UNIVERSITAT DE BARCELONA

Facultat de Medicina

Departament de Ciències Clíniques

Sobreexpressió de l'Antagonista del Receptor d'Interleucina 1 (IL-1Ra) en els illots pancreàtics. Efectes sobre viabilitat, funció i regeneració de les cèl·lules beta.

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RESEARCH ARTICLE

Adenoviral overexpression of interleukin-1 receptor antagonist protein increases β-cell replication in rat pancreatic islets

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The naturally occurring inhibitor of interleukin-1 (IL-1) action, interleukin-1 receptor antagonist protein (IRAP), binds to the type 1 IL-1 receptor but does not initiate IL-1 signal transduction. In this study, we have determined the effects of IL-1β and IRAP overexpression on adult β-cell replication and viability. IL-1β reduced dramatically β-cell replication in adult rat islets both at 5.5 mM (control: 0.29±0.04%; IL-1β: 0.02±0.02%, P<0.05) and 22.2 mM glucose (control: 0.84±0.2%; IL-1β: 0.05±0.05%, P<0.05). This effect was completely prevented in islets overexpressing IRAP after adenoviral gene transfer at 5.5 mM (Ad-IL-1Ra+IL-1β: 0.84±0.1%, P<0.05) and 22.2 mM glucose (Ad-IL-1Ra+IL-1β: 1.22±0.2%, P<0.05). Moreover, overexpression of IRAP increased glucose-stimulated β-cell replication in the absence of IL-1β exposure (Ad-IL-1Ra: 1.59±0.5%, P<0.05). β-Cell death (TUNEL technique) was increased in IL-1β-exposed islets but not in Ad-IL-1Ra-infected islets (control: 0.82±0.2%; control+IL-1β: 1.77±0.2; IRAP: 0.61±0.2%; IRAP+IL-1β: 0.86±0.1%, P<0.05). Comparable results were obtained by flow cytometry. To determine the effect of IRAP overexpression on β-cell replication in vivo, Ad-IL-1Ra-transduced islets were transplanted into streptozotocin diabetic rats. β-Cell replication was significantly increased in IRAP-overexpressing islet grafts (0.98±0.3%, P<0.05) compared to normal pancreas (0.35±0.02%), but not in control islet grafts (0.50±0.1%). This study shows that in addition to the effects of IL-1β on β-cell viability, this cytokine exerts a deleterious action on β-cell replication, which can be prevented by IRAP overexpression, and provides support for the potential use of IRAP as a therapeutic tool.
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Introduction

The capacity of β-cell mass to respond to changes in insulin demand is essential to maintain blood glucose in the normal range, and failure to appropriately increase β-cell mass in response to increased demand results in diabetes.[1] β-Cell mass depends on the changes in β-cell formation, β-cell volume and β-cell death. The specific contribution of replication to β-cell mass adaptation has been shown in different physiological and experimental conditions, among them normal lifespan,[2] pregnancy,[3] glucose infusion,[4] partial pancreatectomy[5,6] and islet transplantation.[7]

Type I diabetes is an autoimmune disease characterized by the destruction of β-cells, and it is believed that proinflammatory cytokines released by activated T cells and macrophages infiltrating the pancreas are important mediators of β-cell damage.[8,9] Among these cytokines, interleukin-1β (IL-1β) is particularly relevant.[10] IL-1β has been conclusively shown to impair glucose-stimulated insulin production in mouse, rat and human islets,[11–13] and to increase β-cell death.[14–16] The effects of IL-1β on β-cell replication have been less studied, but available data indicate that IL-1β decreased DNA synthesis in fetal[17] and in adult islet cells.[18–20] It is basically unknown whether a negative effect of IL-1β on β-cell replication could reduce the capability of β-cells to compensate the increased β-cell loss that takes place in type I diabetes, and contribute to the development of diabetes.

Better understanding of the mechanisms of β-cell damage by IL-1β has opened several therapeutic perspectives, among them IL-1 antagonism. IL-1β binds to the signaling type 1 IL-1 receptor that transmits IL-1 biological actions.[21] The naturally occurring inhibitor of IL-1 action, interleukin-1 receptor antagonist protein (IRAP), binds to this type 1 IL-1 receptor but does not initiate IL-1 signal transduction.[22] *In vitro*, the overexpression of IRAP in human pancreatic islets prevented the deleterious effects of IL-1β on β-cell function and apoptosis.[23] *In vivo*, recombinant IRAP prevented the loss of syngeneic islet transplants in NOD mice.[24] The effects of IRAP on β-cell replication have not been investigated.

In this study, we have determined the effects of IL-1β and of IRAP overexpression on adult β-cell replication. We report that IL-1β reduced dramatically β-cell replication in adult rat islets, and this effect was completely prevented in islets overexpressing IRAP after adenoviral

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gene transfer. Moreover, overexpression of IRAP increased glucose-stimulated β-cell replication independently of islet exposure to IL-1β. Finally, the positive effects of IRAP overexpression on β-cell replication were also found *in vivo* in islets syngeneically transplanted in diabetic rats. These findings support the potential role of IRAP in the prevention of type I diabetes.

Results

Efficiency of transfection

At 24 h after gene transfer, 100% of Ad-GFP-infected islets expressed GFP when observed under the inverted fluorescent microscope. When the efficiency of transfection was determined at the level of individual islet cells by flow cytometry of dispersed islet cells, 20% of islet cells were found to be infected. Finally, when specific β-cell transfection efficiency was determined in Ad-GFP-infected islets stained for insulin, 25.8±1.8% of β-cells expressed the transgene. IRAP expression after 48 h of culture was two-fold higher in Ad-IL-1Ra-infected islets than in control islets (Figure 1).

Islet viability was similar in mock-infected islets and in Ad-LacZ-infected islets. In both cases, fluorescein diacetate (FDA) staining showed 80–90% viability after 48 h in culture. Similar results were obtained with acridine orange/ethidium bromide staining (data not shown). Glucose-stimulated insulin secretion was similar in infected and uninfected islets, indicating that β-cell function was unaffected by the adenoviral infection (Figure 2).

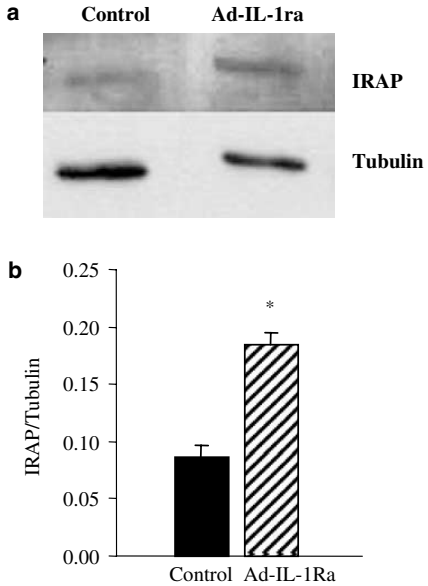

Figure 1 *Western blot of IL-1 receptor antagonist protein and tubulin protein levels in control and Ad-IL-1Ra-infected islets (48 h after infection). (a) Representative blot of the experiments. (b) Densitometric analysis of three independent sets of extracts. Values are means±s.e. *P<0.001.*

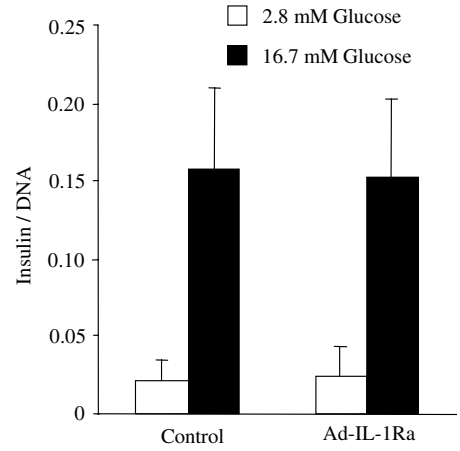

Figure 2 *Glucose-induced insulin secretion in control and Ad-IL-1Ra-infected islets (48 h after infection). Insulin release is expressed as ng of insulin/10 islets/1 h and DNA as ng of DNA/10 islets. Values are means±s.e. of four independent experiments. *P<0.05 between 2.8 and 16.7 mM glucose.*

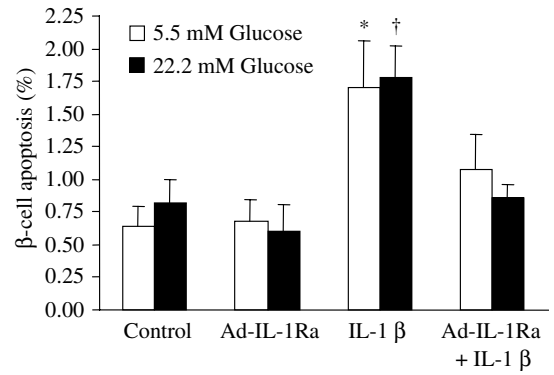

Figure 3 *β-Cell apoptosis, determined by TUNEL, in control islets and in islets overexpressing IRAP (Ad-IL-1Ra groups) incubated at 5.5 or 22.2 mM glucose for 48 h in the presence or absence of 50 U/ml of IL-1β. Values are means±s.e. of six independent experiments. ANOVA, P<0.05 among all groups cultured at 5.5 mM glucose; Fisher PLSD, *P<0.05 between control islets incubated with IL-1β at 5.5 mM glucose, and control islets and Ad-IL-1Ra islets not exposed to IL-1β at 5.5 mM glucose. ANOVA, P<0.005 among all groups cultured at 22.2 mM glucose; Fisher PLSD, †P<0.05 between control islets incubated with IL-1β at 22.2 mM glucose and all other groups incubated at 22.2 mM glucose.*

IRAP overexpression protects islet cells from IL-1β-induced cell death

Islets exposed to 50 U/ml of IL-1β for 48 h showed loss of their spherical and compact structure when visualized under the stereomicroscope. The size of IL-1β-treated islets was reduced compared to those that were not treated.

β-Cell apoptosis, measured by the TUNEL technique, was significantly increased after 48 h exposure to IL-1β, with no differences between islets incubated at 5.5 or 22.2 mM glucose (Figure 3). The overexpression of IRAP protected islets from IL-1β-induced apoptosis, both at low and high glucose, but the degree of protection was

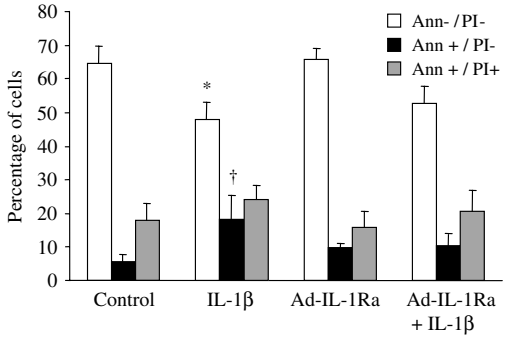

Figure 4 *Islet cell apoptosis, determined by flow cytometry using annexin-V (Ann) and propidium iodide (PI), in control islets and in islets overexpressing IRAP (Ad-IL-1Ra groups) incubated at 22.2 mM glucose for 48 h in the presence or absence of 50 U/ml of IL-1β. Ann−/PI− indicates viable cells, Ann+/PI− apoptotic cells and Ann+/PI+ necrotic cells. Values are means±s.e. of five independent experiments. ANOVA, P<0.05, Fisher PLSD, *P<0.05 Ann−/PI− IL-1β versus Ann−/PI− control group; †P<0.05 Ann+/PI− IL-1β versus Ann+/PI− control group.*

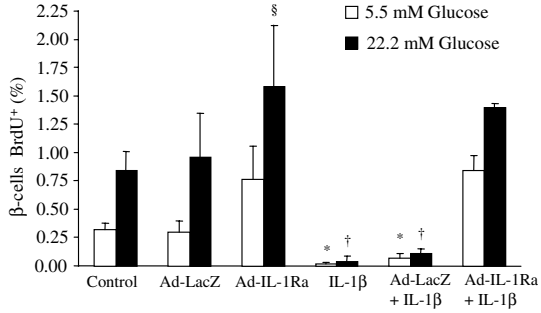

Figure 5 *β-Cell replication in control islets and in islets infected with Ad-LacZ and Ad-IL-1Ra incubated at 5.5 or 22.2 mM glucose for 48 h in the presence or absence of 50 U/ml of IL-1β. Values are means±s.e. of six independent experiments. Kruskal–Wallis, P<0.001 among all groups cultured at 5.5 mM glucose, Mann–Whitney, *P<0.05 control and Ad-LacZ islets exposed to IL-1β versus all other groups incubated at 5.5 mM glucose. Kruskal-Wallis, P<0.001 among all groups cultured at 22.2 mM glucose, Mann-Whitney, †P<0.05 control and Ad-LacZ islets exposed to IL-1β versus all other groups incubated at 22.2 mM glucose; §P<0.05 Ad-IL-1Ra versus all other groups except Ad-IL-1Ra+IL-1β.*

apparently higher at 22.2 mM glucose. In islets exposed to IL-1β at 22.2 mM glucose, β-cell apoptosis was significantly increased in uninfected islets (1.77±0.2%, $P<0.05$) compared with islets overexpressing IRAP (0.86±0.1%) and with control islets not exposed to IL-1β (0.82±0.2%). In islets exposed to IL-1β at 5.5 mM glucose, β-cell apoptosis was intermediate in islets overexpressing IRAP (1.07±0.3%) compared with IL-1β-exposed islets (1.70±0.4%) and with control, non-IL-1β-exposed islets (0.64±0.1%, $P<0.05$ *versus* uninfected IL-1β-exposed islets).

IRAP protection against IL-1β-induced apoptosis was also found when cell death was determined in dispersed islet cells labeled with annexin-V–FITC/propidium iodide and analyzed by flow cytometry. Islets cultured for 48 h with IL-1β had more apoptotic cells than control islets (18.3±6.9 *versus* 5.52±2.1%, $P<0.05$), and IL-1β-induced apoptosis was reduced in Ad-IL-1Ra-transduced islets (10.3±3.8%, $P=$NS) (Figure 4).

IRAP overexpression increases β-cell replication and prevents IL-1β impairment of β-cell replication in cultured islets

β-Cell replication was significantly increased in islets exposed to high glucose concentration (5.5 mM: 0.29±0.04%; 22.2 mM: 0.84±0.2%, $P<0.05$); however, at both glucose concentrations, β-cell replication was almost abolished when islets were exposed to IL-1β (5.5 mM+IL-1β: 0.02±0.02%; 22.2 mM+IL-1 β: 0.05±0.05%). The overexpression of IRAP had a dramatic effect on β-cell replication and completely prevented the deleterious effect of IL-1β both at 5.5 mM (Ad-IL-1ra+IL-1β: 0.84±0.1%) and 22.2 mM glucose (Ad-IL-1ra+IL-1β: 1.22±0.2%) (Figure 5). It is noteworthy that infection of islets with Ad-IL-1Ra did not impair β-cell replication, and in fact β-cell replication was significantly higher in Ad-IL-1Ra-infected islets than in control uninfected islets and Ad-LacZ-infected islets even in the absence of IL-1β exposure (Figure 5).

IRAP overexpression increases β-cell replication in transplanted islets

In order to explore the effect of IRAP overexpression on β-cell replication *in vivo*, control and Ad-IL-1Ra-transduced islets were syngeneically transplanted into streptozotocin (STZ) diabetic rats. Rats were hyperglycemic on transplantation day (Ad-IL-1Ra group: 29.7±1.6 mmol/l; control group: 28.0±0.9 mmol/l), and, as planned, they remained similarly hyperglycemic when the grafts were harvested (Ad-IL-1Ra group: 25.3±0.7 mmol/l; control group: 29.6±1.3 mmol/l).

β-Cell replication was significantly increased in IRAP-overexpressing islet grafts (0.98±0.3%, $P<0.05$) compared to normal pancreas (0.35±0.02%). In contrast, β-cell replication in control islet grafts (0.50±0.1%) was similar to that found in normal pancreas (Figure 6). β-Cell apoptosis was ≈20% lower in IRAP-overexpressing islets than in control islet grafts, but the difference did not reach statistical significance (data not shown).

IL-1β gene expression in cultured and transplanted islets

To investigate whether the increased β-cell replication found in Ad-IL-1Ra-infected islets could reflect the endogenous production of IL-1β by isolated islets and islet grafts, we determined IL-1β gene expression in freshly isolated islets, in 24 h cultured islets and in transplanted islets. IL-1β gene was found to be expressed in freshly isolated islets, and it was increased both in cultured and in transplanted islets (Figure 7).

Discussion

In this study, we have demonstrated that the overexpression of IRAP in cultured adult rat islets protected β-cells from IL-1β-induced reduction in β-cell replication. IRAP protection was found in islets cultured at low and high glucose. Moreover, β-cell replication was increased in islets overexpressing IRAP, even in the absence of

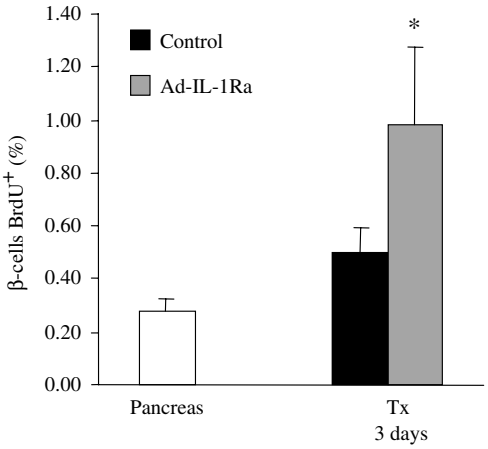

Figure 6 *β-Cell replication in pancreas (n = 6) and in transplanted (Tx) islets. Control islet grafts (n = 4) and Ad-IL-1Ra-infected islet grafts (n = 4) were harvested 3 days after syngeneic transplantation. Values are means ± s.e. ANOVA, P < 0.05 among all three groups, Mann–Whitney *P < 0.05 Ad-IL-1Ra-infected islets versus normal pancreas.*

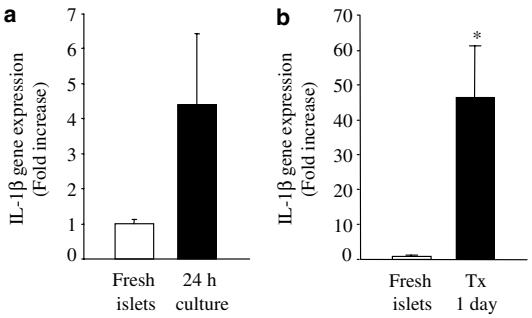

Figure 7 *IL-1β gene expression determined by quantitative real-time PCR using 18S ribosomal gene as an endogenous housekeeping control gene. (a) Freshly isolated islets and in 24 h cultured islets. (b) Freshly isolated and 1 day transplanted islet grafts. Values are means ± s.e. of four independent experiments.*

IL-1β, when compared with control noninfected islets and with Ad-LacZ-infected islets. Increased β-cell replication in IRAP-overexpressing islets was also found *in vivo*, using a syngeneic islet transplantation model.

The limited information previously available on the effects of IL-1β on islet replication, compared to the better known action on β-cell function and death, indicated that IL-1β decreased DNA synthesis in clonal insulinoma cells,[25,26] and in fetal and adult rodent islets.[17–19] However, direct assessment of β-cell replication was not performed and it could not be established whether the results reflected effects on islet non-β-cells. Moreover, variable effects were described on fetal islets depending on the duration of exposure to IL-1.[27] We have now clearly shown that β-cell replication was almost completely suppressed, to less than 10% the replication in normal islets, in islets exposed to IL-1β. The susceptibility to the deleterious effects of cytokines is different among species, with rodent islets showing a higher sensitivity than human islets.[28] However, similar to our results in rat islets, a very strong reduction in β-cell replication has been recently observed in human islets cultured for 5 days in low and high glucose in the presence of IL-1β,[20] suggesting that the very high sensitivity of β-cell replication to IL-1β may be common to rodent and human islets.

β-Cell replication was increased in islets overexpressing IRAP both in *in vitro* and *in vivo* experiments. Giannokaukis and co-workers[23] reported recently that adenoviral gene transfer of IRAP to human islets blocked multiple detrimental effects of IL-1β on islet function, in particular IL-1β-induced NO formation, impaired glucose-stimulated insulin production and Fas-triggered apoptosis, but the effects on β-cell replication were not investigated. Our data indicate that IL-1β-induced impairment of beta replication was totally prevented in cultured islets expressing IRAP. Interestingly, we found that β-cell replication was increased in islets overexpressing IRAP even in the absence of IL-1β exposure. We considered that the increased replication could result from the protection afforded by IRAP against the negative effects of IL-1β produced by the islets themselves. Supporting this hypothesis, we found that the expression of IL-1β gene was four-fold higher 24 h after islet isolation than in freshly isolated islets. These results are in agreement with previous data showing that the isolation procedure induced the expression of inflammatory cytokines, among them IL-1β, in islets,[29,30] and may be related to the apparent reduction in the production of basal NO found in islets infected with Ad-ILRa compared with normal islets.[23]

To further explore the effects of IRAP on β-cell replication, Ad-ILRa-infected islets were transplanted into STZ diabetic rats. In agreement with the *in vitro* data, we found that *in vivo*, β-cell replication was increased in IRAP-overexpressing islet grafts. It is noteworthy that both Ad-ILRa-infected islets and control islets were exposed to the same degree of hyperglycemia after transplantation, and that β-cell replication was increased only in Ad-ILRa-infected islets. It is generally accepted that in the initial days after islet transplantation there is a nonspecific inflammatory reaction at the grafted site, and it has been suggested that proinflammatory cytokines may contribute to it (Ozasa *et al*[31] and our own data). Accordingly, we found that IL-1β gene expression was increased in islet grafts. Thus, we suggest that in transplanted islets, IRAP overexpression protected Ad-IL1Ra-infected islets from the deleterious effects of IL-1β on β-cell replication. We cannot exclude, however, a direct effect of IRAP overexpression on β-cell replication.

IL-1β-induced β-cell apoptosis was prevented in islets overexpressing IRAP. The 48 h exposure to IL-1β, which was sufficient to increase β-cell apoptosis in whole islets, is shorter than the longer (3–9 days) incubation required to induce apoptosis in purified rodent or human islet cells.[8] Compared with single cells, the higher intra-islet NO concentration reached when whole islets are exposed to cytokines can accelerate the induction of β-cell apoptosis and explain these differences.[32] *In vitro*, glucose-induced apoptosis has been described in islets from diabetic-prone animals[33] and in human islets,[34] but not in normal rat islets exposed to high glucose.[33,34] Accordingly, we found that IL-1β-induced apoptosis was

similar in islets cultured at 5.5 and 22.2 mM. Our results suggest, however, that the degree of protection afforded by IRAP was modestly modified by glucose concentration, and was slightly increased in islets cultured at 22.2 mM glucose.

The high molar excess of the antagonist needed in IL-1β competitive binding requires the continuous presence of high levels of IRAP locally,[23] a condition that was met in islets after adenoviral gene transfer. It is known that IRAP is secreted by islet cells infected with Ad-IL-1Ra and it exerts its action by binding to IL-1 receptors of neighboring islet cells.[23] We have found that 24 h after infection with 6.25×10^6 plaque-forming units (PFU) of Ad-GFP, 100% of islets and $\approx$26% of individual β-cells expressed the foreign protein, as directly measured under the fluorescent microscope and also by flow cytometry. We were able to detect the presence of IRAP in noninfected islets, but the protein levels were clearly higher after adenoviral transfection. It is noteworthy that despite the high sensitivity of rat islets to the cytotoxic effects of IL-1β,[28] the level of expression of IRAP achieved in infected islets was sufficient to prevent IL-1β-induced apoptosis and IL-1β-suppressed β-cell replication. Importantly, the beneficial effect was achieved using concentrations of the adenoviral vector that did not result in deleterious effects on β-cells, as demonstrated by the similar viability, replication and glucose-induced insulin secretion of control and Ad-GFP-, Ad-LacZ- or Ad-IL-1Ra-infected islets.

The negative effect of IL-1β on β-cell replication and its prevention in islets overexpressing IRAP may be relevant both to type I and type II diabetes. Proinflammatory cytokines, and IL-1β in particular, are considered important mediators of the β-cell destruction that takes place in type I diabetes.[10] It has been suggested that β-cells do not remain passive to the immune attack, and that survival mechanisms are activated, among them replication, in response to injury.[35] Thus, the degree of β-cell destruction and the eventual development of type I diabetes can be determined by the balance between β-cell damage and death, and β-cell defense and growth. The demonstration that IL-1β specifically reduces β-cell replication suggests that IL-1β may play a dual role in β-cell mass reduction in type I diabetes inducing, on the one hand, β-cell death and, on the other, precluding β-cell compensatory replication. The beneficial effects of IRAP overexpression on β-cell replication that we have found provide further support to the therapeutic potential of IRAP in the prevention of type I diabetes.

β-Cell mass is reduced in type II diabetic patients and in subjects with impaired fasting glucose tolerance, a high risk condition for developing type II diabetes.[36,37] Increased β-cell apoptosis was recently considered the main cause of β-cell mass reduction in type II diabetes.[37] The positive correlation found between age and β-cell replication[37] suggests that replication plays an active role in the maintenance of β-cell mass, and that defects in β-cell replication could also contribute to β-cell mass reduction in type II diabetes.[38,39] Accordingly, insufficient β-cell replication has been reported in several experimental models of diabetes, such as male OLETF rats,[40] STZ-treated neonatal rats[41] or the desert gerbil *Psammomys obesus*.[33] Recent data describing the expression of IL-1β in islets from type II diabetic patients and the glucose-induced production and release of IL-1β in cultured human islets suggest that IL-1β plays a role in type II diabetes.[42] Human islets exposed *in vitro* to high glucose levels showed increased production and release of IL-1β, followed by NF-κB activation, Fas upregulation and DNA fragmentation, indicating that this islet-secreted IL-1β may contribute to increased β-cell apoptosis in type II diabetes. Our data suggest that IL-1β could also reduce β-cell mass throughout the suppression of β-cell replication. Although IRAP has protected cultured human islets from the deleterious effects of high glucose on insulin secretion,[42] it is currently unknown whether IRAP may also have an effect on β-cell replication in human islets.

In summary, this study indicates that in addition to the well-known effects of IL-1β on β-cell death and function, IL-1β exerts a deleterious action on β-cell replication that can be prevented in islets overexpressing IRAP. This effect may be relevant in the development of both type I and type II diabetes, and also in islet transplantation, and provides further support on the potential use of IRAP as a therapeutic tool.

Materials and methods

Recombinant adenoviruses

E1–E3-deleted adenoviral vectors were used for islet transfection. Ad-GFP was used to assess the efficiency of infection, and Ad-LacZ as control of infection. Both adenoviruses were generously provided by Dr Gómez-Foix (University of Barcelona), and Ad-IL-1Ra was a gift from Dr Robbins (University of Pittsburgh).[23] In all adenoviral vectors, the transgene was driven by the CMV promoter.

Islet isolation and gene transfer

Islets from male LEW/SsNHsd rats (Harlan, Horst, The Netherlands) (175–200 g body weight) were isolated by collagenase (Collagenase P; Boehringer Mannheim Biochemicals, Mannheim, Germany) digestion of the pancreas as previously described.[7] Isolated islets were hand-picked under a stereomicroscope two or three times, until a population of pure islets was obtained. Islets were washed in serum-free RPMI 1640 11.1 mM glucose (Sigma Immunochemicals, St Louis, MO, USA) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin before infection. Groups of 200 islets were infected with Ad-LacZ, Ad-GFP or Ad-IL-1Ra at a PFU of 6.25×10^6 in 400 μl of serum-free RPMI 1640 11.1 mM glucose for 2 h at 37°C and 5% CO_2. After infection, islets were washed three times in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), and incubated overnight in a non-tissue-culture-treated plastic ware at 37°C in serum-containing medium at 11.1 mM of D-glucose.

Islet culture

After overnight incubation, islets were maintained in culture for an additional 24 h in RPMI 1640 supplemented with 10% FCS and 11.1 mM D-glucose, and the efficiency of infection and islet viability were determined by flow cytometry and immunohistochemistry. IRAP expression in infected islets was determined by Western blotting.

β-Cell apoptosis and replication were determined in control noninfected islets, Ad-LacZ-infected islets and Ad-IL-1Ra-infected islets cultured for 48 h in RPMI 1640 10% FCS at 5.5 and 22.2 mM D-glucose with or without 50 U/ml of recombinant human IL-1β (BD Pharmingen, Heidelberg, Germany). β-Cell apoptosis was determined by flow cytometry and immunohistochemistry, and β-cell replication by immunohistochemistry.

Islet transplantation

After overnight culture, uninfected and Ad-IL-1Ra-infected islets were transplanted under the kidney capsule of STZ-induced diabetic Lewis rats as described previously.[7] Briefly, Lewis rats were rendered diabetic by a single intraperitoneal injection of 60 mg/kg body weight of STZ (Sigma Inmunochemicals, St Louis, MO, USA). Diabetes was confirmed by the presence of hyperglycemia (>20 mmol/l). Diabetic rats were transplanted with 500 uninfected or Ad-IL-1Ra-infected islets beneath the left kidney capsule, as previously described.[7] This is clearly an insufficient mass to restore normoglycemia, and animals were expected to remain hyperglycemic after transplantation. Grafts were harvested on days 1 and 3 after transplantation to determine IL-1β gene expression and β-cell replication, respectively. Grafts were immediately immersed in lysis buffer for mRNA extraction, or in 4% paraformaldehyde–phosphate-buffered saline (PBS) and processed for paraffin embedding.

The replication in normal pancreas was determined in six pancreases from control Lewis rats. A midlaparotomy was performed, the pancreas was exposed, the animal was killed and the pancreas was immediately excised and fixed in 4% paraformaldehyde–PBS and processed for paraffin embedding.

Flow cytometry

Flow cytometry was used to determine the efficiency of infection, islet viability, and islet cell apoptosis and necrosis in single islet cells.

Efficiency of infection. To determine the efficiency of infection, 24 h cultured Ad-GFP-infected islets were dispersed into single cells by gentle continuous pipetting in trypsin–EDTA for 6 min at 37°C. Enzymatic activity was stopped by the addition of FCS-containing RPMI medium. Single cells were rinsed twice with 11.1 mM glucose RPMI 1640 medium and analyzed on a FACScalibur cytometer (Beckton Dickinson Instruments, Heidelberg, Germany) using 488 nm excitation and a 530 ± 15 nm band-pass filter.

Islet cell viability. To assess islet cell viability after adenovirus infection, FDA (Sigma) assay was used.[43] Viable cells have the capability to incorporate the nonpolar, nonfluorescent compound FDA and rapidly hydrolyze it using acetyl esterase activity to fluorescein, a polar, fluorescent compound that is retained within the cell. Nonviable cells no longer have esterase activity and will not be fluorescently stained. After 24 h culture, Ad-LacZ-infected islets were dispersed into single cells, incubated with FDA (4 μg/μl) at 37°C and 5% CO_2 for 10 min and analyzed on the flow cytometer at 488 nm excitation and a 530 ± 15 nm band-pass filter for FDA detection.

Apoptosis and necrosis. The annexin-V/propidium iodide technique was used to determine islet cell apoptosis and necrosis. FITC-labeled annexin-V is a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for phosphatidylserine that is exposed upon the outer leaflet of the apoptotic cell membrane. Since necrotic and late apoptotic cells also expose phosphatidylserine according to the loss of membrane integrity, necrotic cells were differentiated from apoptotic cells by propidium iodide staining. Dispersed islet cells were washed twice with binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated with annexin-V–fluorescein (Annexin V-FITC Kit, Bender Medsystems, Vienna, Austria) for 30 min. Immediately before cytometric analysis, 0.05 μg/ml propidium iodide was added. Data were analyzed using CellQuest software (Beckton Dickinson Instruments, Heidelberg, Germany).

Western blotting

Whole-cell extracts obtained from 48 h cultured islets were treated with a lysis buffer containing PBS, 1% Triton X-100 (Sigma) and a protease inhibitor cocktail (AEBSF 2 mM, EDTA 1 mM, bestatin 130 μM, E-64 14 μM, leupeptin 1 mM, aprotinin 0.3 μM) (Sigma), and supernatants were collected. A 4 μg portion of protein was fractionated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Sigma) using a Trans-Blot SD semidry transfer cell (Biorad, München, Germany). Immunoblot analyses of IRAP (goat α-human IRAP, 1:500) (R&D Systems Europe Ltd, Abingdon, UK) and tubulin (rabbit α-mouse tubulin, 1:3000) (Sigma) expression were performed using horseradish peroxidase-based chemiluminescence reaction as a secondary detection method (ECL™ Western Blotting Analysis System, Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, UK). Protein production was quantified by densitometry using the Phoretix 1D Advanced software (Nonlinear Dynamics, Newcastle, UK).

Immunohistochemistry

Immunohistochemical staining of whole islets was used to determine efficiency of infection, apoptosis and replication in β-cells. Cultured islets and islets grafts were fixed overnight in 4% paraformaldehyde at 4°C, embedded in paraffin, sectioned and immunostained after deparaffinization and rehydration.

Efficiency of β-cell infection. Sections (3 μm) were incubated overnight with an anti-insulin swine polyclonal antibody (1:500), and detection was performed with a secondary anti-rabbit Cy3-conjugated antibody (1:200). Tissue sections were visualized under the fluorescent microscope. The images were captured and analyzed with an image analytical software (Analysis 3.0; Soft Imaging System, Münster, Germany). The efficiency of β-cell infection was expressed as percentage of total insulin-positive area that was also GFP-positive.

β-Cell apoptosis. Sections were double stained by immunoperoxidase for apoptotic nuclei with the TUNEL technique (*In Situ* Cell Death Detection Kit, ApopTag®, Intergene, Oxford, UK) and by alkaline phosphatase for the endocrine non-β-cells of the islets. A cocktail of

antibodies (Dako, Carpinteria, CA, USA), rabbit anti-swine glucagon (final dilution 1:1000), rabbit anti-human somatostatin (final dilution 1:1000) and rabbit anti-human pancreatic polypeptide (final dilution 1:500), were used as previously described.[44] We stained the endocrine non-β-cells instead of β-cells because high glucose concentrations used in some of the experimental conditions are associated with β-cell degranulation and may result in weak insulin staining. We have previously shown the validity of staining the endocrine non-β-cells to determine β-cell apoptosis.[44] After immunoperoxidase staining, β-cells and TUNEL-positive β-cells were counted using an Olympus BH-2 microscope connected to a video camera with a color monitor. When assessing apoptotic nuclei, we excluded necrotic regions. β-Cell apoptosis was expressed as percentage of TUNEL-positive β-cells. A minimum of 1200 cells per sample were counted; the sections were systematically sampled, all endocrine nuclei were counted, and when needed a second section was included.

β-Cell replication. The thymidine analog 5-bromo-2′-deoxyuridine (BrdU; Amersham) was added to the islet culture for the last 24 h of culture prior to fixation. In the *in vivo* experiments, rats were injected with BrdU, 100 mg/kg body weight intraperitoneally, 6 h before graft or pancreas harvesting. Sections were double stained with immunoperoxidase for BrdU using a Cell Proliferation Kit (Amersham) with a modified protocol,[44] and for endocrine non-β-cells of the islets using the cocktail of antibodies described above. After immunoperoxidase staining, β-cells and BrdU-positive β-cells were counted using an Olympus BH-2 microscope connected to a video camera with a color monitor. β-Cell replication was expressed as percentage of BrdU-positive β-cells, and at least 1200 cells were counted.

Insulin secretion

To determine the effects of adenoviral infection on β-cell function, glucose-stimulated insulin secretion was used as a functional assay. Ad-IL-1Ra-infected and uninfected islets were cultured for 48 h with RPMI 1640 medium supplemented with 11.1 mM glucose and 10% FCS. Cultured islets were then washed twice with Krebs–Ringer–bicarbonate buffer supplemented with Hepes and BSA (KRBH buffer: 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, 0.5% BSA, pH 7.4) with 2.8 mM glucose and preincubated for 1 h at 37°C in triplicate groups of 10 islets in 1 ml of fresh KRBH buffer containing 2.8 mM glucose with continuous shaking. The medium was removed and islets were incubated with 1 ml of KRBH buffer containing 2.8 or 16.7 mM glucose for an additional hour with continuous shaking. The supernatants were removed and stored at −80°C until assayed for insulin content. Insulin was measured by ELISA (Mercodia Rat insulin ELISA, Mercodia AB, Uppsala, Sweden).

DNA content

After the insulin secretion assay, islets were rinsed three times with phosphate buffer (2 M NaCl, 40 mM $Na_2HPO_4 \cdot H_2O$, 2 mM EDTA) to avoid the interfering effect of BSA in the DNA test and were disrupted by sonication. DNA was determined by a fluorimetric assay using Hoechst 33258 (Sigma) (excitation wavelength 356 nm and emission wavelength 448 nm) on a fluorescence spectrophotometer (F-2000, Hitachi Ltd, Tokyo, Japan).

RNA isolation and cDNA synthesis

Total RNA was extracted from 500 freshly isolated, 24 h cultured islets, and islet grafts with the RNeasy Mini Kit (Quiagen, Crawley, UK) according to the manufacturer's instructions and stored at −80°C until use. Before cDNA synthesis, RNA was quantified by spectrophotometry and its integrity visualized in 1% agarose gel. Prior to reverse transcription, DNA from the total RNA sample was digested by RNase-free DNase (RQ1 RNases-Free DNase, Promega, Madison, WI, USA). For the reverse transcription reaction, 1 μg of DNA-free RNA was incubated with 0.5 μg/μl of random hexamer primers (Promega) and 10 mM dNTPs (Invitrogen) for 10 min at 65°C and chilled on ice. cDNA synthesis reaction was carried out by the addition of 400 U of SuperScript™ II RNase H^- reverse transcriptase (Invitrogen), 5 × First-Strand buffer, 0.1 M DTT and 40 U/μl of recombinant ribonuclease inhibitor (RNasin® Ribonuclease Inhibitor, Promega) ending at a final volume of 40 μl. The reaction proceeded for 50 min at 42°C and stopped at 70°C for 15 min. Samples were stored at −80°C until use.

Semiquantitative real time RT-PCR

IL-1β gene expression was determined by real-time quantitative PCR using the ABI Prism 7700 thermocycler (Applied Biosystems, Warrington, UK) in combination with the TaqMan chemistry. For these experiments, we used PDARs (Pre-Developed TaqMan® Assay Reagents, Applied Biosystems, Warrington, UK) designed to have similar amplifying efficiency and allowing the use of the comparative C_T (threshold cycle) method.[45] To correct for variations in input RNA amounts and efficiency of reverse transcription, an endogenous housekeeping gene (18S ribosomal gene) was also quantified. The PCR reaction was carried out with 1 μl of cDNA, 2 × TaqMan® Universal PCR Master Mix, 20 × IL-1β or 18S primers and probe ending up to a final volume of 25 μl. The running conditions were as follows: 10 min at 90°C for DNA denaturation, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C for sequence amplification. Data were analyzed using the SDS software (Applied Biosystems).

Statistical analysis

Results were expressed as means ± s.e. Statistics were performed using SPSS 10.0 for windows, and differences among means were evaluated by one-way analysis of variance (ANOVA). The Fisher's protected least significant difference (PLSD) method was used to determine specific differences among means when determined significant by ANOVA main effects analysis. When results did not show a normal distribution, the Kruskal–Wallis test was used, followed by the Mann–Whitney test. A *P*-value of less than 0.05 was considered significant.

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