Effect of nicotinamide on early graft failure following intraportal islet transplantation

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Abstract

Intraportal islet transplantation (IPIT) may potentially cure Type 1 diabetes mellitus; however, graft failure in the early post-transplantation period presents a major obstacle. In this study, we tested the ability of nicotinamide to prevent early islet destruction in a syngeneic mouse model. Mice (C57BL/6) with chemically-induced diabetes received intraportal transplants of syngeneic islet tissue in various doses. Islets were cultured for 24 h in medium with or without 10 mM nicotinamide supplementation. Following IPIT, islet function was confirmed by an intraperitoneal glucose tolerance test (IPGTT) and hepatectomy. The effects of nicotinamide were evaluated by blood glucose concentration, serum monocyte chemoattractant protein-1 (MCP-1) concentration, and immunohistology at 3 h and 24 h after IPIT. Among the various islet doses, an infusion of 300 syngeneic islets treated with nicotinamide exhibited the greatest differences in glucose tolerance between recipients of treated and untreated (i.e., control) islets. One day after 300 islet equivalent (IEQ) transplantation, islets treated with nicotinamide were better granulated than the un-treated islets (_P_ = 0.01), and the recipients displayed a slight decrease in serum MCP-1 concentration, as compared to controls. After 15 days, recipients of nicotinamide-pretreated islets showed higher levels of graft function (as measured by IPGTT) than controls. The pretreatment also prolonged graft survival ($>$ 100 days) and function; these were confirmed by partial hepatectomy, which led to the recurrence of diabetes. Pretreatment of islet grafts with nicotinamide may prevent their deterioration on the early period following IPIT in a syngeneic mouse model.

Keywords: diabetes mellitus, type 1; hepatectomy; islets of Langerhans; islets of Langerhans transplantation; niacinamide

Introduction

Pancreatic islet transplantation is one of the most effective treatments for type 1 diabetes (Shapiro _et al._, 2000; Goss _et al_, 2002; Markmann _et al._, 2003). The general use of this treatment is limited, however, by the need for a large number of islet transplants (usually obtained from two organ donors) to achieve normoglycemia. In addition, the loss of functional islet mass (50-70%) has been associated with stable islet engraftment (Contreras _et al._, 2004). To investigate the mechanisms of islet graft failure, many studies have used rodent models, most often with the kidney capsule as the transplantation site (Jung _et al._, 2006, 2008). Yet islets transplanted under the kidney capsule are protected from dynamic conditions in surrounding compartments, such as the post-gastrointestinal blood flow through the liver; and therefore data from studies that use this model may not apply directly to intraportal islet transplantation (IPIT) (Hara _et al._, 2004). Islets infused intraportally do not survive as well as transplants under the kidney capsule (Goto _et al._, 2004; Yin _et al._, 2006), which suggests that the intraportal infusions encounter more complex challenges.

Nicotinamide, a form of vitamin B3, participates in diverse biological processes through its major metabolite NAD^+ (nicotinamide adenine dinucleotide), including the production of energy, nutrient metabolism, signal transduction, and maintenance of genome integrity. As an inhibitor of poly

(ADP-ribose) synthetase, nicotinamide may also prevent islet β-cell injury or destruction in nonobese diabetic (NOD) mice (Yamada *et al.*, 1982) and mice with chemically-induced diabetes (Uchigata *et al.*, 1983; Yamada *et al.*, 1990). Importantly, this compound sustains islets in culture (Korsgren *et al.*, 1993), increasing the resistance of β-cells to toxic chemicals, activated macrophages (Kolb *et al.*, 1990), and inflammatory cytokines (Pipeleers and Van de Winkel 1986; Buscema *et al.*, 1992; Kallmann *et al.*, 1992). Based on these observations, nicotinamide has been tested for therapeutic effects in insulin-dependent diabetes of recent onset, in animal models and clinical trials (Elliott and Chase, 1991; Chase *et al.*, 1992; Ueki *et al.*, 1995). Especially, Ueki *et al.* (1995) reported that diabetic rats was injected I.P. with nicotinamide once a day for a 60 days after transplantation, finally, hyperglycemia can be ameliorated with an insufficient under of islet. Furthermore, isolated human or pig islet cells are also cultured in media with 10 mM nicotinamide before transplantation to diabetic recipients

(Korsgren *et al.*, 1993; Beattie *et al.*, 2002; Ichii *et al.*, 2006). These findings suggest that nicotinamide may be used to increase the efficiency and effectiveness of pancreatic islet transplantation.

In the present study, we applied islets cultured with nicotinamide, rather than daily I.P. injection with nicotinamide, to ameliorate hyperglycemia in diabetic mice following infusion into the hepatic portal vein. Using our syngeneic mouse model, we found that islets cultured with nicotinamide effectively prevented as well as decrease the number of islets required for successful transplantation. Moreover, islets cultured with nicotinamide delayed islet destruction and diabetes recurrence in the early post-transplantation phase.

Results

Effect of islet transplantation by intraportal infusion

To characterize the morphology of the implanted islets, we transplanted syngeneic islets (350 IEQ) to STZ-induced diabetic C57BL/6 mice by intra-
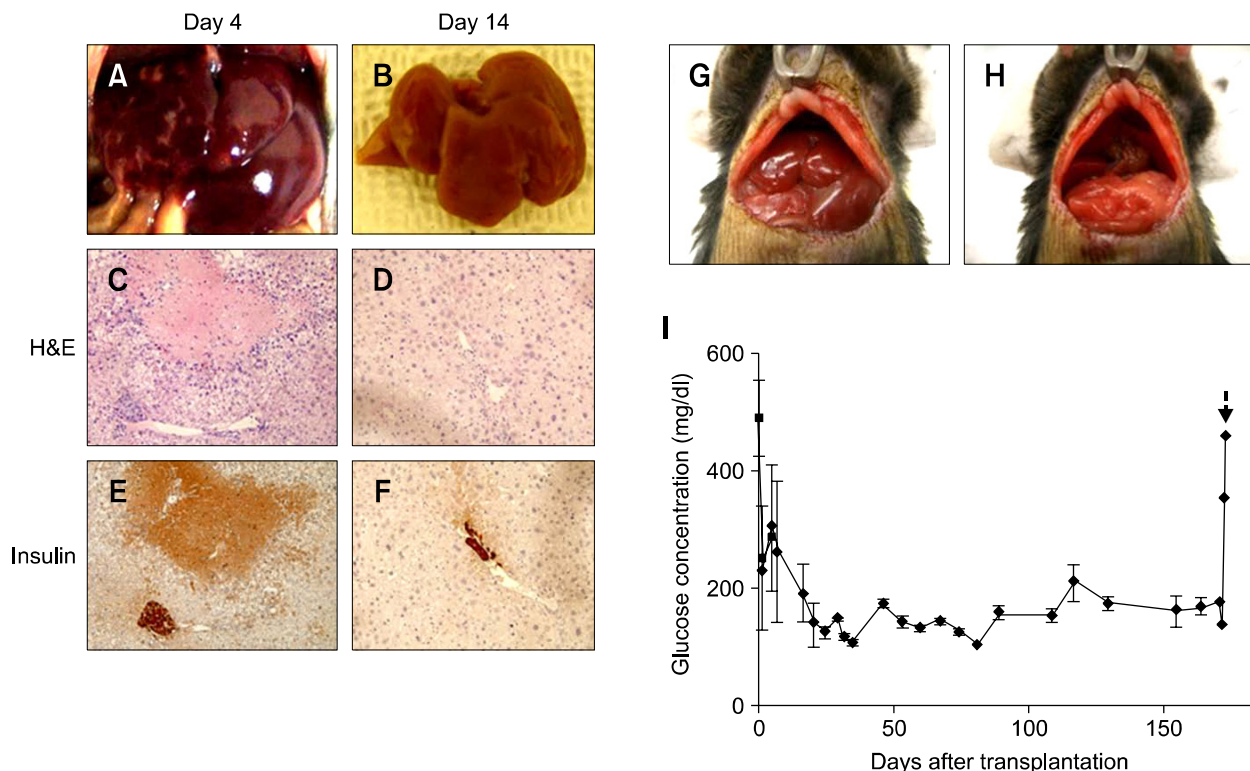

Figure 1. Evaluation of islet grafts early (days 4 and 12) and late (day 178) after intraportal islet transplantation. Four days (A, C, E) and 14 days (B, D, F) after islet transplantation, 70% of the liver was removed. Histological examination revealed multiple foci of necrosis on the liver surface (A, B). Hematoxylin/eosin staining (H&E) (C, D), and immunoperoxidase staining with antibody against insulin (magnification ×200) (E, F) of liver sections, are shown. Histological sections for each marker are representative of three animals from each treatment group. In recipients of 350 islets with long-term graft survival, 70% of the liver was also removed (G, H). Blood glucose levels of recipients were monitored, and normoglycemic mice underwent partial hepatectomy at the time points indicated by arrows in (I) to confirm long-term graft function and acceptance.

portal infusion. Four days after transplantation, we observed significant necrosis on the surface of the recipient liver, but it was resolved by day 14 (Figure 1A and 1B). Islets were noted at day 4 post-transplantation, particularly within the areas of liver necrosis (Figure 1C and 1E). In contrast, no necrosis was observed at day 14 post-transplantation (Figure 1D and 1F).

To confirm the engraftment site following IPIT, the liver was divided into five lobes and the average islet number per cm^2 from each lobe was calculated. In the result, we observed that the transplanted islets were scattered over the five liver lobes (data not shown). To examine the function and acceptance of islets in long-term

surviving grafts, three lobes of recipient livers (about 70%, consisting of the middle lobes and left lobe) were removed on day 172 after the intra-portal transplantation of 350 islets. These partially hepatectomized recipients subsequently displayed high glucose levels and developed recurrent diabetes (Figure 1G-I).

Beneficial effect of nicotinamide treatment on islet grafts

We examined the effect of nicotinamide on engraftment of different islet doses. Purified islets of C57BL/6 mice were cultured for 24 h in M199 medium with or without nicotinamide, and then
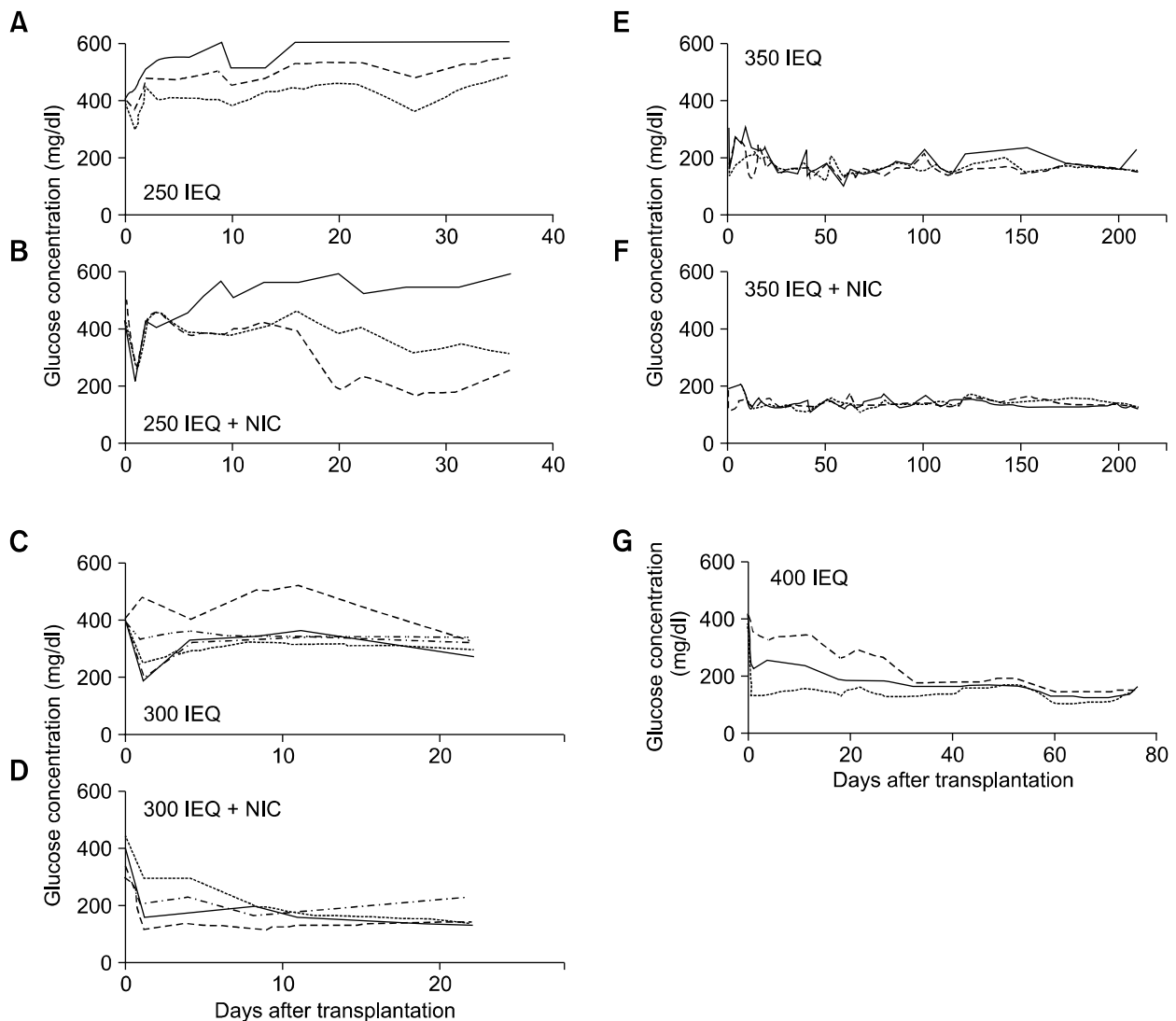

Figure 2. Beneficial effect of nicotinamide on the hyperglycemia in diabetic mice. Recipients received 250 (A, B), 300 (C, D), 350 (E, F), or 400 (G) syngeneic islets that were pretreated without (A, C, E, G) or with (B, D, F) nicotinamide. Individual lines represent the non-fasting plasma glucose levels of each animal. NIC, nicotinamide.

transplanted to diabetic C57BL/6 recipients in aliquots of 250, 300, 350, or 400 islets, respectively. Diabetic recipients receiving 250 islets did not correct the diabetic hyperglycemia, although one of three mice with nicotinamide-treated islets became normoglycemic 20 days after transplantation (Figure 2A and 2B). Mice that received 300 untreated islets maintained high glucose levels. In marked contrast to control mice, nicotinamide-treated islets (300 IEQ) suppressed the recipient's hyperglycemia on of the first day after transplantation (three of four recipients), and all were normalized after 10 days (Figure 2C and 2D). Mice that received 350 or 400 islets not pretreated with nicotinamide showed unstable glucose profiles (Figure 2E and 2G). In contrast, recipients of 350 nicotinamide-treated islets corrected their hyperglycemia and stabilized within one day of transplantation (Figure 2F).

Enhanced glucose tolerance in recipients of nicotinamide-pretreated islet grafts

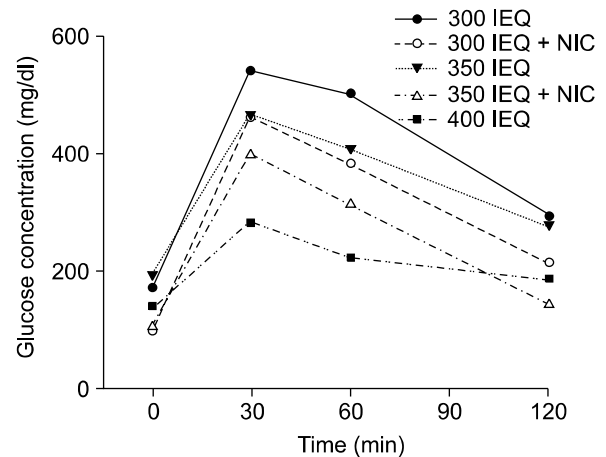To study the effect of nicotinamide-treated islet grafts on the whole-body glucose disposal rate, we

Figure 3. IPGTT in mice receiving islets with or without nicotinamide pretreatment. The IPGTT was performed in diabetic mice at 15 days post-transplantation. Experimental groups included diabetic mice that received 300, 350, or 400 syngeneic, nicotinamide-pretreated or untreated islets. The results for all five groups are expressed as mean plasma glucose concentrations (n = 3). NIC, nicotinamide.

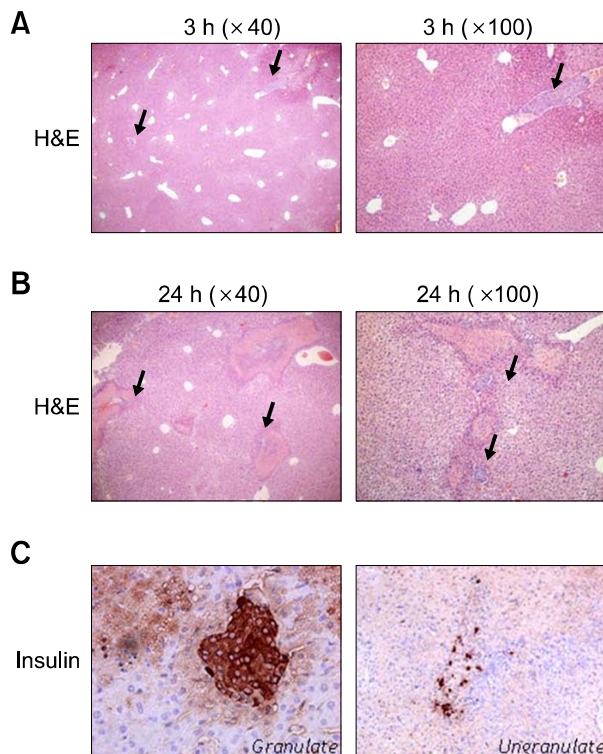performed IPGTTs on recipients of functioning grafts 15 days after transplantation. In recipients of

Figure 4. Morphological changes in the engrafted liver. Liver tissues were collected 3 h (A) and 24 h post-transplantation (B, C), and stained with H&E (A, B) and monoclonal antibody to insulin (C). Insulin-positive cells were morphologically granulated or de-granulated (C, left or right, respectively). The percentage of granulation was based on the number of granulated islets out of the total number of insulin staining cells at 0, 3, and 24 h post-transplantation. The percentage of granulation at 0 h was calculated with islets before transplantation, and no de-granulated islets were observed.
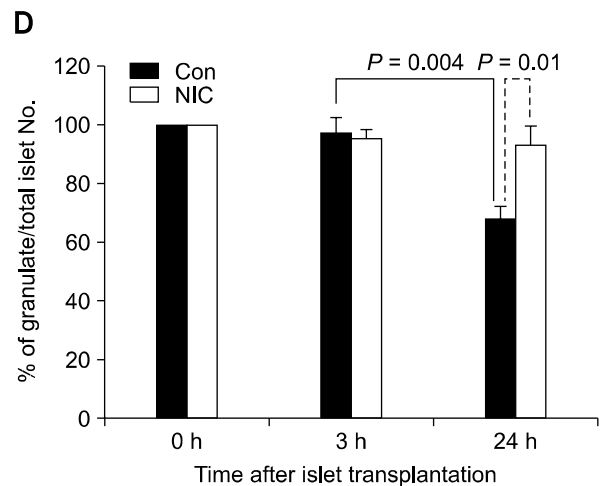
300 or 350 nicotinamide-treated islets, hypergly-cemia induced by glucose injection (2 g/kg, i.p.) declined to the normal range within 2 h (Figure 3). Although recipients of 300 or 350 untreated islets continued to show blood glucose levels in the abnormal range for 2 h, the amplitude of hypergly-cemia and the kinetics of its decline did not differ significantly between groups that received 300 or 350 islets with or without treatment.

Histological evaluation

Following intraportal infusion with 300 untreated or nicotinamide-pretreated syngeneic islets, recipient livers were examined for pathology at 3 h and 24 h (Figure 4). The percentage of granulated islets was calculated by counting the total number of insulin-positive cells and then grouping them into granulated or de-granulated islets according to morphology (Figure 4C).

The morphology was similar in both groups at 3 h after IPIT (Figure 4A and 4D). Most insulin-positive cells were well-granulated, and these healthy islets were located in blood vessels. The condition of the liver was normal or slightly abnormal. After 24 h, however, serious necrosis was observed in the islet recipients' livers. In the control group, more islets were de-granulated (68% granulated) as compared with those after 3 h (96% granulated) (P = 0.004) (Figure 4B and 4D). In contrast, islets pretreated with nicotinamide were consistently well-granulated (94% and 92% granulation at 3 h

and 24 h after transplantation) and significantly different from untreated islets (P = 0.01) (Figure 4D).

Expression of inflammatory mediators in the liver

For RT-PCR and western blot, total RNA and protein were extracted from the recipient livers (lateral right and caudal lobe) at 3 h (Figure 5A and 5C) and 24 h (Figure 5B and 5D) after trans-plantation of 300 islets cultured with or without nicotinamide. At 3 h after IPIT, RNA expression of both TF and TNF-α had increased markedly in both groups; but by 24 h, it had declined (Figure 5A and 5B). IL-1β and MCP-1 were, however, weakly expressed in the liver after both 3 h and 24 h of IPTX. In addition, nicotinamide-pretreatment showed similar expression patterns to non-treatment. And also C3 was detected strongly in all recipient livers of both groups after 3 h in western blot (Figure 5C and 5D). Interestingly, C3 protein was found only a third in the nicotinamide pretreatment mice, whereas it was detected in all control recipients whole experimental period.

Decreased serum MCP-1 concentration

The concentration of serum MCP-1 was deter-mined in recipients of untreated or nicotina-mide-pretreated grafts on day one after intraportal infusion of 300 islets (Figure 6). Serum MCP-1 levels increased in all recipients after IPIT, but
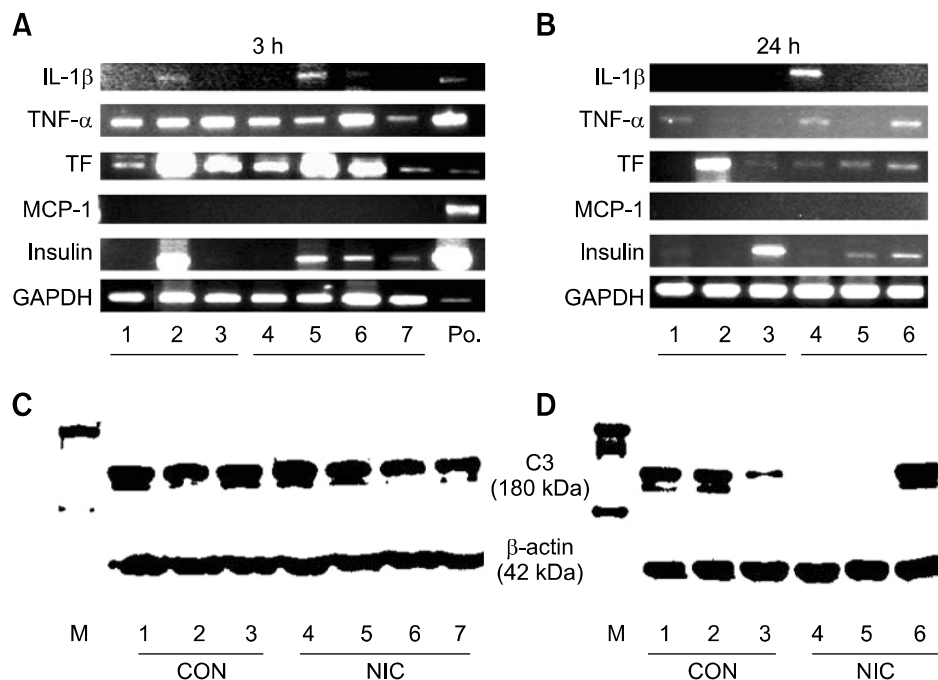

Figure 5. Expression of inflammatory mediators in the liver after IPTX. Islet cells pretreated without (control; lanes 1-3) or with nicotinamide (nicotinamide; lanes 4-6 and/or 7) were intraportally transplanted in the diabetic mouse. After 3 h or 24 h of transplantation, liver of recipient was sacrificed. RNA expressions for IL-1β, TNF-α, TF, MCP-1, insulin, and GAPDH were detected by RT-PCR analysis in the liver 3 h (A) or 24 h (B) after IPTX. C3 and β-actin were detected by Western blot analysis in the liver sample at both times (C, D). Cell line RAW 264.7 activated by LPS was used as a positive control (Po.) of IL-1β, TNF-α, TF, MCP-1, and GAPDH. Cell line MIN 6 was used as a positive control of insulin. NIC, nicotinamide.
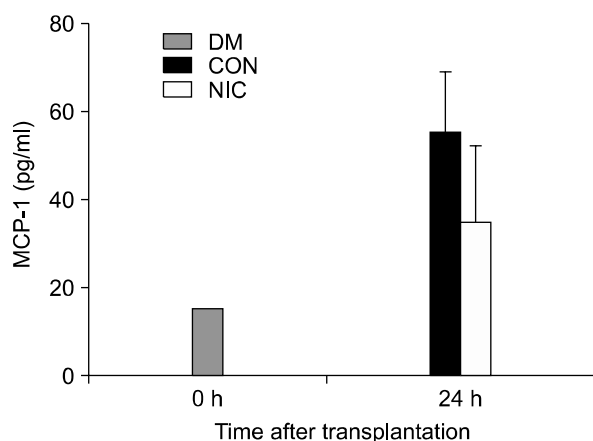
Figure 6. MCP-1 concentrations after intraportal transplantation of nicotinamide-pretreated and untreated islets. Diabetic mice receiving 300 IEQ with or without nicotinamide pretreatment were divided with two groups (CON , control; NIC, nicotinamide. pretreatment). At 24 h post-transplantation, MCP-1 concentrations were analyzed in recipient sera. Serum of diabetic mice (DM) was harvested before islet transplantation for the 0 h measurement.

were lower in the nicotinamide-pretreatment group (although the difference did not reach statistical significance).

Effect of nicotinamide on long-term graft survival

We examined the effect of nicotinamide on the diabetes cure rate and graft maintenance. Syngeneic islet cells (350 IEQ) with or without nicotinamide pretreatment were infused into the portal veins of diabetic mice. All mice receiving 350 nicotinamide-treated islets corrected their hyperglycemia on day one or by day nine (67% or 33% of the recipients, respectively) following transplantation, and all (100%) sustained their grafts for more than 200 days (Figure 7A). Recipients of untreated islet grafts were cured more gradually (33% of recipients each on days 12, 16, and 23), and experienced more rapid recurrence of diabetes (67%) than recipients of nicotinamide-treated islet grafts (0%). Extended function of islets was confirmed by partial hepatectomy at 215 days post-transplantation. Islet cells in long-term surviving grafts were found around blood vessels, some infiltrated by immune cells (Figure 7B). Masson's trichrome stain revealed collagen-rich lesions of islet grafts in the liver (Figure 7C). Fibrosis in recipients with recurrent diabetes and hyperglycemia (67% of control group) was observed in scattered islet clusters. In contrast, fibrotic tissue in surviving grafts of normoglycemic recipients (33% of control group, and all of nicotinamide-treated group) were found around islets.

Discussion

The mechanism for the protective effect of nicotinamide pretreatment on islet tissue transplants is not well understood, although it may involve the scavenging of free radicals or the reduction of immunological injury to beta-cells (Buscema *et al.*, 1992; Andersen *et al.*, 1994). To explore this question, we examined the effect of the nicotinamide pretreatment on graft stability and function in a syngeneic mouse model of diabetes. With respect to glucose tolerance, mice that received nicotinamide-treated islets (300 IEQ) were superior to mice implanted with 300 or 350 untreated islets. The kinetics of glucose control also differed between the groups that received nicotinamide-treated and untreated islets. These observations suggest that this use of nicotinamide may reduce the number of donor islets required to control hyperglycemia in the diabetic recipient mice, at least in this syngeneic model. In addition, the nicotinamide pretreatment markedly reduced the deterioration of islets within the area of necrotic liver one day after islet infusion ($P = 0.01$). Serum MCP-1 expression following intraportal islet infusion was slightly lower in recipients of nicotinamide-treated islets, and the nicotinamide-pretreatment prolonged graft survival. From this we conclude that nicotinamide may reduce the adverse effects of intraportal infusion of syngeneic islets in C57BL/6 mice. In contrast to these results, however, we were unable to replicate the effects of nicotinamide on allogeneic islet transplantation: all transplant recipients remained hyperglycemic after infusion of 350 or 400 IEQ allogeneic islets (data not shown). We suggest that combined with an appropriate immunosuppressive regimen, nicotinamide may also prove beneficial in allogeneic islet transplantation via the portal vein.

In the porcine model, approximately 50% of islet cells are damaged in the first 20 min after the initiation of intraportal allotransplantation (Eich *et al.*, 2007). In a syngeneic rat model of IPIT, an average of 43% of the infused tracer radioactivity was found in the liver 1 h after islet transplantation (Toso *et al.*, 2005). Based on these and other observations, the authors suggested that inflammatory events immediately following IPIT (i.e. instant blood mediated inflammatory reaction, IBMIR) may have long-lasting effect on islet function.

Pretreatment of islets with nicotinamide may potentially suppress the synthesis of triggers for IBMIR, including tissue factor (TF), monocyte chemoattractant protein (MCP)-1, and other inflammatory cytokines (Moberg *et al.*, 2003). The expre-
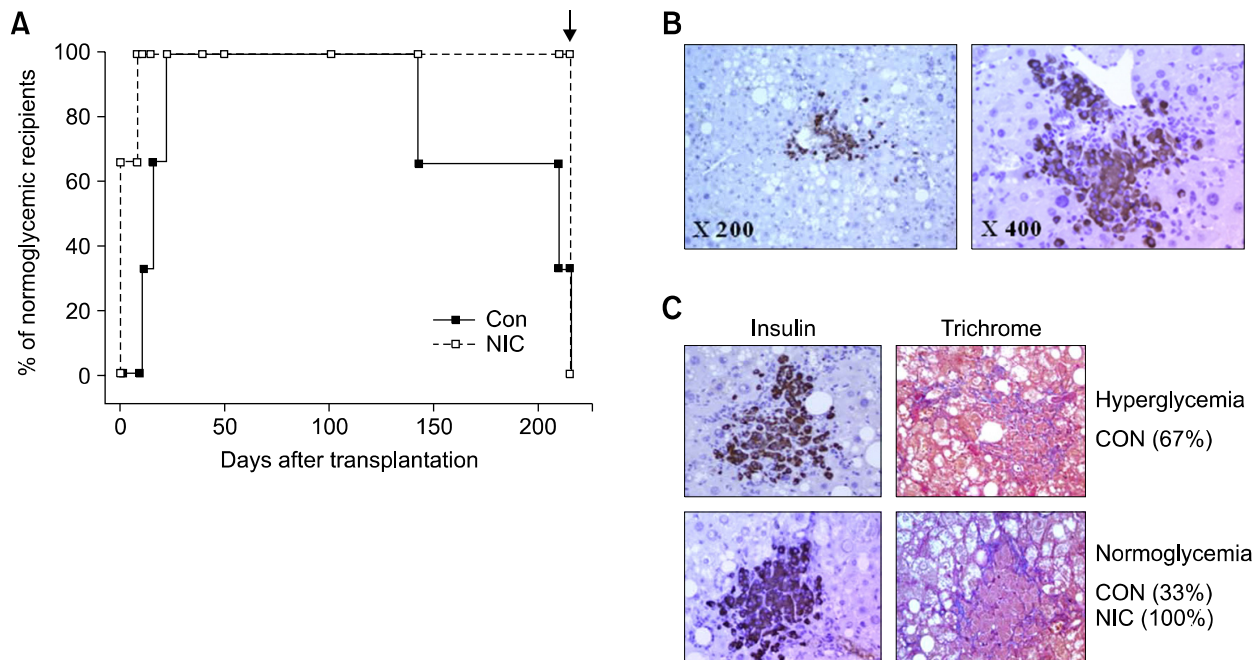
Figure 7. Diabetes cure rate and histology in long-term grafts. Diabetic mice that received 350 IEQ were divided into control (CON) (-■-) and NIC pre-treatment (-□-) groups. Long-term graft survival between groups was described as the percentage of recipients cured of diabetes (A). In order to confirm long-term graft function and acceptance, normoglycemic mice underwent partial hepatectomy of the graft-bearing liver at the time points indicated (arrows). Insulin positive-staining cells were observed near the blood vessels of long-term grafts (B). Islet cells were present in hyperglycemic (recurrent diabetes) and normoglycemic recipients (C: top and bottom panels, respectively). Beta cells were stained with anti-insulin antibody (dark brown color, left panel), and fibrotic lesions were detected by Masson's trichrome staining (blue color, right panel).

ssion of TF was strongly correlated with the level of MCP-1 ($r^2 = 0.98$), and with the ability of islets to initiate IBMIR ($r^2 = 0.94$), with thrombus formation. Moberg *et al.* (2005) also reported that levels of TF expression are strongly correlated with the relative potency of islets in initiating IBMIR in the *in vitro* loop model. Furthermore, the concentrations of TF and MCP-1 in islets show negative correlation with post-transplantation survival, i.e. low levels of these factors promote graft longevity (Piemonti *et al.*, 2002; Berman *et al.*, 2007).

Based on these observations, we evaluated islet loss at 3 h and 24 h after IPIT. At both time points, islets pretreated with nicotinamide were well granulated ($94.3 \pm 3.7\%$ and $92.3 \pm 6.6\%$, respectively) and significantly different from untreated islets ($96.6 \pm 5.9\%$ and $68.2 \pm 3.9\%$, respectively) ($P = 0.01$), suggesting that nicotinamide can prevent or delay islet destruction in the early period after IPIT. We also investigated whether nicotinamide was able to modulate inflammatory mediators such as TF, MCP-1, IL-1β, TNF-α, insulin and complement (C3) following IPIT in RT-PCR and western blot analysis. The expression of TF, TNF-α, and C3 was increased markedly at 3 h after IPTX, while it was rapidly decreased at 24 h in both nicotinamide-pretreatment and non-treatment group.

These results would suggest that inflammatory mediators, such as TF, TNF-α, and C3, were activated in the early phase (up to 3 h) after islet transplantation. Interestingly, serum MCP-1 level of the nicotinamide-pretreatment group was lower than that of non-treatment group on day one after IPTX (not statistically significant), whereas RNA of MCP-1 was weakly expressed in the recipient livers of both groups after 3 h and 24 h of transplantation. In our result, the correlation of between serum MCP-1 concentration and RNA expression of MCP-1 was not significant. Thus, presumably, to understand the role of nicotinamide pretreatment, the quantitative studies including Real-time PCR during early phase (up to 3 h) would be needed.

Nicotinamide suppresses TF expression in several islet transplantation models (Moberg *et al.*, 2002, 2003, 2005; Berman *et al.*, 2007). After activation by an extrinsic pathway, TF induces thrombin formation (Moberg 2005) then thrombin converts fibrinogen to insoluble fibrin (Ozmen *et al.*, 2002; Andrades *et al.*, 2007) and activates the complement system, especially C3 and C5 (Andrades *et al.*, 2007). We speculate that nicotinamide pretreatment may alter expression of these mediators very early (< 3 h) after IPIT. We also suggest that the reduction in TF expression and

thrombin formation following nicotinamide treatment suppresses early C3 activation. Moreover, the reduction of these factors may prolong graft survival and reduce the formation of insoluble fibrin in hyperglycemic recipients, as shown in our study, although the mechanism for the anti-inflammatory action of nicotinamide on TF was not well known.

This action, however, may be highly relevant, not only to the pro-coagulative activity of islets, but also the inflammatory response, in both the early recruitment of immune cells and the activation of specific immunity (Chase *et al*., 1992) that occurs after transplantation (Yamada *et al*., 1990; Buscema *et al*., 1992; Andersen *et al*., 1994). nicotinamide promotes islet regeneration (Yonemura *et al*., 1984), induces differentiation (Hellerstrom 1984; Sandler and Andersson 1988), and enhances insulin biosynthesis (Ye *et al*., 2006). Based on these properties, we suggest that nicotinamide may serve several therapeutic roles in the early post-transplantation period, to prevent IBMIR and promote graft survival after intraportal islet infusion.

We conclude that nicotinamide-pretreated islets suppressed hyperglycemia more efficiently than control islets in a syngeneic mouse model of IPIT. Three hours after transplantation, the pre-treatment group did not differ morphologically from controls; but after 24 h, the degranulated islets in necrotic areas of the liver were markedly reduced in the pretreated grafts. Nicotinamide treatment also prolonged graft survival and reduced the frequency of fibrotic lesions. We propose that nicotinamide pretreatment may function to prevent the deterioration of islet grafts following IPIT in diabetic patients. Further studies are required to completely understand the effects of nicotinamide on IBMIR in intrahepatic islet transplantation. Additional agents will be needed to fully control inflammation and reduce the number of islets required to achieve insulin independence.

Methods

Animals and induction of diabetes

Adult male 12-week-old C57BL/6 (H-2d) mice (Jackson Laboratories, Bar Harbor, ME) served as donors and recipients for IPIT. All animals were housed in accordance with the Samsung Biomedical Research Institute's Principles of Laboratory Animal Care and Guide for the Use of Laboratory Animals. To induce diabetes, mice were given serial intraperitoneal injections of streptozotocin (STZ, 180 mg/kg, Sigma Chemical Co., St. Louis, MO). Blood glucose concentrations were measured in whole blood samples collected from the tail veins of the injected mice with Accu-Chek Advantage Glucose Monitors (Roche Diagnostics, Indianapolis, IN). Diabetic mice that had non-fasting

blood glucose values higher than 400 mg/dl for more than two consecutive days were used as recipients of islet grafts.

Islet isolation and transplantation

Islets were isolated from the C57BL/6 mouse pancreas by digestion with collagenase P (0.8 mg/ml; Roche Diagnostics) followed by purification in a Ficoll gradient (Sigma-Aldrich) (Gotoh *et al*., 1986). Islets were then cultured overnight at 37°C, with 5% CO_2, in M199 medium (Gibco, Long Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Purified islets were cultured for 24 h in this medium, with or without 10 mM nicotinamide (Sigma-Aldrich, St. Louis, MO), and transplanted into the liver via the portal vein with an injector. This injector, modified and developed in our laboratory, was constructed from a 27-gauge needle, 20-gauge catheter, and 1 cc syringe. Blood glucose concentrations in the recipient mice were monitored biweekly after transplantation. Non-fasting blood glucose concentrations greater than 300 mg/dl for two consecutive measurements in a single week were accepted as evidence of graft failure.

Partial hepatectomy

To confirm the success of IPIT in our mouse model, we tested the ability of partial hepatectomy to cause recurrence of diabetes in the longest-surviving mice. We used partial hepatectomy in our IPIT model, in analogy to the diabetogenic effect of partial pancreatectomy in normal mice (Morales *et al*., 2005). The liver has five lobes (lateral left, medial left, medial right, lateral right, and caudal lobe), and 70% partial hepatectomy is routinely performed in animal research (Greene and Puder, 2003). We removed the median and left lateral lobes in the surviving mice, then monitored blood glucose concentration to assess the activity of the islet graft in the control of diabetes.

Intraperitoneal glucose tolerance test

We performed an intraperitoneal glucose tolerance test (IPGTT) in the graft recipients 15 days after IPIT. Briefly, after overnight fasting, unanesthetized mice were injected IP with a 50% glucose solution (2 g/kg body wt). Plasma glucose concentrations were measured at 0, 30, 60, and 120 min thereafter.

Histological analysis

To characterize islet graft morphology, we took liver samples from recipient mice at 3 and 24 h following IPIT, and on day 215, we also performed 70% partial hepatectomies. Between three and five recipients were examined in each experiment. The entire or partial liver was divided into individual lobes, fixed in 10% formalin, and prepared for histological analysis. We prepared 6 μm step-sections at 200 μm intervals, and calculated the total number of islets and the area of each of the three step-sections in each lobe. The islet number in the liver was defined as the

average islet number per cm² from all five lobes. Functional islets were identified using anti-insulin polyclonal antibody (Dako, Carpinteria, CA) to assess insulin expression of islet beta cells. Masson's trichrome staining kit (Sigma) was used to identify collagen-rich fibrotic lesions in the liver. A pathologist, who was blind to the treatment status of the transplanted graft donors, evaluated all islet graft specimens for the presence of inflammatory cells, insulin, glucagons, and apoptotic cells. Controls included the omission of primary antibodies and the use of species-matched nonbinding control antibodies.

RT-PCR

Liver samples were obtained at 3 h and 24 h following transplantation of islets treated with or without nicotinamide and were subject to total RNA extraction using Trizol reagent (Invitrogen). After RNA quantification, 1 μg of total RNA was treated with RNase-free DNase I (Roche), followed by reverse transcription using Omniscript RT Kit (Qiagen) as described by the manufacturer's instructions. GAPDH mRNA was amplified simultaneously as a control. The following primers were used: IL-1β forward (5'-CC-GTGGACCTTCCAGGATGA-3') and reverse (5'-GATCCA-CACTCTCCAGCTGC-3'); TNF-α forward (5'-GGCAGGT-CTACTTTGGAGTCATTGC-3') and reverse (5'- CATTCG-AGGCTCCAGTGAATTCGG-3'); TF forward (5'-CGGGTG-CAGGCATTCCAGAG-3') and reverse (5'-CTCCGTGGG-ACAGAGAGGAC-3'); MCP-1 forward (5'-TCTCTTCCTCC-ACCACCATGCAG-3') and reverse (5'-GGAAAAATGGAT-CCACACCTTGC-3'); insulin forward (5'-TCCTACCCCTG-CTGGCCCTGC-3') and reverse (5'-AGTTGCAGTAGTT-CTCCAG-3'); and GAPDH forward (5'-ACCACAGTCCAT-GCCATCAC-3') and reverse (5'-TCCACCACCCTGTTG-CTGTA-3'). Conditions for the reaction are as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C denaturation (45 s), 59°C annealing (1 min; IL-1β, TF, and MCP-1), and 72°C extension (1 min), with a final extension at 72°C for 5 min. Annealing temperature and time of insulin, TNF-α, and GAPDH is 30 s at 56°C, 1 min at 60°C, and 30 s at 60°C, respectively. RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The murine monocyte/macrophage cell line RAW 264.7 was obtained from the Korean Cell Line Bank (Seoul, Korea), and used as a positive control of inflammatory mediators. To induce expression of inflammatory mediators, cells were activated with 10 ng/ml LPS (Sigma Chemical Co., St. Louis, MO) for 6 h, and then harvested for RNA isolation. Cell line MIN 6 was used as a positive control of insulin.

Western blot analysis

Liver samples were obtained at 3 h and 24 h following IPTX and were homogenized in PBS/Protease Inhibitor cocktail and RIPA lysis buffer. Cellular protein from each sample (50 μg) was mixed with sample buffer (0.25 M Tris, pH 6.8, 8% SDS, 40% glycerol, 2.5% bromphenol blue, and 2% β-mercaptoethanol) and subjected to 10% SDS-PAGE, and then blotted on nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were blocked overnight with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at 4°C and then incubated with monoclonal anti-mouse C3 (1:200; Santa Cruz, Biotechnology, CA) or polyclonal rabbit anti-β-actin (1:1000; Abcam, Cambridge, UK) in 5% non-fat milk in PBS containing 0.1% Tween 20 (PBT) for 4 h at RT. After the blot was washed with TBST, it was incubated for 1 h at RT with secondary HRP-conjugated anti-mouse IgG (1:1000; eBioscience) or anti-rabbit IgG (1:2000; Biolegend). Immunoreactive bands were revealed using an ECL-plus kit. Blots were visualized using the chemiluminescence detection system (Amersham Pharmaceuticals, Amersham, UK).

Serum MCP-1 content

The plasma concentration of MCP-1 was quantified using a commercially available ELISA kit (Quantikine MCP-1; R&D Systems Europe Ltd., Abingdon, United Kingdom). Blood samples were obtained one day after islet transplantation. Plasma was separated, frozen immediately, and stored at -70°C until use.

Data analysis

All values reported represent the mean ± standard deviation. Statistical analysis of survival and granulation among the treatment groups was performed using the Statistical Package for Social Sciences (SPSS for Windows 15.0; SPSS Inc, Chicago, IL) with the Log Rank test. P values < 0.05 were considered to be statistically significant.

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