Protective role of human insulin against the cytotoxicity associated with human mutant S20G islet amyloid polypeptide

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ABSTRACT

Aims/Introduction: Islet amyloid polypeptide (IAPP) is a main component of islet amyloid in type 2 diabetes and cosecreted from β-cell with insulin. Clinical evidence from the patients with S20G mutation of the *IAPP* gene, as well as experimental evidence that insulin could inhibit amyloid formation of IAPP, suggests that a gradual reduction of insulin could be related to the cytotoxicity associated with S20G-IAPP through long-term deterioration of β-cells in type 2 diabetes. Our objective was to show an effect of human insulin on S20G-IAPP associated cytotoxicity.

Materials and Methods: We analyzed the cytotoxicity associated with S20G-IAPP by controlling human insulin expression using adenovirus vectors with micro ribonucleic acid specifically against human insulin in endocrine AtT-20ins cells, which express human insulin permanently. Additionally, we carried out a follow-up study of circulating IAPP and insulin in type 2 diabetic patients.

Results: S20G-IAPP expression was associated with a decrease in viability and an increase in terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling-positive cells in AtT-20ins cells. Furthermore, downregulation of human insulin enhanced the cytotoxicity associated with S20G-IAPP, and induced the cytotoxicity associated with wild-type (WT)-IAPP. Reduction of ubiquitin carboxy-terminal hydrolase L1 activity enhanced cytotoxicity under the downregulation of human insulin expression in both S20G- and WT-IAPP transduced cells. A 5-year follow up of type 2 diabetic patients showed a disproportionate increase of serum fasting IAPP-to-insulin ratio from baseline.

Conclusions: Human insulin plays a protective role against the cytotoxicity associated with S20G-IAPP, as well as WT-IAPP. The findings could suggest long-term deterioration of insulin secretion associates with IAPP linked cytotoxicity in type 2 diabetes.

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KEY WORDS: Insulin, Islet amyloid polypeptide, S20G mutation

INTRODUCTION

Type 2 diabetes is characterized by a slowly progressive deterioration of insulin secretion, as well as deposition of islet amyloid[1]. Human islet amyloid polypeptide (IAPP), a 37 amino acid peptide, is cosecreted with insulin from β-cells and is a main component of islet amyloid deposits[1,2]. Although its association with the loss of β-cell mass in type 2 diabetes is widely accepted[2,3], the detailed physiology of IAPP has not been understood since our isolation and characterization of human *IAPP* complementary deoxyribonucleic acid (cDNA) and genomic DNA[4,5]. To elucidate the mechanisms about cytotoxicity associated with human IAPP, we have highlighted the missense mutant S20G-IAPP.

Clinically, we showed that the type 2 diabetic patients with the S20G mutation in the human *IAPP* gene associate with premature onset diabetes[6] and earlier deterioration of insulin secretion than those without it[7], providing genetic evidence for a role of S20G-IAPP in the pathogenesis of type 2 diabetes. *In vitro*, we also found that S20G-IAPP shows more severe amyloidogenicity and cytotoxicity than human wild-type (WT)-IAPP[8,9]. The results could reflect the clinical findings of severe glucose intolerance in the type 2 diabetic patients with the S20G mutation of the *IAPP* gene.

It is suggested that the intracellular deposition of human IAPP amyloid is accompanied by cell death through apoptosis[1,2,3]. In contrast, rat IAPP does not show amyloidogenicity or cytotoxicity, but could prevent amyloidogenicity of human IAPP, and a number of previous findings using rodent islets or β-cell lines could be influenced by this effect[1,10]. From among a number of candidates concerning the mechanisms for how human IAPP can associate with cytotoxicity[1,3,11], we focused on the role of insulin in cytotoxicity associated with S20G- and WT-IAPP in the β-cell granule, based on the following two reasons. First, human insulin has been found to be a strong inhibitor of human IAPP fibril formation in a number of

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in vitro studies[10,12]; however almost all of these findings are at the test tube level, whereas the number of *in vitro* cell level findings are few, but essential in resolving its mechanism. Second, type 2 diabetic patients with the S20G mutated *IAPP* gene were suggested to show mild glucose intolerance on its own; however, when the mutation is combined with unknown susceptibility genes for late-onset type 2 diabetes, it contributes to the early onset of type 2 diabetes and worsens its severity[6].

Here, we investigated the role of human insulin on cytotoxicity of human mutant S20G- and WT-IAPP as compared with a control rat-IAPP. The investigation was confirmed by using mouse pituitary-derived AtT-20ins cells, which have prohormone convertases and no rodent IAPP, and permanently express human insulin. Furthermore, we studied the mechanism through which human insulin could affect cytotoxicity of S20G-IAPP. Additionally, we followed fasting serum IAPP and C-peptide levels for 5 years in type 2 diabetic patients to estimate the IAPP-to-insulin ratio in β-cell granules.

METHODS

Materials

Anti-IAPP rabbit polyclonal antibodies were purchased from Peninsula Laboratories (San Carlos, CA, USA). Anti-insulin rabbit polyclonal antibodies and anti-GRP 78 rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin rabbit polyclonal antibodies, anti-ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) rabbit polyclonal antibodies, anti-sequestosome1/p62 rabbit polyclonal antibodies, anti-eukaryotic initiation factor2α (elF2α) rabbit polyclonal antibodies and anti-phospho-elF2α (Ser51) rabbit polyclonal antibodies, and anti-78 kDa glucose-regulated protein (GRP78) rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-C/EBP homologous protein (CHOP) rabbit polyclonal antibodies were purchased from BioVision (Milpitas, CA, USA). LDN-57444 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemicals were dissolved in appropriate media or dimethyl sulfoxide (DMSO) and treated at the requisite working dilutions. All chemicals were handled in accordance with the manufacturer's recommendations.

Cell Culture

COS1 cells and AtT-20ins cells were purchased from Riken Cell Bank (Tukuba, Japan) and American Type Culture Collection (Manassas, VA, USA), respectively. INS1 cells were kindly provided by Dr Christopher J Rhodes (University of Chicago, Chicago, IL, USA). COS1 cells were cultured as described before[13]. AtT-20ins cells were cultured in Dulbecco's modified eagle medium containing 25 mmol/L glucose (Life Technologies Japan, Tokyo, Japan) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/mL of penicillin (Life Technologies Japan), 100 μg/mL of streptomycin-sulfate (Life Technologies Japan), 2 mmol/L of L-glutamine (Life Technologies Japan) and 2% sodium bicarbonate at 37°C in 100% humidified air containing 5% CO_2. INS-1 cells were grown in Roswell Park Memorial Institute 1640 containing 11.1 mmol/L glucose, supplemented with 10% FBS, β-mercaptoethanol (50 μmol/L), 100 U/mL of penicillin, 100 μg/mL of streptomycin-sulfate and 2 mmol/L of L-glutamine. All cells in the present study were used within passage 10. Cells were rinsed with phosphate-buffered saline (PBS) and harvested by trypsinization with 0.5 × trypsin-2-([2-{bis(carboxymethyl)amino}ethyl](carboxymethyl)amino)acetic acid solution (Sigma-Aldrich) in PBS.

Antisera

Antisera specific for NH_2-terminal and COOH-terminal flanking regions of human proIAPP were generated in rabbits using peptides corresponding to amino acids 1–11 and 49–62 of human proIAPP by Operon Biotechnologies (Tokyo, Japan). Titers of antibodies were assessed by enzyme-linked immunosorbent assay (ELISA) using the synthetic peptides as standard.

Recombinant Adenoviruses

Human *WT-IAPP*, *S20G-IAPP* and rat-*IAPP* cDNA encoding the full-length human wild type, S20G and rat IAPP precursor protein were subcloned into the expression vector pMT2, respectively, as described previously[13]. Each *WT-proIAPP*, *S20G-proIAPP* and rat-*proIAPP* cDNA was ligated into pIRES-green fluorescent protein (GFP) vector (Clontech, Palo Alto, CA, USA), which is designed for the simultaneous expression of a GFP and each IAPP protein, as described previously[13]. Next, each *proIAPP* cDNA with *IRES-GFP* cDNA was transferred into pAd/CMV/V5-DEST gateway vectors (Life Technologies Japan) according to the manufacturer's instructions. Adenoviruses expressing (each WT, S20G and rat proIAPP [pAd-WT-IAPP, pAd-S20G-IAPP, pAd-rat-IAPP, respectively]) and (the pre-micro ribonucleic acid [miRNA] against human insulin [pAd-miINS], described later) were generated, amplified and purified as to the manufacturer's instructions, using the bacterial recombination method in 293A cells (Life Technologies Japan). All cDNA were verified by sequencing.

miRNA

Duplex single-stranded DNA oligos designed for RNA interference against human insulin (NG_007114.1_1227_top and NG_007114.1_1227_bottom) were purchased from Life Technologies Japan. The oligos were annealed and cloned into pcDNA6.2-GW/EmGFP-miR vector (Life Technologies Japan) as to the manufacturer's instructions. Next, the site of the pre-miRNA expression cassette and GFP coding sequence were transferred into pAd/CMV/V5-DEST gateway vectors as described earlier.

Transduction with Recombinant Adenovirus

AtT-20ins cells at approximately 70% confluency were transduced with each pAd-proIAPP (over 5×10^5 PFU/mL) alone or cotransduced with pAd-miINS (over 5×10^5 PFU/mL). Expression of human (pro)IAPP in transduced AtT-20ins cells

was assessed by detection of GFP expression in cells by fluorescence microscopy 24–72 h after adenoviral transduction.

Cell Viability Assay

Viability of the cells was assessed using a modified 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (Cell Counting Kit; Dojin Kagaku, Kumamoto, Japan) 72 h after the transduction of each adenovirus vector(s), as described previously[13]. Absorbance at 450 nm wavelength, representing the number of viable cells, was measured using a microplate reader (Model 550 Microplate Reader; Bio-Rad laboratories, Hercules, CA, USA), and reference absorption was measured at 650 nm.

Immunostaining of Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling

To detect apoptotic cells, AtT-20ins cells 72 h after the transduction of each adenovirus vector(s) were fixed in 4% paraformaldehyde (20 min), permeabilized with 0.5% Triton X-100 in PBS and incubated with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) reaction mixture (Roche Diagnostics, Laval, Canada) for 1 h at 37°C and then stained with Hoechst-33342 for 10 min, as described previously[14].

Western Blot Analysis

Western blot analysis was carried out with some modifications, as described previously[13]. Briefly, cells were incubated for 48 h after the transduction and harvested as described earlier. Cells were lysed in protein extraction reagent (Tissue Protein Extraction Kit; Pierce, Rockford, IL, USA) containing protease inhibitor cocktail (Protease Inhibitor Cocktail Halt; Pierce). The lysates were extracted by centrifugation (10,000 *g* for 5 min). Equal amounts of protein were loaded per sample in each experiment, separated by sodium dodecyl sulfate-polyaclylamidegel electrophoresis and transferred to polyvinylidene difluoride membranes by electroblotting. The membranes were blocked with 5% (wt/vol) skim milk in PBS for 1 h at ambient temperature, incubated overnight with primary antibodies at 4°C, rinsed with PBS and labeled with peroxidase-conjugated second antibodies for 1 h at ambient temperature. The signals were visualized using LumiGLO Reserve chemiluminescence substrate kit (KPL Inc, Gaithersburg, MD, USA) and recorded by densitometry (Light-Capture; ATTO, Tokyo, Japan). Anti-β-actin antibodies were used for the internal control. To compare the levels of proteins, the density of each signal was evaluated by image analysis software (CS Analyzer; ATTO).

Downregulation of UCH-L1 Function

To evaluate the effect of UCH-L1 downregulation on cell viability[11], we treated AtT-20ins cells with UCH-L1 inhibitor, LDN-57444 (LDN), 24 h after transduction of each adenovirus vector for 24 h at different concentrations.

Five-Year Follow Up of Fasting Serum IAPP and Insulin Level in Type 2 Diabetic Patients

We followed 30 type 2 diabetic outpatients at Wakayama Medical University who had not had any changes in treatment for 5 years to investigate fasting serum C-peptide and IAPP. Type 2 diabetes was diagnosed based on the criteria of the Japan Diabetes Society[15,16]. The local Ethical Committee of Wakayama Medical University approved the present study. We excluded patients who had an endocrine, hepatic or renal disorder (serum creatinine >9.2 μmol/L) and severe obesity (body mass index [BMI] ≥30 kg/m^2). We also excluded patients who had anti-insulin antibodies among the insulin-treated patients. Informed consent was obtained from all of the patients, and procedures were carried out in accordance with the Declaration of Helsinki as revised in 2000. Serum C-peptide was measured by ELISA (LUMIPULSE C-peptide; Fujirevio, Tokyo, Japan). Both the within-run and day-to-day precisions (coefficients of variation) were <10%. IAPP was also measured by ELISA from Peninsula Laboratories. Both the within-run and day-to-day precisions (coefficients of variation) were <10%. The value for A1c (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula A1c (%) = hemoglobin A1c (HbA_{1c}; Japan Diabetes Society [JDS]; %) + 0.4%, considering the relational expression of HbA_{1c} (JDS; %) measured by the previous Japanese standard substance and measurement methods and A1c (NGSP)[16,17].

Statistics

Data are given as means ± standard deviation (SD) for the number of experiments indicated. Differences between means were evaluated using analysis of variance or paired *t*-test appropriately. A *P*-value <0.05 was considered statistically significant.

RESULTS

Expression of IAPPs and Human Insulin

In western blot analysis, both WT-, S20G- and rat-IAPP were equally detected by anti-IAPP antibodies (Figure 1a). Human insulin was detected in AtT-20ins cells and the expression level was suppressed by approximately 55% in pAd-miINS transduced AtT-20ins cells (Figure 1b,c). Next, we analyzed the processing level of human IAPP in WT-IAPP transduced AtT-20ins cells by comparing the human IAPP level in COS1 cells and in INS1 cells. The NH_2-terminal human-proIAPP level in AtT-20ins cells transduced by pAd-WT-IAPP was the same as in INS1 cells transduced by pAd-WT-IAPP (Figure 1d). In contrast, expression of COOH-terminal human-proIAPP was not detected in either AtT-20ins cells or INS1 cells transduced by pAd-WT-IAPP (Figure 1e).

Viability and Apoptosis in AtT-20ins Cells Expressing WT- and S20G-IAPP Under the Control of Human Insulin

We tested the viability of AtT-20ins cells using a modified MTT assay 72 h after transduction of each pAd-IAPP with and without transduction of pAd-miINS. Under the condition

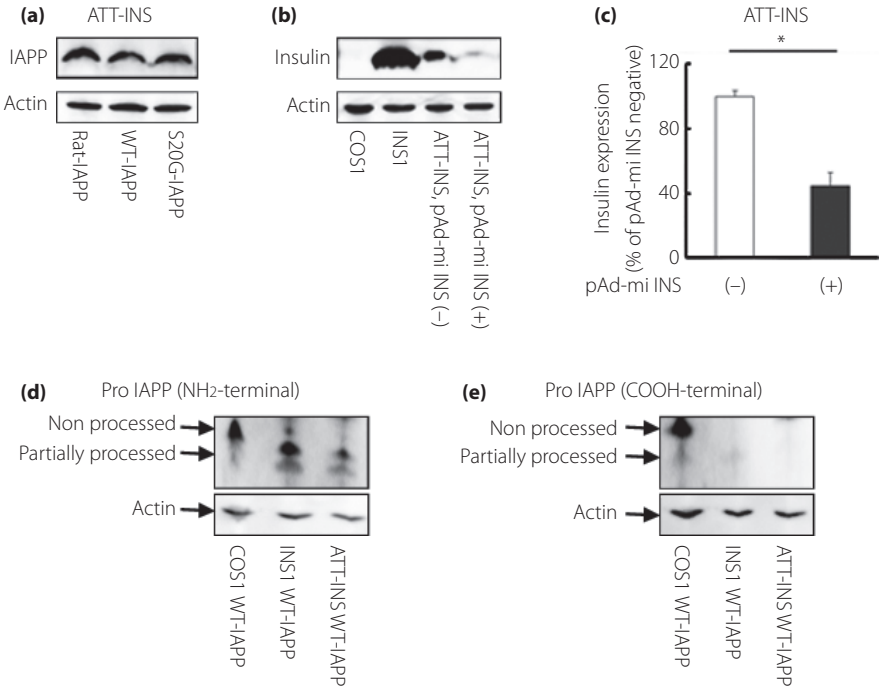

Figure 1 | Western blot analysis of each islet amyloid polypeptide (IAPP) and human insulin in AtT-20ins cells. (a) Rat-, wild-type (WT)- and S20G-IAPP were equally detected by western blot analysis by anti-human IAPP antibodies in AtT-20ins cells transduced with pAd-rat-IAPP, pAd-WT-IAPP and pAd-S20G-IAPP, respectively. (b,c) The expression of insulin in COS1, INS1 and AtT-20ins cells was detected by anti-human insulin antibodies. The density of insulin was attenuated by transduction of pAd-miINS (insulin expression of the cells without pAd-miINS [the open bar] vs the cells with pAd-miINS [the black bar]; 100 ± 3.9% vs 45 ± 8.7% of the cells without pAd-miINS, respectively, $*P < 0.0001$, $n = 3$). Values represent the mean ± standard deviation. Density of insulin was attenuated by 48-h transduction of pAd-miINS. (d) Partially processed human proIAPP were detected in WT-IAPP expressing AtT-20ins cells at the same level as in that expressing INS1 cells by anti-NH_2-terminal proIAPP antibodies. (e) Human proIAPP was not detected in both WT-IAPP expressing AtT-20ins cells and INS1 cells by anti-COOH-terminal proIAPP antibodies.

of non-transduction of pAd-miINS, a significant reduction of cell viability was found in S20G-IAPP expressing cells as compared with rat-IAPP expressing cells (Figure 2a). In both S20G- and WT-IAPP expressing cells, the viabilities were reduced by the transduction of pAd-miINS (Figure 2b–d), which contrasted with the rat-IAPP expressing cells. We also tested the apoptosis level of AtT-20ins cells using staining of TUNEL 72 h after transduction of each pAd-IAPP with and without the transduction of pAd-miINS. Under the condition of non-transduction of pAd-miINS, a significant increase of TUNEL-positive cells was found in S20G-IAPP expressing cells as compared with rat-IAPP expressing cells (Figure 2e). In WT- and S20G-IAPP expressing cells, TUNEL-positive cells were induced by the downregulation of human insulin, which contrasted with the rat-IAPP expressing cells (Figure 2f–h). These findings could indicate that exogenous S20G-IAPP expressions were associated with cytotoxicity in AtT-20ins cells, and downregulation of human insulin enhanced the cytotoxicity associated with S20G-IAPP and induced the cytotoxicity associated with WT-IAPP.

IAPP and Degradation System

We studied the expression level of IAPP-related degradation proteins by western blot. We tested protein levels of ubiquitin and UCH-L1 as markers of ubiquitin proteasome system[1,11], p62 as a marker of autophagy related proteins[13,18], and GRP78, CHOP, eIF-2α and p-eIF-2α as markers of endosplasmic reticulum stress (Figure 3a)[1,11]. Ubiquitin and UCH-L1 levels were reduced only in S20G-IAPP expressing AtT-20ins cells with pAd-miINS as compared with those without it (Figure 3a–e). In contrast, we did not detect the significant differences of P62, GRP78, CHOP, eIF-2α and p-eIF-2α levels in rat-, WT- and S20G-IAPP expressing AtT-20ins cells with pAd-miINS as compared with those without it (Figure 3a). Furthermore, we investigated the effect of UCH-L1 inhibitor, LDN, on AtT-20ins cells transduced by each pAd-IAPP with pAd-miINS. In WT- and S20G-IAPP expressing cells treated with LDN (100 μmol/L), viability was reduced as compared with those treated with DMSO, different from rat-IAPP expressing cells (Figure 4a–c).

Five-Year Follow Up of Fasting Serum C-peptide and IAPP in Type 2 Diabetic Patients

We followed 30 (16 males and 14 females) type 2 diabetic patients for 5 years without change of treatment (lifestyle modification 9, sulfonylurea 11, insulin 10). The characteristics of the patients are shown in Table 1. We compared A1c, BMI,

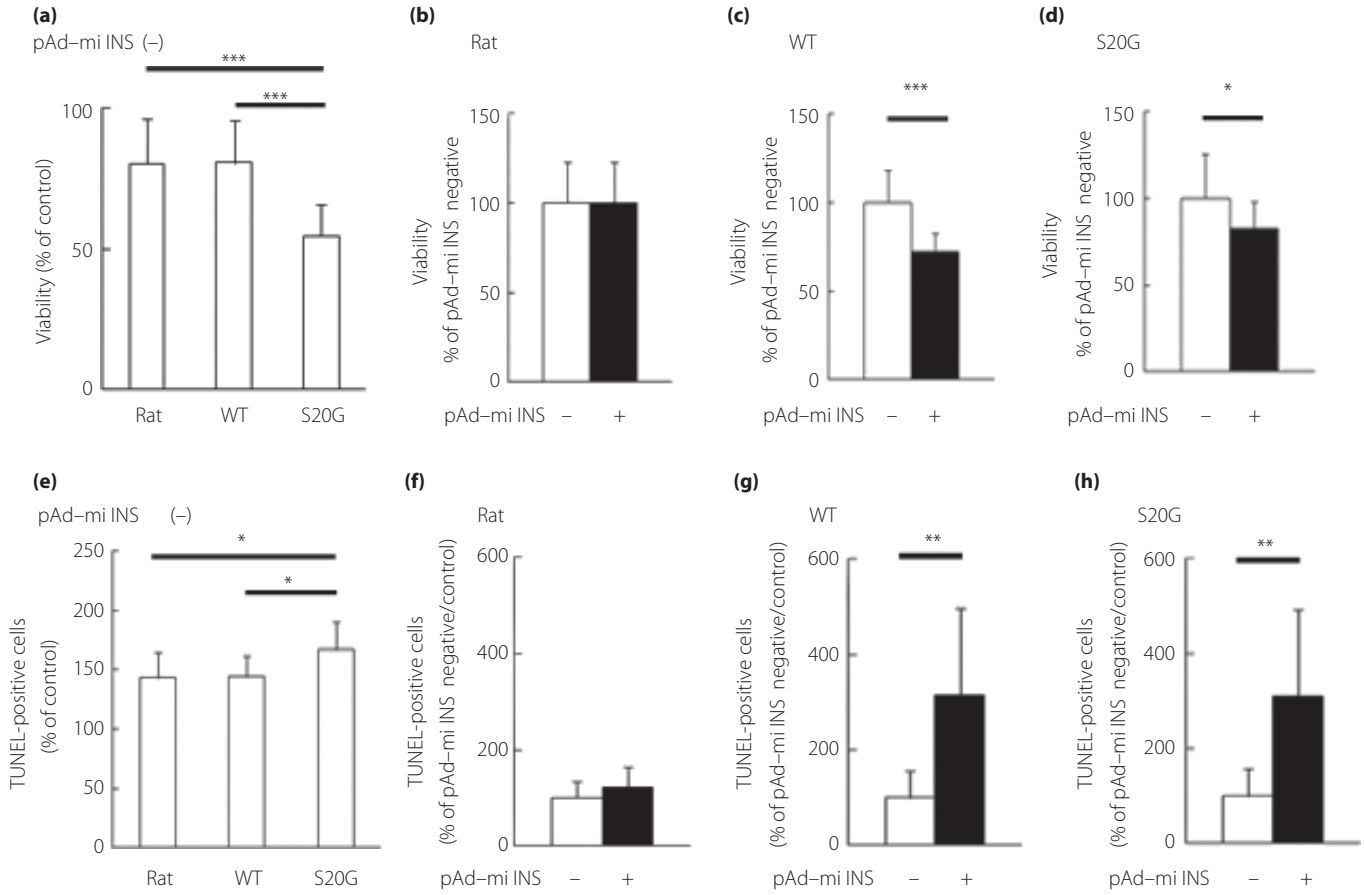

Figure 2 | Cytotoxicity associated with wild-type (WT)- and S20G islet amyloid polypeptide (IAPP) with or without downregulation of human insulin by micro ribonucleic acid. (a) Viabilities 72 h after the transduction of each pAd-IAPP were detected by modified 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assays in AtT-20ins cells transduced with pAd-S20G-IAPP without the transduction of pAd-miINS. The viability was normalized by the viability in non-transduced AtT-20ins cells (n = 7). The viability in AtT-20ins cells transduced each pAd-IAPP with or without the transduction with pAd-miINS. (b) pAd-rat-IAPP, (c) pAd-WT-IAPP and (d) pAd-S20G-IAPP (n = 7). (e) Numbers of transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)-positive cells normalized by control cells 72 h after the transduction of each pAd-IAPP (n = 8). The number of TUNEL-positive cells in AtT-20ins cells transduced each pAd-IAPP with or without the transduction with pAd-miINS. (f) pAd-rat-IAPP, (g) pAd-WT-IAPP and (h) pAd-S20G-IAPP (n = 7). All values represent the mean ± standard deviation. The open bars represent the viability or number of TUNEL-positive cells without the transduction of pAd-miINS and the black bars represent the viability or number of TUNEL-positive cells with the transduction of pAd-miINS. *P < 0.05, **P < 0.01 and ***P < 0.001.

fasting serum C-peptide, fasting serum IAPP and fasting serum IAPP with C-peptide molar ratio at baseline with those at the 5-year follow-up point. As compared with baseline, the IAPP-to-C-peptide molar ratio had increased at the 5-year follow-up point (Table 1).

DISCUSSION

In the present study, we showed that loss of human insulin enhances the cytotoxicity associated with human mutant S20G-IAPP in AtT-20ins cells, and induces the cytotoxicity associated with human WT-IAPP. These findings are consistent with the suggestion from the clinical evidence showing that a combination of the mutation with unknown susceptibility genes for late-onset type 2 diabetes could generate severe glucose intolerance[6,7]. Furthermore, the results agree with a number of previous studies providing evidence that insulin could inhibit amyloid formation of human WT-IAPP in a test tube[1,10,12].

AtT-20ins cells have the following advantages as compared with rodent islet. First, the AtT-20ins cells permanently express human insulin, but do not express rodent IAPP[1,10]. Because rodent IAPP can inhibit the amyloidogenicity of human IAPP, there is the possibility that previously-reported *in vitro* models with rodent islet or cell lines and *in vivo* models with human IAPP transgenic rodent could not develop the typical deposits of amyloid with human IAPP[1,3,19]. Second, the cells have a unique possession pattern of prohormone convertases; the cells express prohormone convertase (PC)1/3, but little PC2[20].

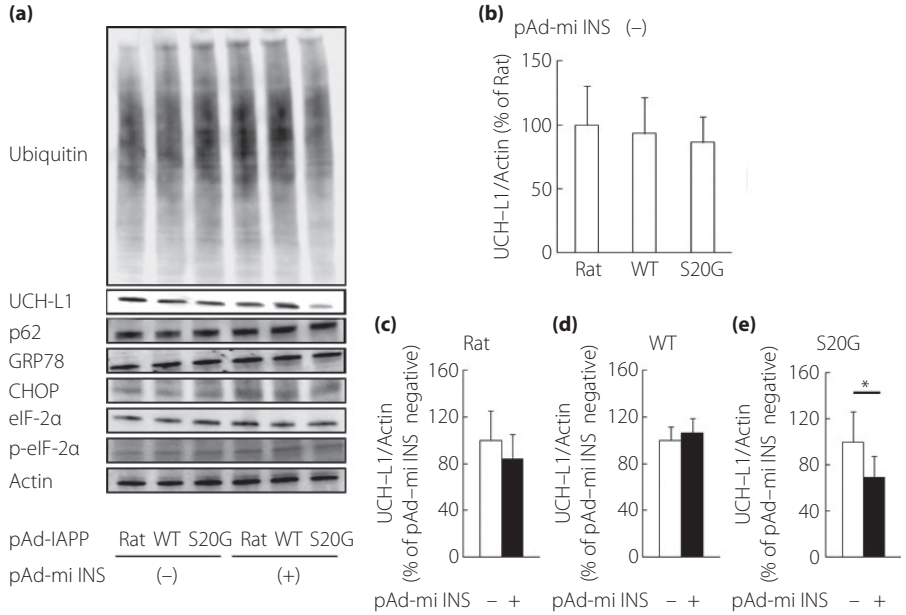

Figure 3 | (a) Proteins mediating cytotoxicity associated with human islet amyloid polypeptide (IAPP) in AtT-20ins cells 40 h after the transduction of each pAd-IAPP with or without pAd-miINS. One of three independent experiments is shown. (b) Density of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) was qualified by densitometry and normalized to that of β-actin (n = 3). Density ratio of UCH-L1/β-actin with or without the transduction of pAd-miINS in AtT-20ins cells transduced with (c) pAd-rat-IAPP (n = 3), (d) pAd-wild-type(WT)-IAPP (n = 3) and (e) pAd-S20G-IAPP (n = 3). All values represent the mean ± standard deviation. The open bars represent for the UCH-L1/β-actin density ratio in the cells without the transduction of pAd-miINS and the black bars represent for the UCH-L1/β-actin density ratio in the cells with the transduction of pAd-miINS. *P < 0.05.

Human proIAPP has the cleavage site for PC1/3 at the COOH-terminal and the cleavage site for PC2 at the NH_2-terminal[1]. Comparing the western blot pattern of COS1, INS1 and AtT-20ins cells transduced by pAd-WT-IAPP blotted by anti-NH_2-terminal or anti-COOH-terminal proIAPP antibodies, the results actually showed impaired processing of NH_2-terminal proIAPP. Several reports shown an important role of the unprocessed human proIAPP, especially NH_2-terminal unprocessed IAPP, on amyloid formation and cytotoxicity using GH3 cells, with expression of both human-proIAPP and PC1/3, and human-IAPP$^{+/+}$/PC2$^{-/-}$ mice[1,14,21]. The proIAPP is first cleaved by PC1/3 early in the secretary pathway, whereas it is secondarily cleaved by PC2 later in the pathway, mainly in mature granules[22]. The later step is thought to be more susceptible to impairment, particularly when the secretary demand increases[14]. To support this hypothesis, chronic exposure to high glucose concentration is shown to increase the proportion of the NH_2-terminal unprocessed IAPP intermediate in the cultured human islet β-cells[23]. In addition, the N-terminal flanking peptide of proIAPP, but not COOH-terminal, was immunoreactively found to be present in human islet deposit[24]. Considering these findings, this AtT-20ins model could be an ideal culture model for examining the role of human NH_2-terminal unprocessed IAPP that could induce amyloid formation and cytotoxicity.

We showed that impairment of the ubiquitin proteasome system could be linked to the mechanisms of how human insulin could have a protective effect against cytotoxicity associated with human mutant and WT-IAPP. Some types of failure in protein degradation in β-cells are reported to be possibly attributed to misfolded human IAPP and could lead to the cytotoxicity[1,11,13]. For example, we recently showed that autophagy has a protective role on human IAPP-associated cytotoxicity *in vitro* cell lines[13]. In contrast, Costes *et al.*[11] reported that β-cell dysfunctional endoplasmic reticulum-associated degradation in type 2 diabetes is mediated by human IAPP-induced UCH-L1 deficiency. The present results from analysis of UCH-L1 inhibitor support loss of UCH-L1 function could be associated with human WT- and S20G-IAPP mediated cytotoxicity under the condition of reduction of human insulin expression. However, the present findings revealed the discrepancy that UCH-L1 level with loss of human insulin decreased only in S20G-IAPP expressing cells (Figure 3e), and slightly increased in WT-IAPP expressing cells, though it was not significant (Figure 3d). The reason for the discrepancy might be that human insulin supports the activation of a protective role of the ubiquitin proteasome system from human WT- and mutant S20G-mediated misfolding of IAPPs leading to cytotoxicity, and it can, in part, salvage an accumulation of misfolding protein that induces cell death in the human WT case; however, in the mutant S20G case, a failure of the system cannot treat an aggressive and accumulating misfolding protein because of its overloaded and severe amyloidogenicity. This failure of

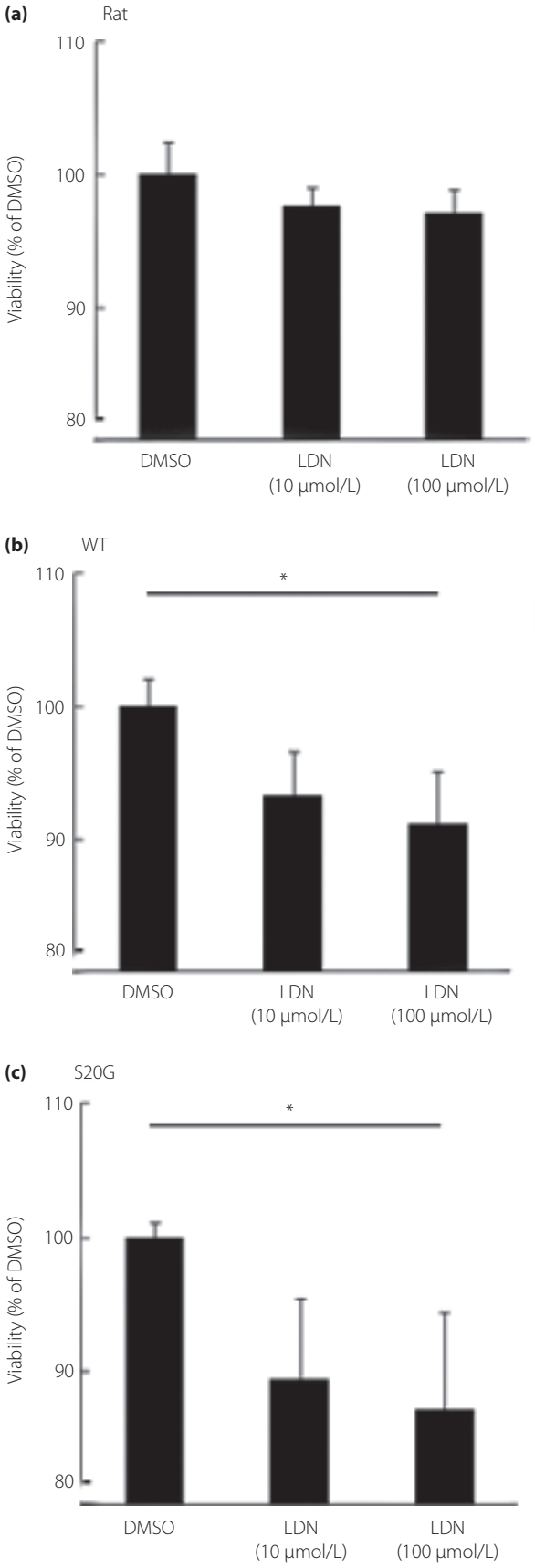

Figure 4 | Effect of inhibition of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) activity on viability in AtT-20ins cells cotransduced with each pAd-islet amyloid polypeptide (IAPP) and pAd-milNS in (a) rat-IAPP expressing cells, (b) wild-type (WT)-IAPP expressing cells and (c) S20G-IAPP expressing cells. AtT-20ins cells were treated for 24 h with UCH-L1 inhibitor, LDN-57444 (LDN), 24 h after the cotransduction with each pAd-IAPP and pAd-milNS. All values represent the mean ± standard deviation (n = 4). *P < 0.05, significant difference vs cells treated with control dimethyl sulfoxide (DMSO).

Table 1 | Circulating islet amyloid polypeptide and C-peptide level in type 2 diabetic patients at baseline and the 5-year follow-up point

n = 30	Baseline	5-year follow-up	P-value
Age (years)	64.8 ± 10.4	69.8 ± 10.4	ND
BMI (kg/m^2)	24.3 ± 3.6	24.2 ± 3.9	0.913
Duration of diabetes after the diagnosis (years)	9.50 ± 7.17	14.5 ± 7.17	ND
A1c (%)	7.25 ± 1.15	7.56 ± 1.05	0.276
Fasting serum C-peptide (nmol/L)	0.53 ± 0.20	0.48 ± 0.20	0.306
Fasting serum IAPP (nmol/L)	0.33 ± 0.25	0.48 ± 0.30	0.044
IAPP/C-peptide (molar ratio)	0.75 ± 0.78	1.42 ± 1.50	0.036

Data are presented as mean ± standard deviation. BMI, body mass index; IAPP, islet amyloid polypeptide; ND; not detected.

the system can be shown as both UCH-L1 deficiency and reduction of ubiquitin proteins, which is salvaged from digestion in proteasome or lysosome by UCH-L1[1,11].

The molar ratio between IAPP and insulin in the granule as a whole is estimated to be approximately 1–2:50, although the concentrations in the halo region are not known[1]. The exact observation of the IAPP-to-insulin ratio in β-cell cell granules *in vivo* is extremely difficult, because human IAPP is known to be aggressively amyloidogenic under experimental conditions[3,25]. It is generally suggested that even minor changes in the relative proportions of IAPP to insulin and other halo components can initiate aggregation and start fibrillation leading to the cytotoxicity[1,26]. Our results *in vitro* agreed with the assumption by showing that relatively increased IAPP-to-insulin ratio can induce cytotoxicity. In addition, though there are limitations, our results in humans might suggest the long-term deterioration of β-cells could have an association with IAPP-to-insulin ratio. Although most cross-sectional studies with serum samples suggest the parallel deterioration of IAPP and insulin secretion in several stages of type 2 diabetes[27,28,29], little is known about the longitudinal change. Our 5-year longitudinal design might prove advantageous in the detection of a subtle change in IAPP-to-insulin ratio in β-cell granules. In addition, we previously showed that the concentration ratio of serum IAPP to insulin in affected patients with the S20G mutation of the *IAPP* gene was even significantly higher than those

in type 2 diabetic patients without its mutation[6]. These findings could emphasize the importance of a subtle change of IAPP-to-insulin ratio in β-cell granules to initiate the cytotoxicity in type 2 diabetic patients and, much further, in those with the S20G mutation of the *IAPP* gene.

The present study had the following limitations. First, we could not detect the process of the hIAPP oligomer and amyloid formation. A recent report suggested the importance of the early stage of the oligomerization process of hIAPP for cytotoxicity. However, they also pointed out the difficulty of detecting the process, because the progression of oligomerization is too early to detect precisely[1,3]. Second, we should carefully interpret the results of *in vitro* experiments, because the experimental data are the results of artificially-induced loss of insulin; the clinical situation is definitely complicated and heterogeneous, and the issue of whether insulin deficiency is the cause or the result of β-cell deterioration in type 2 diabetic patients still remains to be further investigated.

In summary, we showed the protective role of human insulin against cytotoxicity associated with human S20G-IAPP and WT-IAPP in unique AtT-20ins cells, and provided additional 5-year follow-up data of the fasting serum IAPP-to-insulin ratio in type 2 diabetic patients. These findings could have the potential to show the mechanisms of the long-term deterioration of β-cell through cytotoxicity associated with IAPP, not only in type 2 diabetic patients with the S20G mutation, but also in those without it, and suggest the important role of the IAPP-to-insulin ratio in β-cell granules in this mechanism. The intervention of the mechanism might have the therapeutic potential to prevent the critical β-cell deterioration in type 2 diabetic patients.

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