diabetes, β-cell mass is not static but in a constant process of cell death and renewal. Inactivating mutations in transcription factor 1 (tcf-1/hepatocyte nuclear factor 1α (hnf1α) result in decreased β-cell mass and HNF1A–maturity onset diabetes of the young (HNF1A-MODY). Here, we investigated the effect of a dominant-negative HNF1A mutant (DN-HNF1A) induced apoptosis on the regenerative capacity of INS-1 cells.

RESEARCH DESIGN AND METHODS—DN-HNF1A was expressed in INS-1 cells using a reverse tetracycline-dependent transactivator system. Gene(s)/protein(s) involved in β-cell regeneration were investigated by real-time quantitative RT-PCR, Western blotting, and immunohistochemistry. Pancreatic stone protein/regenerating protein (PSP/reg) serum levels in human subjects were detected by enzyme-linked immunosorbent assay.

RESULTS—We detected a prominent induction of PSP/reg at the gene and protein level during DN-HNF1A–induced apoptosis. Elevated PSP/reg levels were also detected in islets of transgenic HNF1A-MODY mice and in the serum of HNF1A-MODY patients. The induction of PSP/reg was glucose dependent and mediated by caspase activation during apoptosis. Interestingly, the supernatant from DN-HNF1A–expressing cells, but not DN-HNF1A–expressing cells treated with zVAD.fmk, was sufficient to induce PSP/reg gene expression and increase cell proliferation in naïve, untreated INS-1 cells. Further experiments demonstrated that annexin-V–positive microparticles originating from apoptosing INS-1 cells mediated the induction of PSP/reg. Treatment with recombinant PSP/reg reversed the phenotype of DN-HNF1A–induced cells by stimulating cell proliferation and increasing insulin gene expression.

CONCLUSIONS—Our results suggest that apoptosing INS-1 cells shed microparticles that may stimulate PSP/reg induction in neighboring cells, a mechanism that may facilitate the recovery of β-cell mass in HNF1A-MODY. Diabetes 59:2799–2808, 2010

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t-glutamine, 1 mmol/l pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES (pH 7.4), and 50 mmol/l 2-mercaptoethanol (Sigma, Dublin, Ireland). For experiments investigating glucose dependence of PSP/reg induction, expression of DN-HNF1A was induced for 24 h and continued for a further 2 h in medium supplemented with glucose at 3, 6, 12, and 18 mmol/l. For caspase inhibition, cells were preincubated with 100 nmol/l N-BQ (Nikon, broad-spectrum caspase inhibitor zVAD.fmk) (Bachem, St. Helen's, U.K.) for 45 min prior to induction.

Real-time quantitative RT-PCR. cDNA synthesis was performed using 1.5 μg total RNA as the template and Superscript II reverse transcriptase (Invitrogen, Paisley, U.K.) primed with 50 pmol random hexamers (New England Biolabs, Ipswich, MA). Real-time PCR was performed using the LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN) and the Quantitect SYBR Green PCR kit (Qiagen). Specific PCR primers were designed using Primer3 software (sequences available on request). For absolute quantification of hnf1a copy number, a gene-specific PCR amplion of known concentration was prepared as a standard. Melting curve analysis and gel electrophoresis was utilized to verify specificity of all PCR products. The data were analyzed using LightCycler software, version 4.0, with all samples normalized to β-actin.

Western blotting. For Western blotting analysis, 25 μg protein lysates were obtained from INS-1 cells overexpressing WT-HNF1A and DN-HNF1A as described (9). The rabbit polyclonal anti-PSpreg antibody (22) was diluted 1:20,000 in Tris-buffered saline containing 1% bovine serum albumin. The primary mouse monoclonal anti–β-actin antibody was utilized at a 1:1,000 dilution in PBS, pH 7.4, and the secondary antibody was obtained from Pierce and detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and imaged using a FujiFilm LAS-3000 imaging system (Fuji, Shiedfeld, U.K.).

Immunohistochemistry. Paraflin-embedded pancreatic sections from rat insulin promoter (RIP)-DN-HNF1A and control wild-type C57BL/6JBomTac mice (13) were deparaffinized and incubated overnight at 4°C with the rabbit polyclonal anti–PSpreg/reg antibody diluted 1:20 (22). Specificity of the antibody was confirmed by Western blotting experiments using recombinant human PSP/reg protein as a control. Slides were incubated for 1 h at room temperature in rhodamine-labeled anti-rabbit secondary antibody (1:100; Jackson Immunoresearch, Suffolk, U.K.). Slides were incubated again overnight at 4°C with the second primary guinea pig anti-insulin antibody (1:20; DakoCytomation A1064) followed by 1 h at room temperature in fluorescein isothiocyanate (FITC)-labeled anti-goat secondary antibody (1:100; Jackson Immunoresearch) and mounted in Vectashield with DAPI (Vector Shield; Vector Labs, Burlingame, CA). Images were taken with a Zeiss LSM710 confocal microscope equipped with a 40 × 1.3NA oil-immersion objective (Carl Zeiss, Jena, Germany). FITC was excited at 488 nm with an argon laser, using a 485/543-nm multichrome beam splitter, and the emission was collected at a 490- to 550-nm bandpass. Rhodamine was excited at 543 nm with a helium–neon laser, using the same beam splitter, and the emission was collected at a 570- to 650-nm spectral band. DAPI was excited at the 405 nm DPSS laser and a 405-nm dichroic beam splitter. DAPI fluorescence was detected in the 420- to 480-nm spectral band. Images were processed using Zeiss LSM 4.2 software (Carl Zeiss). Rhodamine-positive cells in the periphery and interior of the islets of Langerhans were quantified as a percentage of total islet cells using Image J software.

Determination of caspase-3–like protease activity. INS-1 cells were seeded at 1 × 105 per ml on a 24-well plate and allowed to settle overnight. Following treatment cells were lysed in 200 μl lysis buffer (10 mmol/l HEPES, pH 7.4; 42 mmol/l KCl; 5 mmol/l MgCl2; 1 mmol/l phenylmethylsulfonyl fluoride; 0.1 mmol/l EDTA; 0.1 mmol/l EGTA; 1 mmol/l diithothreitol [DTT]; 1 μg/ml peptatin A; 1 μg/ml leupeptin; 5 μg/ml aprotinin; and 0.5% [3-cholamidopropyl]dimethylammonio]-1-propane sulfonate [CHAPS]). Fifty microliters of this lysate was then added to 150 μl reaction buffer (25 mmol/l HEPES, 1 mmol/l EDTA, 0.1% CHAPS, 10% sucrose, and 3 mmol/l DTT, pH 7.5) containing 10 mmol/l Ac-DEVD-AMC, which is efficiently cleaved by the apoptotic executioner caspases 3 and 7, and among others (23). Cleavage of the substrate DEVD-AMC resulting in the accumulation of fluorescent AMC was measured on a Genios fluorescence plate reader (Tecan) using 355 nm excitation and 460 nm emission wavelengths as previously described (9). Protein content was determined using the Pierce Coomassie Plus Protein assay reagent (Perbio) and activity expressed as change in fluorescent units per microgram of protein per hour.

Detection of nuclear apoptosis. Cells were stained using DAPI to visualize nuclear morphology. Condensed and/or fragmented nuclei were considered apoptotic. For enumeration an Eclipse TE 300 inverted microscope (Nikon, Dusseldorf, Germany) and a 20 × dry objective, with DAPI excitation at 340 ± 25 nm and the emission collected between 450 and 500 nm.

5-Bromo-2-deoxyuridine cell proliferation assay. Cellular proliferation was assessed by means of bromodeoxyuridine (5-bromo-2-deoxyuridine [BrDu]) incorporation using the BrdU Cell Proliferation Assay (Calbiochem). BrdU was used to cells for 24 h, and cells were fixed following treatment. Following incubations with the primary and secondary antibodies, the peroxidase-labeled secondary antibody was detected by addition of substrate solution for 15 min and the reaction stopped. Absorbance was read using a Bio-Rad 550 microplate reader at dual wavelengths of 450/540 nm. The values for the background wells were subtracted from all values. The data were normalized to noninduced control.

Microparticle isolation and purification. Cell supernatants were centrifuged (500g; 15 min) to remove floating cells and medium containing microparticles released from INS-1 cells collected following an established protocol (24). The medium was filtered using 0.2-μm filters (VWR International, Dublin, Ireland) in order to remove microparticles. Microparticles were analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Villepinte, France) as previously described (25). Regions corresponding to microparticles were identified in forward light scatter and side-angle light scatter dot plots using a logarithmic gain. The gate for microparticles was defined as events with a 0.1- to 1-μm diameter, in comparison with calibrator beads (Megamix fluorescent beads of 0.5, 0.9, and 3 μm in diameter; Biocyttex, Marseille, France), and plotted on an FLF/SC dot plot to determine the microparticles. Microparticles were defined as membrane vesicles <1 μm in diameter and expressing externalized phosphatidylinositol. Phosphatidylinositol was labeled using FITC-conjugated annexin V (Roche Diagnostics, Meylan, France) in the presence or absence (negative control) of CatC (5 mmol/l) as reported earlier (25).

Optimization of real-time human HNF1A/PSP preg PCR. The recombinant human PSP/reg protein was generated as previously described (22,26). Briefly, the coding region of PSP/reg was cloned into a transfer vector (pPIC9; Invitrogen) containing the signal sequence of the yeast a-factor mating factor to drive the protein into the secretory pathway.

Subjects and clinical and laboratory measurements. Sixteen diabetic patients (PSP/reg positive: P291fsinsC, P243His, and Ser353) (n = 7) were identified from an Irish MODY collection. Sequencing of the HNF1A gene was performed by IntegraGen (Bonn, Germany). Control subjects were first-degree relatives of HNF1A-positive subjects who were nontype 2 diabetes and negative for the known HNF1A mutation in their pedigree (n = 7). In addition, 10 GAD- and/or islet antibody–positive patients with type 1 diabetes were recruited in the Mater Misericordiae University Hospital. Anthropometric measurements including weight, height, and BMI were obtained. The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio). The plasma glucose concentration was measured using an YSI analyser (Yellow Springs, Ohio).

PSP/reg enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay (ELISA) to quantify human PSP/reg was performed using anti-sera from rabbits and guinea pigs immunized with recombinant human PSP/reg protein as previously described (22,27). Serum was centrifuged, and the IgG were purified by affinity chromatography (Menarini HAB1-10, Rome, Italy). Anti-GAID was analyzed using competitive fluid-phase radioimmunoassay by the neuroscience group at John Radcliffe Hospital, Oxford, U.K., and islet cell antibodies by University College London Medical School by similar means. Study groups were matched for age, sex, and BMI as shown in Table 1 and approved by the Mater Misericordiae University Hospital Ethics committee.

TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>HNF1A-MODY</th>
<th>HNF1A-MODY negative</th>
<th>Type 1 diabetes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>10</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>(female:male)</td>
<td>10:6</td>
<td>4:3</td>
<td>6:4</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.8 ± 20</td>
<td>27.9 ± 11</td>
<td>31.8 ± 4.9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.4 ± 4</td>
<td>26.6 ± 7.9</td>
<td>25.5 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>A1C (%)</td>
<td>7.6 ± 0.3*</td>
<td>5.4 ± 0.3</td>
<td>7.8 ± 0.3*</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>PSP (ng/ml)</td>
<td>18.8 ± 2.7*</td>
<td>8.3 ± 5.8</td>
<td>17.3 ± 1.7*</td>
<td>P &lt; 0.05*</td>
</tr>
</tbody>
</table>

Data are means ± SE. Patients were age, sex, and BMI matched. *P < 0.05 vs. HNF1A-MODY-negative group.
PSP/reg levels were compared with standard amounts of protein of recombinant human PSP/reg.

**Statistical analysis.** Results were expressed as means ± SE. Statistical analysis was conducted using the SPSS version 15.0 software package for Windows (SPSS). Differences between treatments were analyzed by Student *t* test, as well as one-way ANOVA and a subsequent Tukey tests. Nonparametric data were analyzed by Mann-Whitney *U* test. Differences were considered to be significant at *P* < 0.05.

**RESULTS**

The inducible expression of DN-HNF1A leads to a potent induction of PSP/reg mRNA and protein. Inducible, dominant-negative suppression of HNF1A function in INS-1 cells reduces the expression of HNF1A target genes involved in glucose and lipid homeostasis (4) and induces a caspase-dependent apoptosis (8). To investigate the relationship between DN-HNF1A expression, activation of apoptosis, and induction of the regenerative PSP/reg gene in particular, INS-1 cells stably transfected with respective rTA systems were induced to express either DN-HNF1A or WT-HNF1A. We observed a time-dependent induction of DN-HNF1A or WT-HNF1A mRNA in the respective INS-1 cells (Fig. 1A). This correlated with a significant reduction in mRNA levels of *pdk1*, a known HNF1A target gene (7), in response to DN-HNF1A expression but not WT-HNF1A (Fig. 1B). Examination of the expression of the Reg family of genes in INS-1 cells demonstrated that DN-HNF1A led to a potent induction of the *PSP/reg* gene at 24 h (15-fold) and 48 h (30-fold). Induction of WT-HNF1A for up to 48 h did not significantly regulate *PSP/reg* expression (Fig. 1C).

We next investigated PSP/reg protein levels after induction of DN-HNF1A. Cleavage of PSP/reg converts the 16-kDa proteins into a 14-kDa insoluble fibrillar protein (28). Western blot analysis demonstrated a significant upregulation of the PSP/reg protein (16 kDa) following DN-HNF1A induction and a time-dependent increase in the 14 kDa form of PSP/reg (Fig. 1D).

**High extracellular glucose levels potentiates PSP/reg mRNA induction.** We next determined whether the induction of *PSP/reg* mRNA was glucose dependent. Interestingly, we found that the DN-HNF1A induction of *PSP/reg* mRNA expression was modulated by varying extracellular glucose concentrations for 2 h following DN-HNF1A induction. The increase in *PSP/reg* mRNA expression was potentiated in a concentration-dependent manner, with the highest induction evident at 18 mmol/l (Fig. 2A).

**Elevated PSP/reg levels in an HNF1A-MODY animal model and in the serum of HNF1A-MODY patients.** We analyzed paraffin-embedded pancreatic sections from 5-month-old diabetic (Fig. 2B) mice expressing DN-HNF1A in β-cells and compared them with wild-type C57BL/6JBonTac mice. Sections were costained for PSP/reg and insulin. PSP/reg was expressed at low levels in wild-type islets (36 ± 1 PSP/reg-positive cells per islet; *n* = 8 islets from *n* = 2 animals) and was found almost exclusively in peripheral islet cells. In contrast, DN-HNF1A islets showed significantly elevated PSP/reg-positive islet cells (82 ± 2 PSP/reg-positive cells per islet; *n* = 8 islets from *n* = 2 animals). Moreover, PSP/reg-positive cells were found to be widespread throughout the transgenic islets and were also detectable in insulin-negative islet cells. Strong expression was also observed in the surrounding acinar cells (Fig. 2B). Interestingly, high magnification analysis suggested that many islet cells with elevated PSP/reg immunoactivity were positioned in the vicinity of cells displaying apoptotic nuclear morphology (Fig. 2C).

We next sought to determine whether increased PSP/Reg1a levels could also be detected in HNF1A-MODY diabetic subjects. We analyzed serum PSP/Reg1a levels in a group of 16 diabetic subjects with HNF1A-MODY using ELISA. Results were compared with serum levels in seven HNF1A-MODY–negative, nondiabetic family members. PSP/Reg1a levels were significantly elevated in HNF1A-MODY subjects (Fig. 2D). Interestingly, we also detected elevated PSP/Reg1a serum levels in the serum of patients with type 1 diabetes (*n* = 10 patients) (Fig. 2D).

**Induction of PSP/reg mRNA and protein by DN-HNF1A function involves the activation of caspases.** Previously we demonstrated that DN-HNF1A–induced apoptosis of INS-1 cells required caspases and involved the activation of the mitochondrial apoptosis pathway (8). To assess whether the activation of caspase 3/7 activity monitored by monitoring the cleavage of a fluorogenic caspase substrate. In agreement with our earlier report (8), there was a significant increase in caspase activity in the time frame of 12–18 h post induction. Expression of WT-HNF1A or doxycycline treatment of parental INS-1 cells did not activate caspases (Fig. 3A). Furthermore, DN-HNF1A induction mediated nuclear morphological changes indicative of apoptosis with condensation and fragmented nuclei. Pretreatment with the pancaspase inhibitor zVAD.fmk inhibited the occurrence of this apoptotic morphology confirming the caspase dependence (Fig. 3B).

Given the similar kinetics of *PSP/reg* mRNA induction and caspase activation, we explored the possibility that caspase activation regulated *PSP/reg* expression. Indeed, DN-HNF1A induction of *PSP/reg* expression was completely inhibited by pretreatment with zVAD.fmk (Fig. 3C and D). Next we addressed whether DN-HNF1A induction of *PSP/reg* mRNA could be sufficiently explained by its ability to activate apoptosis. Indeed, induction of apoptosis in INS-1 cells with the topoisomerase inhibitor, etoposide, was sufficient to increase *PSP/reg* mRNA levels in a caspase-dependent manner (Fig. 3E).

**Conditioned medium from DN-HNF1A–induced INS1 cells results in a prominent increase in PSP/reg mRNA expression in naïve INS1 cells.** Executioner caspases such as caspase 3 inactivate transcription and translation processes (29), suggesting that apoptosing cells were unlikely to be the source of increased *PSP/reg* mRNA during DN-HNF1A–induced apoptosis. We investigated whether this increased expression was due to apoptosing cells sending a paracrine signal to neighboring cells. After 48 h of DN-HNF1A induction in the presence and absence of zVAD.fmk, the conditioned medium was added directly to naïve INS-1 cells resulting in a 16-fold increase in *PSP/reg* mRNA levels in the naïve INS-1 cells (Fig. 4A). This induction was absent in cultures incubated with the conditioned medium of zVAD.fmk pretreated DN-HNF1A–induced cells (Fig. 4A) or heat-inactivated conditioned medium (Fig. 4B), supporting the hypothesis that apoptotic cells secrete heat-sensitive factor(s) that stimulate(s) *PSP/reg* gene induction in neighboring cells.
Removal of microparticles inhibits \( P\text{S}P\text{/reg} \) gene induction. Microparticles are small (100 nm) membrane vesicles originating from blebbing membranes of apoptotic cells and can be identified by annexin V staining. These microparticles may contain nuclear proteins as well as nucleic acids (30,31). We examined if microparticles were present in our conditioned medium and, if so, whether these could modulate \( P\text{S}P\text{/reg} \) expression. After 48 h of DN-HNF1A induction in the presence and absence of zVAD.fmk, we enriched for microparticles in the supernatant and evaluated their impact on \( P\text{S}P\text{/reg} \) expression.

**FIG. 1.** The inducible expression of DN-HNF1A leads to a potent induction of \( P\text{S}P\text{/reg} \) mRNA and protein. A: The time course of the \( hnf1a \) gene induction in DN-HNF1A and WT-HNF1A induced INS-1 cells, represented by absolute quantitative PCR (qPCR). Data are presented as cDNA copy number per microliter. Data are represented as means ± SE from \( n = 3 \) cultures. The experiment was repeated three times with similar results. *\( P < 0.05 \) indicates the difference from noninduced controls. B: Quantification of the pyruvate dehydrogenase kinase (\( Pdk1 \)) gene expression. DN-HNF1A and WT-HNF1A INS-1 cells were induced with doxycycline from 0 to 48 h. \( Pdk1 \) mRNA expression was examined using real-time qPCR relative to \( \beta\)-actin. Data shown are the means ± SEM of \( n = 3 \) cultures. The experiment was repeated four times with similar results. *\( P < 0.05 \), difference from noninduced controls. C: Quantification of \( P\text{S}P\text{/reg} \) gene expression following DN-HNF1A and WT-HNF1A induction in INS-1 cells. INS-1 cells were treated with 500 ng/ml doxycycline from 0 to 48 h. mRNA expression of \( P\text{S}P\text{/reg} \) was examined using real-time qPCR relative to \( \beta\)-actin. Expression levels were normalized to control cells and data represent means ± SEM from \( n = 3 \) cultures. *\( P < 0.05 \), difference from noninduced controls. Experiments were repeated six times with similar results (D) whole cell lysates (25 μg) were analyzed by Western blotting on 15% SDS-PAGE. Membranes were probed with a polyclonal antibody recognizing \( P\text{S}P\text{/reg} \). The 14-kDa fragment represents a \( P\text{S}P\text{/reg} \) cleavage product. \( \beta\)-Actin served as a loading control. Approximate molecular weights are provided on the right side of the figure. Similar results were obtained in two separate experiments.
High extracellular glucose potentiates PSP/reg mRNA induction in DN-HNF1A expressing INS-1 cells. A: INS-1 cells were induced to express DN-HNF1A for 24 h. Cultures were continued for 2 h at either 3, 6, 12, and 18 mmol/l glucose concentrations. After this period, mRNA expression of PSP/reg was analyzed by real-time qPCR. Expression levels were normalized to noninduced control cells and data are represented as means ± SEM from $n = 3$ cultures. #P < 0.05, difference from non induced controls; *P < 0.05, difference from induced cultures at 3 mmol/l glucose. Experiment was repeated three times with similar results. B: PSP/reg immunoreactivity in islet cells in an in vivo mouse model of DN-HNF1A–induced apoptosis. Paraffin-embedded pancreatic sections on slides from 5-month-old wild-type C57BL/6J BomTac control mice and RIP-DN-HNF1A transgenic mice were double stained using antibodies against PSP/reg and insulin. Primary antibodies were recognized by secondary antibodies coupled to Rhodamine (red) for PSP/reg and to FITC (green) for insulin, respectively. Cell nuclei were recognized by secondary antibodies coupled to Rhodamine (red) for using antibodies against PSP/reg and insulin. Primary antibodies were at least 150-fold more abundant in unfiltered compared with filtered conditioned medium with a significantly reduced level in zVAD.fmk pretreated unfiltered medium (Fig. 4D). These results suggest that caspases are required for microparticle formation, and their removal abolishes the conditioned medium–induced PSP/reg mRNA expression in naïve INS-1 cells.

Treatment of INS-1 cells with recombinant PSP/reg protein reverses the DN-HNF1A–induced decrease in cell proliferation and increases insulin mRNA levels. Animal models of diabetes have demonstrated that recombinant PSP/reg (rPSP/reg) increases β-cell ductal cell proliferation (21,32). DN-HNF1A induction led to significant decreases in cell proliferation as detected by BrdU incorporation, which was reversed by rPSP/reg treatment (Fig. 5A). Decreased cell proliferation has been attributed to DN-HNF1A–induced expression of the cell cycle inhibitor p27Kip1 (8,9). Induction of DN-HNF1A led to increased p27Kip1 mRNA expression but did not affect p21WAF1 expression (data not shown), with rPSP/reg cotreatment reducing DN-HNF1A–induced p27Kip1 mRNA expression (Fig. 5C). Furthermore, rPSP/reg treatment rescued the decrease in insulin gene expression induced by DN-HNF1A expression (Fig. 5B). Taken together, our data suggest that rPSP/reg treatment reverses the DN-HNF1A–induced phenotype. Indeed, BrdU incorporation was also significantly elevated in naïve INS-1 cells treated with conditioned cultured medium from DN-HNF1A–induced cells compared with those induced in the presence of zVAD.fmk (Fig. 5D).

**DISCUSSION**

The present study provides evidence that β-cell apoptosis may stimulate β-cell regeneration. We demonstrate 1) that dominant-negative suppression of HNF1A leads to apoptosis and a potent induction of PSP/reg; 2) this induction was potentiated by increased extracellular glucose concentrations; 3) the induction of PSP/reg was inhibited by the broad-spectrum caspase inhibitor, zVAD.fmk, which also inhibited DN-HNF1A–induced apoptosis; 4) conditioned medium from DN-HNF1A–induced cells was sufficient to induce PSP/reg expression in naïve INS-1 cells, suggesting that apoptosing INS-1 cells secrete factors that stimulate PSP/reg induction in neighboring cells; 5) removal of

PSP/reg mRNA

(ΔN-HNF1A)


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staining of nuclear chromatin. Apoptotic cell morphology was assessed by DAPI staining. Treatment with the broad spectrum caspase inhibitor zVAD.fmk (100 µmol/l) inhibits apoptosis after induction of DN-HNF1A. Cultures were simultaneously treated with doxycycline and zVAD.fmk or vehicle (DMSO; zVAD.fmk) for 48 h. Apoptotic cell morphology was assessed by DAPI staining of nuclear chromatin. C: zVAD.fmk inhibits PSP/reg mRNA induction. The mRNA expression of PSP/reg was examined using real-time qPCR. Expression levels were normalized to noninduced plus noninduced zVAD.fmk–treated control cells. Data are represented as means ± SE from n = 3 cultures. *P < 0.05, difference compared with doxycycline alone–treated cultures. The experiment was repeated three times with similar results. 

**FIG. 3.** Activation of caspases during DN-HNF1A–induced apoptosis triggers PSP/reg induction. A: Time course of caspase-3–like protease activity in whole-cell extracts. INS-1 cells were induced to overexpress WT-HNF1A or DN-HNF1A for 0, 16, 24, and 48 h. As a control, parental INS-1 cells were treated with doxycycline for 0, 16, 24, and 48 h. Caspase protease activity was measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC (10 µmol/l). Activities are represented as increase in AMC fluorescence (in AU) per 1 h per microgram of protein. Data represent means ± SE from n = 6 cultures. Experiments were repeated two times with similar results. *P < 0.05, difference from noninduced controls. B: Treatment with the broad spectrum caspase inhibitor zVAD.fmk (100 µmol/l) inhibits apoptosis after induction of DN-HNF1A. Cultures were simultaneously pretreated with doxycycline and zVAD.fmk or vehicle (DMSO; zVAD.fmk) for 48 h. Apoptotic cell morphology was assessed by DAPI staining of nuclear chromatin. C: zVAD.fmk inhibits PSP/reg mRNA induction. The mRNA expression of PSP/reg was examined using real-time qPCR. Expression levels were normalized to noninduced plus noninduced zVAD.fmk–treated control cells. Data are represented as means ± SE from n = 3 cultures. *P < 0.05, difference from noninduced controls. #P < 0.05, difference compared with doxycycline alone–treated cultures. The experiment was repeated three times with similar results. D: PSP/reg protein expression was detected by Western blotting. A duplicate experiment showed similar results. Membrane was stripped and reprobed with anti–β-actin as a loading control. E: PSP/reg gene induction in parental INS-1 cells treated with etoposide is caspase dependent. INS-1 cells were simultaneously treated with etoposide (20 µmol/l) or vehicle (0.1% DMSO) for 24 h in the presence and absence of zVAD.fmk (100 µmol/l). Following treatment, mRNA expression of PSP/reg was examined using real-time qPCR. Expression levels were normalized to control cells. Data are represented as means ± SE from n = 3 separate cultures. *P < 0.05, difference compared with etoposide alone–treated cultures. Data shown are the mean of n = 3 separate experiments.

**Regulation of β-cell mass in HNF1A-MODY.** Evidence is growing that functional β-cell mass is decreased in animal models of MODY and in type 2 diabetes, with an increased apoptosis rate largely contributing to this decrease (12,13,33). However, a decrease in β-cell mass does not necessarily implicate that new β-cells are not generated during disease progression. Indeed, β-cell mass is a result of a homeostatic balance between the generation of new β-cells and the rate of their apoptosis (34). As HNF1A-MODY and type 2 diabetes evolve over several years or decades, the destruction or apoptosis rate of β-cells may eventually exceed the rate of β-cell generation during disease progression.

Although β-cell regeneration may occur during disease progression, it is not clear whether this happens through self-replication of mature β-cells, through neogenesis of progenitor cells residing in the pancreas or recruited from
Conditioned medium from DN-HNF1A–induced INS-1 cells results in a prominent increase in PSP/reg mRNA expression in naïve INS1 Cells. A: INS-1 cells were induced with 500 ng/ml of doxycycline in the presence and absence of zVAD.fmk (100 μmol/l) at 0.05% serum in 6 mmol/l glucose for 48 h. The conditioned culture medium was added to the noninduced, naïve cells for a further 24 h. After treatment, mRNA expression of PSP/reg was analyzed by real-time qPCR. Expression levels were normalized to noninduced controls. Data are represented as means ± SE from n = 3 cultures. *P < 0.05, difference from noninduced controls. Experiments were repeated six times with similar results. B: Duplicate experiments of INS-1 cells were induced for 48 h. Conditioned culture medium was heat inactivated at 95°C for 15 min and added to the naïve cells for a further 24 h. PSP/reg gene expression was analyzed by real-time qPCR. Data are represented as means ± SE from n = 3 cultures. *P < 0.05, difference from noninduced controls. Experiments were repeated four times with similar results. C: The conditioned medium from cells treated as described in A above was centrifuged and filtered through 0.20 μm filters and added to naïve INS-1 cells for 24 h. Unfiltered conditioned medium served as a control. PSP/reg gene expression was analyzed by real-time qPCR. Data are represented as means ± SE from n = 3 cultures. *P < 0.05, difference from noninduced controls. Experiments were repeated three times with similar results. D: INS-1 cells were induced with doxycycline in the presence and absence of zVAD.fmk. The conditioned culture medium was added to naïve cells for a further 24 h. Supernatants were collected and detached cells were precipitated at (300 g), followed by additional 800g centrifugation. Microparticles were quantified as described in research design and methods.

FIG. 4. Conditioned medium from DN-HNF1A–induced INS-1 cells.
detected increased expression of PSP/reg in insulin-positive islet cells in 5-month-old transgenic HNF1A-MODY mice, confirming previous findings that PSP/reg is expressed in islets during injury and regeneration processes (17,19). Moreover, PSP/reg was also found in insulin-negative islet cells (Fig. 2B). In this context, a recent study has suggested α-cells to be the major source of β-cell regeneration/conversion in mice in vivo (41). We also detected increased PSP/reg levels in the serum of HNF1A-MODY patients. We can only speculate whether the source of PSP/reg in the serum of HNF1A-MODY patients was the endocrine or exocrine pancreas (i.e., islet or acinar cells). We can likewise not exclude that these levels came from other organs potentially affected in HNF1A-MODY, such as the liver, where PSP/reg is also expressed (42). However, we also detected elevated levels of PSP/reg in subjects with type 1 diabetes, arguing for a role for pancreatic injury and β-cell apoptosis as a source for elevated PSP/reg serum levels.

**Microparticles as signal transducers or enablers.** In our study, we detected that apoptotic INS-1 cells released annexin V-positive microparticles into the medium in a caspase-dependent manner. Microparticles are being increasingly described as efficient vehicles for the release of signaling molecules. Microparticles enable communication between different cell types (43) and also deliver proteins to cells that do not normally express them (44). Microparticles deriving from apoptotic cells have been shown to carry nuclear proteins as well as nucleic acids (31,45). Filtration experiments suggested that the absence of microparticles abolished the effect of the conditioned medium to induce PSP/reg mRNA in naive INS-1 cells. This suggested that cytokines, peptide hormones, or other soluble messengers present in the conditioned medium did not mediate this effect. Interestingly, heat inactivation of the conditioned medium was sufficient to inhibit PSP/reg mRNA expression in naive INS-1 cells. It is therefore tempting to speculate that microparticles may contain signaling molecules or nuclear proteins which, upon uptake by neighboring cells, enhanced PSP/reg gene expression in these cells.

In conclusion, our study demonstrates that the execution of caspase-dependent apoptosis in INS-1 cells stimulates the induction of regenerative genes in neighboring cells, suggesting a role for MPs and PSP/reg in this process. These findings provide new mechanistic insights into a fundamental aspect of β-cell mass regulation.
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