Deletion of *Fas* protects islet beta cells from cytotoxic effects of human islet amyloid polypeptide

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Abstract

Aims/hypothesis Islet amyloid, which is mainly composed of human islet amyloid polypeptide (hIAPP), is a pathological characteristic of type 2 diabetes and also forms in cultured and transplanted islets. We used islet beta cells as well as two ex vivo models of islet amyloid formation, cultured human islets and hIAPP-expressing transgenic mouse islets with or without beta cell *Fas* deletion, to test whether: (1) the aggregation of endogenous hIAPP induces *Fas* upregulation in beta cells; and (2) deletion or blocking of *Fas* protects beta cells from amyloid toxicity.

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Methods INS-1, mouse or human islet cells were cultured with hIAPP alone, or with amyloid inhibitor or *Fas* antagonist. Non-transduced islets, and human islets or hIAPP-expressing mouse islets transduced with an adenovirus that delivers a human proIAPP-specific small interfering RNA (siRNA) (Ad-ProhIAPP-siRNA) were cultured to form amyloid. Mouse islets expressing hIAPP with or without *Fas* were similarly cultured. Beta cell *Fas* upregulation, caspase-3 activation, apoptosis and function, and islet IL-1 β levels were assessed.

Results hIAPP treatment induced *Fas* upregulation, caspase-3 activation and apoptosis in INS-1 and islet cells. The amyloid inhibitor or *Fas* antagonist reduced apoptosis in hIAPP-treated beta cells. Islet cells with *Fas* deletion had lower hIAPP-induced beta cell apoptosis than those expressing *Fas*. Ad-ProhIAPP-siRNA-mediated amyloid inhibition reduced *Fas* upregulation and IL-1 β immunoreactivity in human and hIAPP-expressing mouse islets. Cultured hIAPP-expressing mouse islets with *Fas* deletion had similar amyloid levels, but lower caspase-3 activation and beta cell apoptosis, and a higher islet beta:alpha cell ratio and insulin response to glucose, compared with islets expressing *Fas* and hIAPP.

Conclusions/interpretation The aggregation of biosynthetic hIAPP produced in islets induces beta cell apoptosis, at least partially, via *Fas* upregulation and the *Fas*-mediated apoptotic pathway. Deletion of *Fas* protects islet beta cells from the cytotoxic effects of endogenously secreted (and exogenously applied) hIAPP.

Keywords Amylin · Amyloid · Beta cell apoptosis · *Fas* receptor · Islet amyloid polypeptide · Type 2 diabetes

Abbreviations

Ad-Cont-siRNA	An adenovirus that delivers a random non-specific small interfering RNA
Ad-Proh <i>IAPP</i> -siRNA	An adenovirus that delivers a human pro <i>IAPP</i> -specific small interfering RNA
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
hIAPP	Human islet amyloid polypeptide
rIAPP	Rat islet amyloid polypeptide

Introduction

Islet amyloid formation is a pathological characteristic of the pancreas in type 2 diabetes and contributes to progressive beta cell dysfunction and death in this disease [1–3]. Islet amyloid also forms in human islets during culture [4] and following transplantation into mouse models of type 1 diabetes [5–7]. Importantly, widespread amyloid deposition was reported in human islets that had been transplanted into a patient with type 1 diabetes [8]. Amyloid formation in transplanted islets is associated with beta cell dysfunction and recurrence of hyperglycaemia in animal models of type 1 diabetes [7, 9]. Thus, in addition to its role in the pathogenesis of type 2 diabetes, islet amyloid formation may also play a role in islet graft failure in patients with type 1 diabetes.

Islet amyloid is mainly formed by the aggregation of islet amyloid polypeptide (amylin) [10, 11], a 37-amino acid hormone that is co-localised and co-secreted with insulin in response to beta cell secretagogues [12, 13]. Despite considerable study during the past decade, it is still not clear why normally soluble human islet amyloid polypeptide (hIAPP) molecules form toxic aggregates in type 2 diabetes. The following have been proposed to contribute to the aggregation of hIAPP molecules in type 2 diabetes: (1) presence of an amyloidogenic sequence in the hIAPP molecule [2, 14]; (2) elevated hIAPP production and secretion from beta cells, associated with an increased demand for insulin [13, 15]; and (3) defects in trafficking and processing of proIAPP, associated with beta cell dysfunction [13, 15–17]. The rapid formation of amyloid in cultured and transplanted human islets may be related to poor clearance of released hIAPP from beta cells due to the disrupted vasculature [18] and/or to the impaired proIAPP processing and secretion that are associated with beta cell dysfunction induced by islet isolation, culture and transplantation [13, 19].

Aggregates of hIAPP, including small oligomeric species, are toxic to beta cells and have been shown to induce beta cell dysfunction and apoptosis in isolated human islets [4, 20]. Previous *in vitro* studies have suggested different mechanisms for the beta cell apoptosis mediated by hIAPP aggregates, including disruption of membrane integrity and

formation of non-selective ion-channel-like structures [21–23], activation of the caspase pathways [24–27] and interaction of hIAPP fibrils with components of beta cell membranes such as heparan sulphate proteoglycans [28, 29] or touch receptors [30]. It is, however, not clear which of these mechanism(s) contribute(s) to hIAPP-induced beta cell death in primary islets, in which the level of endogenously produced hIAPP (pmol/l) is several fold lower [1, 31] than that used *in vitro* (μmol/l). Recent studies suggest that endoplasmic reticulum stress [32–34], oxidative stress [35] and disruption in the autophagy–lysosomal pathway [36] due to formation of intracellular hIAPP aggregates also contribute to the cytotoxic effects of hIAPP. It therefore appears that, at least *in vitro*, hIAPP-induced beta cell apoptosis involves several mechanisms, some of which may share the same apoptotic signalling pathways.

Fas (CD95/APO-1) is a transmembrane receptor protein belonging to the tumour necrosis factor superfamily of receptors [37–39]. The extrinsic apoptotic pathway initiated by the interaction between Fas and Fas ligand (FasL/CD95L) has been implicated in autoimmune-mediated beta cell death in type 1 diabetes [40–42] and non-immune-mediated beta cell death in type 2 diabetes [43, 44]. Islet beta cells constitutively produce Fas ligand, but do not normally produce Fas at detectable levels [40, 43]. However, conditions associated with beta cell stress, such as exposure to elevated glucose, leptin or cytokines (e.g. IL-1β), can induce upregulation of Fas and apoptosis in isolated human islets [43–45].

In the present study, we tested whether hIAPP aggregates derived from biosynthetic hIAPP secreted from islets can induce beta cell apoptosis via upregulation of Fas. We used islets from humans and transgenic mice with beta cell-specific hIAPP expression and *Fas* deletion, as well as transformed and primary islet beta cells, to investigate the role of Fas in mediating islet amyloid-induced beta cell toxicity and to examine whether deletion of *Fas* can protect beta cells from hIAPP aggregates derived from endogenously secreted and exogenously applied hIAPP.

Methods

Materials Synthetic hIAPP and rat islet amyloid polypeptide (rIAPP) (1–37) were from Bachem (Torrance, CA, USA) and Fas antagonist (Kp7-6) was from EMD Chemicals (Gibbstown, NJ, USA). Thioflavin S, dithizone, BSA, Congo red, HEPES buffer, 2-mercaptoethanol, sodium bicarbonate, poly-L-lysine, Triton X-100, DMSO, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), tribromoethanol and collagenase (Type XI) were obtained from Sigma-Aldrich (Oakville, ON, Canada). RPMI-1640, FBS, penicillin, streptomycin, gentamycin, cell dissociation buffer enzyme-free Hanks'-based solution, Ham's-F10, trypsin-EDTA and

glutamax were from Invitrogen Canada (Burlington, ON, Canada). CMRL culture medium was from Mediatech (Herndon, VA, USA).

Human islets Human islets isolated from deceased pancreas donors were provided by the Ike Barber Human Islet Transplant Laboratory (Vancouver, BC, Canada) in accordance with approved procedures and guidelines of the Clinical Research Ethics Board of the University of British Columbia. Hand-picked human islets (purity >90%) were cultured for 7 days at 37°C in CMRL supplemented with 11.1 mmol/l glucose, 10% (vol./vol.) FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 50 µg/ml gentamycin. Culture procedures were in humidified 5% CO₂/95% air.

Animal models Hemizygous C57BL/6 *hIAPP* transgenic mice with beta cell-specific *hIAPP* expression (*hIAPP*^{+/-}) were kindly provided by S. Kahn (Department of Medicine, University of Washington, Seattle, WA, USA) and maintained by breeding with DBA/2J mice (Jackson Laboratory, Bar Harbor, ME, USA). Male *hIAPP*-expressing mice form islet amyloid in vivo and develop diabetes in the presence of a predisposing factor such as a high-fat diet [46]. Mice with beta cell-specific *Fas* deletion (*RIPcre*⁺*Fas*^{fl/fl}) were generated using the Cre/loxP recombinase system as previously described [47]. *RIPcre*⁺*Fas*^{fl/fl} mice were maintained by breeding *RIPcre*⁺*Fas*^{+/fl} mice from the Ontario Cancer Institute. To generate mice with beta cell-specific *hIAPP* expression and *Fas* deletion, *hIAPP*^{+/-} mice were cross-bred with *RIPcre*⁺*Fas*^{fl/fl} mice to produce *RIPcre*⁺*Fas*^{+/fl}/*hIAPP*^{+/-} mice, which were then bred together to generate *RIPcre*⁺*Fas*^{fl/fl}/*hIAPP*⁺ and *RIPcre*⁺*Fas*^{+/+}/*hIAPP*⁺ mice. The first generation of these offspring was used for the studies. Islets from *RIPcre*⁺*Fas*^{fl/fl} animals have normal islet mass and enhanced insulin secretion [47]. All mice were fed mouse chow containing 9% (wt/wt) fat (Purina 5021; LabDiet, Richmond, IN, USA). Animals were cared for in accordance with the Guidelines and Principles of Laboratory Animal Care and the standard procedures established by the Canadian Council on Animal Care and the University of British Columbia's Animal Policy and Welfare Committee.

Mouse islet isolation and culture Islets from wild-type (*hIAPP*^{-/-}) and *hIAPP*^{+/-} mice (8–12 weeks old) or different genotypes of *RIPcre*⁺*Fas*/*hIAPP* mice (8–18 weeks) were isolated as previously described [27] with some modifications. Briefly, animals were anaesthetised with tribromoethanol (0.02 ml/g body weight, i.p.) and killed by cervical dislocation. The abdominal cavity was opened and ice-cold collagenase (Type XI; Sigma) in 2.5 ml calcium-free Hanks' solution (final concentration 1,000 U/ml) was injected via the common bile duct. The removed pancreas was incubated with collagenase and Hanks' solution (1,000 U/ml) in a

shaker water bath (14 min, 37°C, 120 rev/min). Digestion was stopped by addition of ice-cold Hanks' solution containing 1 mmol/l CaCl₂. Digested pancreatic tissues were rinsed with the same solution, resuspended in Ham's-F10 and filtered through a 70 µm mesh cell strainer (BD Biosciences, Oakville, ON, Canada). Hand-picked islets (purity >95%) were cultured in Ham's-F10 supplemented with 16.7 mmol/l glucose, 0.5% (wt/vol.) BSA and antibiotics as described for human islets. For all islet studies, the culture medium was changed every 2 days.

Adenoviral transduction of human and mouse islets As previously described [4], we used human islets or *hIAPP*-expressing mouse islets transduced overnight with an adenovirus that delivers a human *proIAPP*-specific small interfering RNA (Ad-Pro*hIAPP*-siRNA) (multiplicity of infection 20), or transduced with an adenovirus that delivers a random non-specific small interfering RNA (Ad-Cont-siRNA) (multiplicity of infection 20). Adenoviral-siRNA transduced human or *hIAPP*-expressing mouse islets were then rinsed and cultured in CMRL (11.1 mmol/l glucose, 7 days) or Ham's-F10 (16.7 mmol/l, 6 days), respectively.

Transformed beta cells and islet cell culture INS-1 (832/13) cells, a transformed rat beta cell line, were a gift from C. Newgard (Duke University Medical Center, NC, USA). Cells were grown in RPMI-1640 containing 11.1 mmol/l glucose supplemented with 10% (vol./vol.) FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 50 µmol/l 2-mercaptoethanol. Human or mouse islets (~200) were dissociated as previously described [27] and cell viability assessed by Trypan blue. Islet cells were cultured in 8-well chamber slides (BD Biosciences) pre-coated with poly-L-lysine; the culture process was in CMRL (human) or Ham's-F10 (mouse) containing 5.5 and 10 mmol/l glucose, respectively.

Treatment studies with *hIAPP*, *Fas* antagonist and amyloid binding dye Lyophilised *hIAPP* or *riAPP* was dissolved in HFIP and incubated at room temperature (1 h); aliquots were frozen (-80°C) and lyophilised. INS-1 or dispersed islet cells were treated with *hIAPP* or *riAPP* that had been prepared freshly by dissolving lyophilised peptide in medium; they were then cultured for different times as detailed. The amyloid-binding dye Congo red or *Fas* antagonist Kp7-6 was added to the culture medium 30 min before addition of *hIAPP* at final concentrations of 25 µmol/l and 10 mmol/l, respectively.

Assessment of beta cell function in cultured islets Islets (25 islets per mouse; duplicate, four to six mice per group) were pre-incubated (1 h) at 37°C in 300 µl KRB containing 10 mmol/l HEPES (pH 7.4), 0.25% (wt/vol.) BSA and 1.67 mmol/l glucose, followed by 1 h of incubation in 150 µl

KRB containing 1.67 mmol/l glucose (basal insulin release) and by 1 h of incubation in KRB with 16.7 mmol/l glucose (stimulated insulin release). Islets were lysed in 100 μ l of 1 mol/l acetic acid and 0.1% (wt/vol.) BSA lysis buffer, followed by 10 min incubation at 100°C. Incubation media and islet lysates were centrifuged and supernatant fractions frozen (−20°C) until assayed. Insulin and proinsulin levels were measured using mouse-specific insulin and proinsulin ELISA kits (ALPCO Diagnostics, Salem, NH, USA).

Immunolabelling, TUNEL and thioflavin S staining Paraffin-embedded islet sections (5 μ m) were de-waxed, rehydrated and blocked in 2% (vol./vol.) normal goat and/or donkey serum (Vector Laboratories, Burlingame, CA, USA). Fixed islet sections (following antigen retrieval with citrate buffer) or cells were incubated overnight with guinea pig anti-insulin alone (islets 1:750, cells 1:200; Dako, Carpinteria, CA, USA) or with each of rabbit anti-IL-1 β (1:100; Santa Cruz, Santa

Cruz, CA, USA), anti-glucagon (1:750; Dako), anti-cleaved caspase-3 (1:100; Cell Signaling, Pickering, ON, Canada) and anti-Fas (mouse islets 1:50, cells 1:100, Santa Cruz; human islets 1:100, Cell Signaling). Islet sections or cells were then incubated for 1 h with Texas red-conjugated anti-guinea pig (1:750; Jackson Laboratories, West Grove, PA, USA) and Alexa 488-conjugated anti-rabbit (1:200; Molecular Probes, Eugene, OR, USA). For triple immunostaining, islet cells were immunolabelled overnight for insulin (1:200) and Fas (1:100), followed by incubation with aminomethylcoumarin acetate-conjugated anti-guinea pig (1:200; Jackson) and Texas red-conjugated anti-rabbit (1:100; Jackson), and then with Alexa 488-conjugated cleaved caspase-3 antibody (1:10; Cell Signaling). The specificity of the Fas antibody was evaluated by immunolabelling of IL-1 β - or hIAPP-treated INS-1 cells after antibody blocking using recombinant Fas (electronic supplementary material [ESM] Fig. 1). For double insulin and TUNEL or thioflavin S staining, after immunolabelling

Fig. 1 Exposure to synthetic hIAPP induces Fas upregulation, associated with caspase-3 activation and apoptosis in INS-1 and mouse islet beta cells. INS-1 cells and dispersed mouse islet cells were treated with 10 μ mol/l synthetic hIAPP or non-fibrillogenic rIAPP (as control) at different time points. **a** Immunolabelling of INS-1 cells for insulin (red) and Fas (green) after 8 and 12 h of exposure to hIAPP or rIAPP. **b** Immunolabelling of dispersed mouse islet cells for insulin (red) and Fas (green), or cleaved (active) caspase-3 (green) following exposure to hIAPP for 8, 12 or 16 h. The squares (dashed white lines) denote regions enlarged and depicted as insets at bottom right of the relevant images.

c The proportion of TUNEL-positive islet cells and **(d)** beta cell:total islet cell ratio in dispersed mouse islets following culture with or without hIAPP for 24 h. The proportion of apoptotic beta cells **(c)** was quantified by manual counting of double insulin- and TUNEL-positive islet cells in a minimum of ten microscopic fields each containing 100 to 150 dispersed islet cells. The beta cell:total islet cell ratio **(d)** represents the mean of insulin-positive islet cells divided by total islet cells in each microscopic field. Results **(c, d)** are expressed as means $\pm$ SEM of three independent studies performed in triplicate. * p <0.05 by Student's t test vs non-treated group

for insulin, islet sections (or cells) were incubated for 30 min at 37°C with TUNEL reaction mixture (Roche Diagnostics, Laval, QC, Canada) or for 5 min at room temperature with 0.5% (wt/vol.) thioflavin S solution.

Statistical analysis Data are expressed as means±SEM. Statistical analyses were performed using one-way ANOVA followed by Newman–Keuls test or by Student's *t* test as appropriate. A value of $p < 0.05$ was taken as significant. All experiments were performed in duplicate or triplicate and repeated at least three times.

Results

Exogenously applied hIAPP induces Fas upregulation, caspase-3 activation and apoptosis in transformed INS-1 cells and in primary human and mouse islet beta cells Transformed INS-1 cells and dispersed mouse or human islet cells were treated with synthetic hIAPP (10 µmol/l) for 4, 8 and 12 h. Fas was not detectable in non-treated INS-1 cells by immunolabelling, but treatment with hIAPP induced Fas upregulation in a time-dependent manner, with low levels of Fas present after 8 h and higher levels detectable after 12 h

Fig. 2 Co-localisation of Fas and active caspase-3 in human and mouse islet cells treated with synthetic hIAPP. **a** Dispersed human or **(b)** mouse islet cells cultured without (control) or with synthetic hIAPP (10 µmol/l, 12 h) as indicated were immunolabelled for insulin (blue), Fas (red) and cleaved (active) caspase-3 (aCASP-3) (green). The

squares (white outline) denote regions enlarged in each image. Note the higher number of double Fas- and cleaved caspase-3-positive islet beta cells in human and mouse islet cells treated with hIAPP compared with non-treated islet beta cells. Images represent three independent human or mouse studies performed in duplicate

(Fig. 1a). Treatment with non-fibrillogenic rIAPP did not have any noticeable effect on Fas abundance. Similarly, exogenously applied hIAPP induced Fas upregulation in primary islet cells, mainly in beta cells, this being detectable after 8 and 12 h (Fig. 1b). Upregulation of Fas was associated with an increased number of active caspase-3 and TUNEL-positive (apoptotic) beta cells (Fig. 1b, c), resulting in a lower islet beta:total cell ratio in hIAPP-treated than in non-treated islet cells (Fig. 1d). The time point of Fas upregulation preceded that of caspase-3 activation (16 h) and apoptosis (24 h). Moreover, immunolabelling studies showed co-localisation of Fas and active caspase-3 in hIAPP-treated human and mouse islet beta cells (Fig. 2). Unlike INS-1 cells, a low number of Fas-positive beta and non-beta islet cells was present in non-treated dispersed mouse and human islets (Figs 1b and 2a).

Preventing the interaction between hIAPP aggregates and beta cells markedly reduces beta cell apoptosis in hIAPP-treated INS-1 cells We examined whether the interaction between hIAPP fibrils and islet beta cells is required for induction of Fas upregulation. INS-1 beta cells were cultured without or with hIAPP or non-fibrillogenic rIAPP (as control) in the presence or absence of the amyloid binding dye, Congo red, for 12 h. We have previously shown that Congo red at this concentration does not have any adverse effects on beta cell survival [27]. Treatment with Congo red markedly reduced Fas upregulation, caspase-3 activation and apoptosis in hIAPP-treated INS-1 cells (Fig. 3a–c). Treatment with non-fibrillogenic rIAPP did not have any detectable effect on beta cell apoptosis (Fig. 3c).

Blocking or deletion of Fas reduces the beta cell apoptosis induced by exogenously applied hIAPP INS-1 cells or primary islet cells were cultured with or without synthetic hIAPP in the absence or presence of a Fas antagonist (Kp7-6) for 12 h. INS-1 and primary mouse islet cells treated with hIAPP and the Fas antagonist had significantly lower beta cell apoptosis than cells treated with hIAPP in the absence of the Fas antagonist (Fig. 3b–d). Treatment with the Fas antagonist alone did not have any effect on beta cell apoptosis in non-treated cells (Fig. 3d). Furthermore, dispersed wild-type and $RIP^{cre+}Fas^{fl/fl}$ mouse islet cells were treated with synthetic hIAPP. There was no significant difference in basal beta cell apoptosis between wild-type and $RIP^{cre+}Fas^{fl/fl}$ mouse islet cells (Fig. 3e, f). Interestingly, hIAPP-treated $RIP^{cre+}Fas^{fl/fl}$ islets had markedly lower beta cell death than hIAPP-treated wild-type islet cells, suggesting that deletion of *Fas* reduces beta cell death induced by the aggregation of exogenously applied hIAPP.

Inhibition of islet amyloid formation reduces IL-1 β levels, Fas upregulation and beta cell apoptosis in human and

hIAPP-expressing transgenic mouse islets Human or hIAPP-expressing mouse islets were transduced with Ad-ProhIAPP-siRNA to suppress hIAPP production, thereby preventing amyloid formation [4]. Non-transduced and transduced human and mouse islets were then cultured in 11.1 and 16.7 mmol/l glucose, respectively, to potentiate amyloid formation. Islets transduced with Ad-Cont-siRNA, which had a non-specific siRNA, were used as a control to detect any potential adverse effects of adenoviral transduction on beta cells. As expected [16, 48], hIAPP-expressing mouse islets formed amyloid during culture and had a higher rate of beta cell apoptosis than wild-type islets (Fig. 4). Interestingly, the aggregation of endogenously produced hIAPP in cultured hIAPP-expressing mouse islets was associated with Fas upregulation in beta cells (Fig. 4a) and increased islet IL-1 β levels (Fig. 4b). Adenoviral siRNA-mediated suppression of hIAPP expression markedly reduced amyloid formation, islet IL-1 β levels, beta cell Fas upregulation and apoptosis. Similarly, the formation of hIAPP aggregates in human islets during culture was associated with the upregulation of Fas in beta cells and increased islet IL-1 β levels (Fig. 5), both of which were reduced by adenoviral-siRNA-mediated suppression of amyloid formation. Transduction with control adenovirus (Ad-Cont-siRNA) had no noticeable effect on amyloid formation or beta cell survival in human or hIAPP-expressing mouse islets.

Fig. 3 Culture with islet amyloid inhibitor or a Fas antagonist, or deletion of *Fas* in islet beta cells markedly reduces apoptosis induced by exogenously applied hIAPP. **a** INS-1 cells treated with hIAPP (10 μ mol/l, 12 h) without or with Congo red (CR; 25 μ mol/l) were immunolabelled for insulin (red) and Fas (green). **b** INS-1 and mouse islet cells were treated with hIAPP alone (24 h), or with CR or Kp7-6 (10 mmol/l), and immunolabelled for insulin (green) and TUNEL (red). The squares (dashed white lines) denote regions enlarged and depicted as insets at bottom right of the relevant images. **c** The proportion of cleaved caspase-3 (CASP-3) and TUNEL-positive INS-1 cells treated with hIAPP (or rIAPP) in the absence and presence of CR or Kp7-6. Black bars, non-treated; white bars, rIAPP; grey bars, hIAPP; dotted bars, hIAPP+CR; hatched bars, hIAPP+Kp7-6. **d** The proportion of apoptotic mouse islet cells treated with hIAPP alone or with Kp7-6 for 24 h. **e** hIAPP-treated $RIP^{cre+}Fas^{fl/fl}$ and wild-type ($RIP^{cre+}Fas^{+/+}$) mouse islet cells immunolabelled for insulin (red) and Fas (green). The squares (white outline) denote regions enlarged in each image. Fas-positive beta cells were present in hIAPP-treated wild-type islet cells, but not in mouse islet cells with beta cell *Fas* deletion. A low number of Fas-positive non-beta cells was detected in wild-type and $Fas^{fl/fl}$ islet cells. **f** The proportion of apoptotic beta cells in mouse islet cells with and without *Fas* deletion after treatment with hIAPP (24 h). The proportion of apoptotic beta cells was quantified by manual counting of double insulin- and cleaved caspase-3- or TUNEL-positive INS-1 or islet cells in a minimum of ten microscopic fields, each containing 200 to 300 INS-1 cells or 100 to 150 dispersed islet cells. Results (**c**, **d**, **f**) are expressed as means $\pm$ SEM of three independent studies performed in triplicate; * p <0.05 by one-way ANOVA vs non-treated groups; † p <0.05 by one-way ANOVA vs hIAPP alone group; ‡ p <0.05 by one-way ANOVA vs *Fas*-expressing hIAPP-treated group

Beta cell-specific deletion of Fas in hIAPP-expressing transgenic mouse islets markedly reduces apoptosis induced by the aggregation of endogenously produced hIAPP during culture We generated a mouse model with beta cell-specific

expression of hIAPP and deletion of *Fas* ($RIP^{cre+}Fas^{fl/fl}/hIAPP^{+}$) by cross-breeding hIAPP-expressing mice with $RIP^{cre+}Fas^{fl/fl}$ mice. Our aim was to further examine the role of Fas in beta cell apoptosis induced by the hIAPP

Fig. 4 Suppression of islet amyloid formation in cultured *hIAPP*-expressing mouse islets reduces IL-1 β levels, Fas upregulation and beta cell apoptosis. Islets isolated from *hIAPP*⁺ transgenic mice (8 to 12 weeks old) were transduced overnight with Ad-Proh*IAPP*-siRNA or Ad-Cont-siRNA (as control). Non-transduced and transduced islets were cultured in Ham's-F10 (16.7 mmol/l glucose) for 6 days to allow amyloid formation. Paraffin-embedded islet sections were immunolabelled for: **(a)** insulin (Ins; red) and thioflavin S (Thio; blue), or insulin (red) and Fas (green) as indicated; and **(b)** insulin (red) and thioflavin S (blue), or insulin (red) and IL-1 β (green) as indicated. The squares (dashed white outline) denote regions enlarged and depicted as insets at bottom right of the relevant images. **c** The percentage of thioflavin S (amyloid)-positive islets in non-transduced and transduced islets with Ad-Proh*IAPP*-siRNA or Ad-Cont-siRNA after 6 days of culture. **d** The proportion of apoptotic beta cells in islets as above (c). The number of double insulin- and TUNEL-positive islet cells was counted in each islet in a total of 15 to 20 islets from each condition. Results are expressed as means $\pm$ SEM of three independent studies; * p <0.05 by one-way ANOVA vs corresponding non-transduced group

aggregates that are formed by endogenously produced *hIAPP* in islets. Islets from *RIPcre*⁺*Fas*^{+/+}/*hIAPP*^{-/-} and *RIPcre*⁺*Fas*^{fl/fl}/*hIAPP*^{-/-} mice were used as controls to detect any *hIAPP*-independent effects of *Fas* deletion on beta cell apoptosis. Cultured islets from *RIPcre*⁺*Fas*^{fl/fl}/*hIAPP*⁺ and *RIPcre*⁺*Fas*^{+/+}/*hIAPP*⁺ mice had comparable levels of amyloid formation (Fig. 6a, b). Basal beta cell death in *RIPcre*⁺*Fas*^{fl/fl} mice was somewhat lower than in wild-type mice, but this difference was not statistically significant (Fig. 6c). Despite similar levels of amyloid formation, islets from *RIPcre*⁺*Fas*^{fl/fl}/*hIAPP*⁺ mice had a markedly lower

number of active caspase-3 and TUNEL-positive beta cells than *RIPcre*⁺*Fas*^{+/+}/*hIAPP*⁺ islets, but a comparable number of alpha cells (Fig. 6a, c), resulting in a higher islet beta:alpha cell ratio in *RIPcre*⁺*Fas*^{fl/fl}/*hIAPP*⁺ islets (Fig. 6d). Islet IL-1 β immunoreactivity was markedly higher in *hIAPP*-expressing mouse islets with amyloid formation than in wild-type islets (Fig. 6a).

Reduced amyloid-induced beta cell death in hIAPP-expressing transgenic mouse islets with Fas deletion is associated with enhanced beta cell function Amyloid formation in *hIAPP*-

Fig. 5 Adenoviral-siRNA-mediated suppression of islet amyloid formation in cultured human islets reduces IL-1 β levels and Fas upregulation, and enhances beta cell survival. Isolated human islets were transduced overnight with Ad-ProhIAPP-siRNA or Ad-Cont-siRNA (as control). Transduced islets were cultured for 7 days in CMRL containing 11.1 mmol/l glucose to allow amyloid formation. Paraffin-embedded islet sections from cultured islets transduced as indicated were immunolabelled for **(a)** insulin (Ins; red), Fas (green) and thioflavin S (Thio; blue), and **(b)** for insulin (Ins; red), IL-1 β (green) and thioflavin S (Thio; blue). Note the higher Fas and islet IL-1 β immunoreactivity in thioflavin S (amyloid)-positive islets compared with islets in which amyloid formation was suppressed. Images are representative of studies performed on three human islet preparations

expressing mouse islets during culture was associated with lower islet insulin content and glucose-stimulated insulin release, and higher proinsulin release than in wild-type islets (Fig. 7). This resulted in a higher proinsulin:insulin secretion ratio from hIAPP-expressing islets than from wild-type islets. Glucose-stimulated insulin release was increased by 28% and proinsulin release was reduced by 18% in hIAPP-expressing mouse islets with beta cell *Fas* deletion compared with islets expressing *Fas*. These findings suggest that *Fas* deletion in hIAPP-expressing mouse islets enhances insulin secretion and improves the ratio of proinsulin:insulin released from islets. Deletion of *Fas* in hIAPP-expressing mouse islets did not have any significant effect on islet

insulin or proinsulin content during 7 days of culture. Finally, glucose-stimulated insulin release was somewhat higher in non-transgenic (hIAPP^{-/-}) islets with *Fas* deletion than in non-transgenic islets expressing *Fas*, but this difference was not statistically significant.

Discussion

Growing evidence suggests that the formation of hIAPP aggregates in pancreatic islets of patients with type 2 diabetes, cultured human islets and human islet grafts is associated with progressive beta cell dysfunction and death [1–3]. Beta cell-toxic effects of hIAPP aggregates are likely to be mediated through similar mechanisms in all conditions associated with islet amyloid formation. As yet, however, the molecular mechanisms by which the aggregation of biosynthetic hIAPP induces beta cell apoptosis are not well understood. Previous studies have shown that amyloid formation in human [4] and hIAPP-expressing mouse islets [16] increases beta cell apoptosis, and that prevention of amyloid formation enhances survival and function of human islets [4]. We further demonstrated that prevention of caspase-3 activation protects islet beta cells from apoptosis induced by fibrillogenesis of hIAPP produced and secreted from islet beta cells [27].

In the present study, using two ex vivo models of islet amyloid formation, cultured human islets and hIAPP-expressing mouse islets with beta cell-specific *Fas* deletion, we show that *Fas* has a key role in the beta cell apoptosis induced by aggregation of the biosynthetic hIAPP produced by islet beta cells. We also show that prevention of hIAPP-induced *Fas* upregulation or deletion of *Fas* protects islet beta cells from the hIAPP aggregates formed during in situ islet culture. These findings suggest that aggregation of biosynthetic hIAPP induces beta cell apoptosis, at least partially, through the *Fas* receptor and the *Fas*-mediated apoptotic pathway.

Consistent with a previous in vitro study performed on beta cells [26], we found that micromolar concentrations of synthetic hIAPP induces *Fas* upregulation in INS-1 cells, as well as in human and mouse islet beta cells, in a time-dependent manner. The majority of *Fas*-positive islet cells were beta cells, suggesting that hIAPP-induced upregulation of *Fas* is beta cell-specific. Furthermore, our immunolabeling studies showed a clear co-localisation of *Fas* and active caspase-3 in INS-1 and dispersed islet beta cells. Finally, culture in the presence of a *Fas* antagonist or deletion of *Fas* in islet beta cells significantly reduced hIAPP-induced apoptosis. Taken together, these findings suggest that in transformed and primary islet beta cells, exposure to hIAPP aggregates derived from exogenously applied hIAPP induces upregulation of *Fas* and that hIAPP-induced *Fas* upregulation

Fig. 6 Beta cell-specific deletion of *Fas* in *hIAPP*-expressing transgenic mouse islets markedly reduces apoptosis induced by the aggregation of endogenously produced *hIAPP* during culture. Freshly isolated islets from wild-type (*RIPcre⁺Fas^{+/+}*), *RIPcre⁺Fas^{fl/fl}*, and *hIAPP* transgenic mice expressing (*RIPcre⁺Fas^{+/+}/hIAPP⁺*) or lacking (*RIPcre⁺Fas^{fl/fl}/hIAPP⁺*) *Fas* were cultured for 7 days. **a** Paraffin-embedded sections of cultured islets from wild-type and *hIAPP*-expressing mice (8 to 18 weeks old) with or without *Fas* deletion were double immunolabelled as indicated for: insulin (Ins; red), Fas (green), and thioflavin S (Thio; blue); insulin (red) and cleaved (active) caspase-3 (aCASP-3; green); insulin (red), IL-1β (green) and thioflavin S (blue); insulin (green) and TUNEL (red); and insulin (red) and glucagon (Glu; green). The squares (dashed white outline) denote

regions enlarged and depicted as insets at bottom right of the relevant images. **b** The percentage of thioflavin S (amyloid)-positive *hIAPP*-expressing islets with or without *Fas* deletion after 7 days of culture. **c** The proportion of TUNEL-positive beta cells and **(d)** the islet beta:alpha cell ratio were quantified following triple insulin/TUNEL/DAPI or insulin/glucagon/DAPI staining, respectively. The number of double insulin- and TUNEL-positive islet cells **(c)** was counted in each islet in a total of 15 to 25 islets per animal from each genotype (six to eight animals per group). Islet beta:alpha cell ratio **(d)** represents the mean of insulin-positive cells divided by glucagon-positive cells in each islet. Results **(b–d)** are presented as mean ± SEM; **p*<0.05 by one-way ANOVA vs wild-type group; †*p*<0.05 by one-way ANOVA vs corresponding *Fas*-expressing group

Fig. 7 Reduced amyloid-induced beta cell death in *hIAPP*-expressing transgenic mouse islets with *Fas* deletion is associated with enhanced beta cell function. **a** Islet insulin response to glucose stimulation (16.7 mmol/l), **(b)** insulin content, **(c)** glucose-stimulated proinsulin release and **(d)** proinsulin content in 7-day cultured wild-type and *hIAPP*-expressing mouse islets with or without *Fas* deletion were assessed by the glucose-stimulated insulin secretion test as described (Methods). Islet insulin and proinsulin content are reported as percentage of insulin or proinsulin content in cultured wild-type islets taken as 100%. Glucose-stimulated insulin or proinsulin release is depicted as fold increase over basal release (1.67 mmol/l glucose). Results are expressed as means $\pm$ SEM of four to six animals per genotype; * p < 0.05 by one-way ANOVA vs wild-type group; † p < 0.05 by one-way ANOVA vs corresponding *Fas*-expressing group

is associated with caspase-3 activation and subsequent beta cell apoptosis.

Under normal conditions, INS-1 cells did not produce Fas at levels detectable with the immunolabelling techniques used by us. Unlike INS-1 cells, however, low numbers of beta and non-beta Fas-positive islet cells were present in dispersed human and mouse islets. It is possible that cytokines or other Fas-inducing factors that are released from islet cells and other cells in the digested pancreatic tissue during islet isolation and culture contribute to the low levels of Fas detected in non-treated islet cells. Consistent with our findings, IL-1 β and elevated glucose have been reported to induce Fas upregulation in islet beta cells [43, 44]. Finally, islet amyloid and elevated glucose, both of which contribute to beta cell death in type 2 diabetes, may induce Fas upregulation in an additive manner.

Treatment of INS-1 cells with the amyloid binding dye, Congo red, which prevents amyloid formation and its interaction with beta cell membranes, markedly reduced *hIAPP*-induced Fas upregulation and the proportion of active

caspase-3 and apoptotic beta cells. These findings support the notion that interaction of extracellular *hIAPP* aggregates with beta cell membranes is important for Fas upregulation and subsequent beta cell apoptosis, and that inhibitors of islet amyloid may provide a way of preventing amyloid-induced Fas upregulation.

To examine whether the *hIAPP* aggregates formed by biosynthetic *hIAPP* produced and secreted from islet beta cells can induce Fas upregulation, we used human and transgenic mouse islets with beta cell *hIAPP* expression as two ex vivo models of islet amyloid formation [4, 16, 48]. Human and *hIAPP*-expressing mouse islets, but not wild-type mouse islets formed *hIAPP* aggregates during culture, this being associated with upregulation of Fas. Immunolabelling studies showed that Fas-positive islet areas colocalised with thioflavin S (amyloid)-positive islet areas, indicating the correlation between amyloid formation and Fas upregulation. Furthermore, the prevention of amyloid formation in human and *hIAPP*-expressing mouse islets by Ad-Pro*hIAPP*-siRNA markedly reduced Fas upregulation and subsequent beta cell apoptosis.

We also generated a mouse model expressing *hIAPP* with beta cell-specific *Fas* deletion to further investigate the role of Fas in mediating the beta cell-toxic effects of endogenously produced *hIAPP* aggregates in islets. Islets from *hIAPP*-expressing mice with or without *Fas* deletion had comparable levels of amyloid formation, confirming that the absence of Fas as such does not have any significant effect on amyloid formation in *hIAPP*-expressing mouse islets. Interestingly, *hIAPP*-expressing mouse islets lacking Fas had significantly lower numbers of active caspase-3 and apoptotic beta cells, a higher islet insulin response to glucose and lower proinsulin release than islets expressing *hIAPP* and *Fas*, suggesting that deletion of *Fas* protects islet beta cells from endogenously produced *hIAPP* aggregates and improves their survival and function. Enhanced insulin secretion in *hIAPP*-expressing mouse islets with beta cell *Fas* deletion is likely to be due to the reduction of *hIAPP*-induced beta cell apoptosis mediated by Fas upregulation and to the effects of *Fas* deletion on beta cell secretion [47]. Importantly, despite significant increases in the proportion of active caspase-3 and apoptotic beta cells, the number of alpha cells remained unchanged in cultured *hIAPP*-transgenic mouse islets expressing *Fas*, resulting in a lower beta:alpha cell ratio in *hIAPP*-expressing mouse islets with *Fas* expression than in those lacking *Fas*.

The formation of *hIAPP*-aggregates and upregulation of Fas in human and *hIAPP*-expressing mouse islets was associated with increased islet IL-1 β immunoreactivity, which was reduced by adenoviral-siRNA-mediated prevention of amyloid formation, suggesting that IL-1 β may contribute to *hIAPP*-induced Fas upregulation. The source of IL-1 β detected by immunolabelling in thioflavin S (amyloid)-positive islets

might be islet beta cells, non-beta cells or non-islet cells. In support of this notion, beta cells have been shown to produce IL-1 β in conditions such as exposure to elevated glucose resulting in upregulation of Fas [44]. Furthermore, two recent studies have demonstrated the release of IL-1 β from non-islet cells such as macrophages activated by hIAPP aggregates [49, 50]. Taken together, these findings support a role for IL-1 β in hIAPP-induced Fas upregulation, although further studies are required to identify the cell types that release IL-1 β in conditions associated with islet amyloid formation.

In summary, our studies show that hIAPP-induced beta cell death is mediated, at least partially, via upregulation of Fas and activation of the Fas-mediated apoptotic pathway. We also show that deletion of *Fas* protects islet beta cells from the cytotoxic effects of endogenously secreted (and exogenously applied) hIAPP. Preventing the interaction between hIAPP aggregates and beta cells, or blocking Fas could constitute new ways to avoid amyloid toxicity and thereby enhance survival and function of human islets in conditions associated with islet amyloid formation, such as type 2 diabetes, islet culture, and transplantation.

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