Activation of c-Jun NH₂-terminal kinase (JNK) pathway during islet transplantation and prevention of islet graft loss by intraportal injection of JNK inhibitor

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Abstract

Aims/hypothesis Although application of the Edmonton protocol has markedly improved the outcome for pancreatic islet transplantation, the insulin independence rate after islet transplantation from one donor pancreas has remained low. During the isolation process and subsequent clinical transplantation, islets are subjected to severe adverse

conditions that impair survival and ultimately contribute to graft failure. The aim of this study was to map the c-Jun NH₂-terminal kinase (JNK) pathway that mediates islet loss during islet transplantation and to clarify whether intraportal injection with JNK inhibitor during islet transplantation can prevent islet graft loss.

Methods We measured JNK activity in the liver, fat and muscle of diabetic mice and in the liver immediately after islet transplantation. We examined the effect of intraportal injection of JNK inhibitory peptide at islet transplantation. **Results** JNK activity became progressively higher at least until 24 h after transplantation. The cell-permeable peptide of JNK inhibitor was delivered not only in the liver but also in other insulin target organs, preventing JNK activation in the liver at least until 24 h after transplantation and reducing JNK activity in these insulin target organs. Moreover, the peptide inhibitor prevented islet graft loss immediately after transplantation and improved islet transplant outcome.

Conclusions/interpretation These findings suggest that control of the JNK pathway is extremely important in islet transplantation and that intraportal injection of JNK inhibitor during islet transplantation (addition of JNK inhibitor to transplant media) could prevent the impairment of islet cells, leading to improved outcome for pancreatic islet transplantation.

Keywords Cell-permeable peptide · Islet transplantation · JNK inhibitory peptide · c-Jun NH₂-terminal kinase

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Abbreviations

FITC	fluorescein isothiocyanate
IBMIR	instant coagulation/inflammation reaction
IE	islet equivalent
IPGTT	intraperitoneal glucose tolerance test

JNK	c-Jun NH ₂ -terminal kinase
PTD	protein transduction domain
11R-JNKI	JNK peptide
11R-mJNKI	mutant JNK peptide
STZ	streptozotocin

Introduction

Application of the Edmonton protocol has markedly improved the outcome for pancreatic islet transplantation as a therapeutic strategy for type 1 diabetes [1–3]. However, the insulin independence rate after islet transplantation from one donor pancreas remains low. The low frequency of islet grafting is dependent on poor islet recovery from donors [4] and early islet loss during the first hours after grafting [5, 6], termed primary graft non-function. Islet transplantation exposes cells to a variety of stressful stimuli, notably proinflammatory cytokines that encourage beta cell death and lead to early graft failure [7–9]. The reduction in islet mass immediately after transplantation implicates beta cell death by apoptosis and the prerecruitment of intracellular death-signalling pathways [10–12].

Intrahepatic infusion of isolated islets is the preferred site for clinical islet transplantation. However, instant coagulation/inflammation reaction (IBMIR), which is characterised by activation of the coagulation and complement systems, rapid binding and activation of platelets, leucocyte infiltration into the islets [13, 14], and activation of endothelial cells, occurs regularly during this procedure, even without clinical signs of intraportal thrombosis [15–17]. Finally, these effects contribute to the disruption of islet morphology, islet dysfunction and death. When islets come into direct contact with blood in the portal vein, islet-produced tissue factor triggers IBMIR. Furthermore, non-specific activation and dysfunction of intrahepatic endothelial cells, characterised by the upregulation of intracellular adhesion molecule-1 and P-selectin, along with the production of proinflammatory cytokines such as TNF- α , IL-1 β , interferon- γ and nitric oxide, have been demonstrated in vitro and in vivo after islet transplantation. These effects contribute to the complex network of events that ultimately lead to early graft loss [16–20].

Although the production of proinflammatory cytokines such as TNF- α , IL-1 β and interferon- γ , has been demonstrated after islet transplantation, intracellular stress-signalling pathways such as extracellular signal-regulated kinases [21], p38 mitogen-activated protein kinase [22] and c-Jun NH₂-terminal kinases (JNKs) [23] during the islet transplant process are not well known. Multiple stresses, including proinflammatory cytokines and reactive oxygen

species, lead to both JNK and p38 activation. In type 1 diabetes, JNK plays a central role in the intracellular events that signal beta cell loss after exposure to the proinflammatory cytokine IL-1 β [24, 25]. JNK is abnormally elevated in various tissues under diabetic conditions due to the phenomenon known as glucose toxicity, and it has been demonstrated that activation of the JNK pathway interferes with insulin action [26–28] and beta cell function [29, 30], as well as with insulin biosynthesis [29]. Islet transplantation involves the exposure of islets to several forms of stress, not only as a result of pancreas preservation and islet isolation, but also of inflammation and glucose toxicity after transplantation.

In this study, we have mapped the JNK pathway that mediates islet loss during islet transplantation and clarified whether intraportal injection with JNK inhibitor during islet transplantation can prevent islet graft loss immediately after transplantation.

Materials and methods

Animals BALB/c mice (8–10 weeks old), purchased from CLEA Japan (Meguro, Tokyo, Japan), were used for the experiments. Chemical diabetes was induced in the recipients by a single (220 mg/kg) intraperitoneal injection of streptozotocin (STZ) (Sigma, St Louis, MO, USA). Hyperglycaemia was defined as a glucose level of >19.4 mmol/l detected twice consecutively after STZ injection. Mouse studies were approved by the review committee of Kyoto University Graduate School of Medicine.

JNK activity assay JNK activity was measured using a JNK activity assay kit (KinaseSTAR; BioVision Research Products, Mountain View, CA, USA). The cells were lysed in JNK extraction buffer. Next, 2 μ l JNK-specific antibody was added to the cell lysate and rotated for 45 min at room temperature. Protein A Sepharose was resuspended, and kinase assay buffer was added to the washed Protein A beads, after which 2 μ l c-Jun Protein/ATP mixture was added and the mixture was incubated at 30°C for 1 to 4 h. Finally, the supernatant fraction was collected and loaded on 12% SDS-PAGE and subjected to western blotting using the rabbit anti-Phospho-c-Jun (Ser73) specific antibody at 1:1000 dilution as described previously [31, 32]. The cell lysates from beta-cell-derived MIN6 cells treated with and without 1 μ g/ml anisomycin for 1 h were used as positive and negative controls, respectively.

Peptide synthesis JNK peptide (11R-JNKI), which we reported previously to be a selective JNK inhibitor [31], and mutant JNK peptide (11R-mJNKI), which we reported previously to be a control peptide [31], and fluorescein

isothiocyanate (FITC)-conjugated peptides were prepared by Fmoc-solid-phase synthesis followed by FITC-labelling and treatment with trifluoroacetic acid. Here, γ -amino-butyric acid was used as a linker to connect FITC and the peptide segments. The peptides thus obtained were then purified by preparative reversed-phase HPLC, with >95% purity as analysed by HPLC. The structure of the peptides was confirmed by mass spectrometry.

Western blotting After treatment with 11R-JNKI or 11R-mJNKI, whole-cell extracts obtained from various insulin target tissues (liver, fat and muscle) from BALB/c mice were fractionated by 10% SDS-PAGE and transferred to reinforced cellulose nitrate membrane. After blocking, the membranes were incubated overnight at 4°C in TBS buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl) containing a 1:1,000 dilution of rabbit anti-IRS-1 or anti-IRS-1-pSer307 antibody (Upstate Biotechnology, Iowa City, IA, USA), and then incubated for 1 h at room temperature in TBS containing a 1:1,000 dilution of rabbit antibody to IgG coupled to horseradish peroxidase (Cell Signaling, Danvers, MA, USA). Immunoreactive bands were visualised by incubation with LumiGLO (Cell Signaling) and exposed to light-sensitive film.

Islet isolation We injected 2 ml of Hanks' balanced salt solution (Life Technologies, Rockville, MD, USA) containing 2 mg/ml collagenase (Sigma; type V) into the common bile duct of mice. The distended pancreas was removed and incubated at 37°C for 16 min. The islets were purified by centrifugation (244 g, 5 min) on Ficoll gradients comprising four different densities (25, 23, 21.5, 11%). The tissue fragments at the interface of the 21.5% and 23% layers were collected, washed and resuspended in Hanks' balanced salt solution. Individual islets, free of attached acinar, vascular and ductal tissue were selected and removed with a Pasteur pipette under a dissecting microscope, yielding highly purified islets for transplantation.

Islet transplantation The freshly isolated islets were picked up with an infusion set. Intraportal islet transplantation, with or without JNK inhibitor, was directed into the whole hepatic lobe. Using a 27-gauge needle infusion set, the injection was made into the ileo-colic vein. After removal of the needle from the vein, haemostasis was easily achieved with gentle pressure using a cotton tip. In our preliminary experiments, approximately 20% of STZ-induced diabetic mice became normoglycaemic when 250 islet equivalents (IE) of fresh (non-cultured) islets were transplanted into the liver (data not shown). For this study, we therefore considered 250 IE islets to be a marginal mass, and 250 IE islets were therefore used for the transplant experiments.

Assessment of graft function During the 30-day post-transplantation period, the non-fasting blood glucose levels were monitored three times per week. Normoglycaemia was defined when two consecutive blood glucose level measurements showed less than 11.1 mmol/l. An intraperitoneal glucose tolerance test (IPGTT) was performed 30 days after transplantation. The mice were fasted overnight, after which glucose (2.0 g/kg body weight) was injected intraperitoneally. The blood glucose levels were measured before and 5, 15, 30, 60, 90 and 120 min after injection. No statistical differences in pre-transplantation blood glucose levels or pre-transplantation body weight were observed among each group of mice.

Morphological examination The islet grafts in the liver were examined morphologically 30 days after transplantation. After fixation in 10% neutral-buffered formalin, the specimens were embedded in paraffin, and 3- to 5- μ m thick sections were stained with haematoxylin and eosin. Immunohistochemical staining for insulin was performed with the avidin-biotin-peroxidase complex method [32, 33]. The immunoreaction was visualised with 3-amino-9-ethylcarbazol. All sections were slightly counterstained with Mayer haematoxylin. As a negative control, the primary antibody was replaced with the appropriate normal serum. Porcine pancreas served as a positive control.

Statistics Differences in the duration of graft survival between groups were evaluated with the Kaplan-Meier log-rank test. A *p* value of <0.05 was considered statistically significant.

Results

JNK activity in liver, fat and muscle under diabetic conditions We first measured JNK activity in the liver, fat and muscle from diabetic or non-diabetic mice. Chemical diabetes was induced in the recipients by a single intraperitoneal injection of STZ and liver, fat and muscle were removed 3 weeks after STZ injection. JNK activity became higher in tissues from diabetic than in tissues from non-diabetic mice. In particular, the activity in fat and muscle was extremely high, while activity in the liver was relatively low (Fig. 1). These data suggest that the diabetic state leads to the sustained activation of JNK. This certainly has profound implications for insulin resistance.

JNK activity in the liver immediately after islet transplantation We next measured JNK activity in the liver immediately after islet transplantation. Diabetic mice induced by STZ injection were transplanted 3 weeks after

Fig. 1 JNK activity in liver, fat and muscle under diabetic conditions. Chemical diabetes (DM) was induced in the recipients by a single intraperitoneal injection of STZ, and liver, fat and muscle were removed 3 weeks later. JNK activity was measured using a JNK activity assay kit. The cell lysates from beta-cell-derived MIN6 cells treated with and without 1 μ g/ml anisomycin for 1 h were used as positive and negative controls, respectively. Data are expressed with JNK activity of positive and negative controls arbitrarily set at 100 and 0, respectively. All assessments were made in triplicate and the data (mean $\pm$ SE) are expressed as a percentage of the control values in each experiment. ** $p < 0.01$

STZ injection. Samples were collected from the liver before transplantation and 1, 3, 6 and 24 h after transplantation. Although JNK activity was relatively low in the liver at 1 h after transplantation, its activity became progressively higher at least until 24 h (Fig. 2). These data suggest that islet transplantation in the liver leads to the strong activation of JNK. This has profound implications for the release of proinflammatory cytokines, and thus islet apoptosis.

Intraportal injection of JNK inhibitory peptide We reported previously that cell-permeable 11R-JNKI peptide is a selective JNK inhibitor and significantly prevented JNK activation compared with that in untreated, or control peptide-treated groups using beta-cell-derived MIN6 cells [31]. A 20-amino-acid sequence derived from the JNK-binding

Fig. 2 JNK activity in the liver immediately after islet transplantation. Diabetic mice induced by STZ injection were transplanted 3 weeks after STZ injection. Samples were collected from the liver before transplantation (Pre Tx) and 1, 3, 6 and 24 h after transplantation. Measurement of JNK activity, details on positive and negative controls, and definition of relative JNK activity as in Fig. 1. All assessments were made in triplicate and the data (mean $\pm$ SE) are expressed as a percentage of the control values in each experiment. ** $p < 0.01$ vs Pre Tx

domain of islet-brain-1 (RPKRPTTLNLFQVPRSQDT) was covalently linked at its C terminus to an 11-amino acid arginine carrier peptide. To examine the delivery of the 11R-JNKI in vivo, diabetic mice were injected intraportally with the FITC-conjugated 11R-JNKI peptide (10 mg/kg), which showed fluorescence signals not only in the liver, but also in other insulin target organs such as fat and muscle (Fig. 3).

Effect of JNK inhibitory peptide To examine the effect of the intraportal injection of JNK inhibitory peptide during islet transplantation, diabetic mice induced by STZ injection were transplanted with 10 mg/kg of 11R-JNKI or 11R-mJNKI 3 weeks after STZ injection. Samples were collected from the liver before transplantation and 1, 3, 6 and 24 h after transplantation. Compared with 11R-mJNKI treatment, the activity of JNK in cells treated with 11R-JNKI maintained an extremely low level in the liver at least until 24 h after transplantation (Fig. 4a). We also examined the activity of JNK in insulin target organs (fat and muscle) 24 h after islet transplantation with 11R-JNKI treatment. Activity in both fat and muscle was reduced 24 h after islet transplantation with 11R-JNKI treatment but not with 11R-mJNKI (Fig. 4b). Phosphorylation of IRS-1 Ser307 on fat and muscle was lower in 11R-JNKI-treated mice than in 11R-mJNKI-treated mice (Fig. 4c), although no differences were seen in phosphorylation in liver (data not shown). These data suggest that JNK inhibitory peptide prevented JNK activation immediately after islet transplantation in the liver and reduced JNK activity in insulin target organs.

Fig. 3 Transduction of 11R-peptides in vivo. **a** Sequence of the JNK inhibitory peptide (11R-JNKI) and mutant JNK peptide (11R-mJNKI). The sequence of mJNKI is mutated at three conserved residues of JNK-binding domain of islet-brain-1 and -2. Two glycines were added between the 11R and JNKI or mJNKI sequences. **b–g** Intraportal injection of 11R-JNKI. FITC-conjugated 11R-JNKI (10 mg/kg) was injected intraportally. One hour after treatment, 11R-JNKI was observed as fluorescence signals in liver (**e**), fat (**f**) and muscle (**g**) treated with FITC-11R-JNKI, but not in liver, fat and muscle tissue treated by vehicle (**b–d** respectively)

Fig. 4 Effect of JNK inhibitory peptide. **a** JNK activity after islet transplantation in the liver. Diabetic mice induced by STZ injection were transplanted with 10 mg/kg of 11R-JNKI (filled bars) or 11R-mJNKI (open bars) 3 weeks after STZ injection. Samples were collected from the liver before transplantation (*Pre Tx*) and 1, 3, 6 and 24 h after transplantation. $**p < 0.01$. **b** JNK activity in fat and muscle with 11R-JNKI treatment. The activity in fat and muscle was examined 24 h after islet transplantation with 11R-JNKI or 11R-mJNKI treatment. The samples were loaded on 12% SDS-PAGE and subjected to western blotting using the rabbit anti-Phospho-c-Jun (Ser73)-specific antibody. Measurement of JNK activity, details on positive and negative controls, and definition of relative JNK activity as in Fig. 1. All assessments were made in triplicate and the data (mean $\pm$ SE) are expressed as a percentage of the control value in each experiment. $**p < 0.01$. **c** Effect of 11R-JNKI on insulin signalling in mice. After treatment with 11R-JNKI or 11R-mJNKI, cell extracts were obtained from fat and muscle in BALB/c mice; then expression of total and serine-phosphorylated forms of IRS-1 (Ser307) were examined by western blot analysis. Similar findings were obtained in three independent experiments

Transplantation experiments To determine whether an intraportal injection of 11R-JNKI with islet transplantation enhances islet graft survival *in vivo*, 250 IE islets with or without 11R-JNKI in transplant media were transplanted in the liver of STZ-induced diabetic mice through the portal vein. The blood glucose levels of 9 of the 11 mice (81.8%) transplanted with 10 mg/kg of 11R-JNKI decreased gradually and reached normoglycaemia (Fig. 5a,c), remaining stable thereafter (Fig. 5a). On the other hand, only 2 of the 11 mice transplanted without 11R-JNKI became normoglycaemic (Fig. 5b,c). The difference in attainment of posttransplantation normoglycaemia was statistically significant ($p < 0.05$). Morphological studies showed the presence of islets in the liver of all mice 30 days after transplantation. While the islet grafts

Fig. 5 Transplant experiments. 250 IE islets were transplanted into the liver of STZ-induced diabetic mice through the portal vein. Non-fasting blood glucose levels of individual 11R-JNKI-treated mice (**a**) and of individual vehicle (PBS without 11R-JNKI)-treated mice (**b**). **c** Normoglycaemic rate of STZ-induced diabetic mice after islet transplantation. Normoglycaemia was defined as two consecutive posttransplant blood glucose levels showing less than 11.1 mmol/l (vehicle $n = 11$; 11R-JNKI $n = 11$)

transplanted with 10 mg/kg 11R-JNKI in the normoglycaemic mice showed intense insulin staining, those in control mice with hyperglycaemia showed less staining (Fig. 6a,b). For IPGTT, fasting blood glucose levels of mice transplanted with islets plus 10 mg/kg 11R-JNKI were lower than those of control groups before glucose injection and at 5, 15, 30, 60, 90 and 120 min after injection ($p < 0.01$) (Fig. 6c).

Discussion

In this study, we have demonstrated for the first time that JNK is activated after intraportal islet transplantation. In addition, JNK inhibitor delivered by the protein transduction system to the portal vein clearly prevents this activation and improves the results of islet transplantation. Although we used a syngeneic transplantation

Fig. 6 Immunostaining and glucose tolerance test. **a, b** Immunostaining of islets grafted into the liver of transplanted mice. The liver-transplanted islets were examined histologically on day 30 after transplantation. The islet grafts treated with 10 mg/kg of 11R-JNKI in normoglycaemic mice showed intense insulin staining (**b**); staining was less intense in vehicle-treated mice (**a**). **c** Intraperitoneal glucose

tolerance test 30 days after transplantation. The mice were fasted overnight before the test and injected with glucose (2.0 g/kg body weight) intraperitoneally. Blood glucose levels were measured before and 5, 15, 30, 60 and 120 min after injection (vehicle $n=3$; 11R-JNKI $n=3$) in normoglycaemic mice after islet transplantation. Filled circles, vehicle; filled squares, 11R-JNKI

model without immune suppression according to the Edmonton protocol, the present findings suggest that JNK inhibition could have beneficial effects for allogeneic transplantation.

It is known that beta cell destruction by cytokines such as IL-1 β [34, 35] can be prevented by inhibition of the JNK pathway [24, 36], implying that JNK plays a role in the autoimmune beta cell destruction found in the early stage of type 1 diabetes. In addition, the JNK pathway is activated in various tissues, including pancreatic islets, in the diabetic state, and suppression of that pathway reduces insulin resistance and ameliorates glucose intolerance in type 2 diabetic animal models [37]. In islet transplantation, we and other groups have previously shown that JNK was activated during pancreas preservation and islet isolation and its inhibition improved the transplant outcome [12, 31]. Moreover, we have shown here that JNK was also activated in the liver (and naturally transplanted islets) in the transplant process. Therefore, we assume that JNK is involved in the pathophysiology of islet transplantation, since islets are exposed not only to mechanical, enzymatic, osmotic and ischaemic stress in pancreas preservation and islet isolation, but also to auto- and allo-immunity, glucose toxicity and inflammatory cytokines at islet transplantation.

Protein transduction systems have been employed for the delivery of full-length proteins into many cell types [38–47]. A series of protein transduction domains (PTDs, also known as cell-penetrating peptides), such as antennapedia [39, 40], VP22 [41], TAT [42–46] and poly-arginine [31, 33, 47], have been shown to cross biological membranes efficiently and to promote the delivery of peptides and proteins into cells. These properties make protein transduction potentially useful for islet transplantation. The inclusion of TAT-PTD derivatives of anti-apoptotic-antioxidative proteins, such as haem oxygenase-1, Bcl-XL and BH4 in the culture media can improve islet viability [45, 46]. Moreover, it has been reported that TAT-PTD fusion

proteins can be delivered efficiently to islets and pancreatic tissues without impairing islet function [44].

In this study, the intraportal injection of JNK inhibitory peptide along with islets improved the outcome of islet transplantation. The injection of JNK inhibitory peptide has a transient effect and the intraportal injection of JNK inhibitory peptide without islets to STZ-induced diabetic mice did not improve blood glucose levels (data not shown). Although the effect of JNK inhibition on lowering blood glucose after transplantation is due both to prevention of islet loss and to increased insulin sensitivity in target organs, its main effect in improving transplant outcome might be protection of the islets.

In conclusion, the intraportal injection of JNK inhibitory peptide prevents the apoptosis of isolated islet cells in the liver and also improves islet graft function. Ours is the first study to show that JNK is activated at islet transplantation and that an intraportal injection of JNK inhibitory peptide enhances the graft survival of isolated islets. The prevention of islet apoptosis due to several forms of stress at transplantation may make it possible to transplant islets into one or more recipients using the pancreas from one single donor. Delivery of anti-apoptotic peptides by means of PTDs could protect cells during the critical time before transplantation without the need for genetic modifications. Control of the JNK pathway is extremely important at islet transplantation, and protein transduction systems offer a significant opportunity for determining the true applicability of this technology in islet transplantation.

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Duality of interest The authors declare that they have no conflict of interest in relation to the contents of this manuscript.

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