Beneficial Effects of Activated Protein C on Amelioration of Hyperglycemia in Streptozotocin−induced Diabetic Mice Receiving Intrahepatic Syngenic Islets From a Single Donor

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Abstract The inability to achieve successful islet transplantation from one donor to one recipient has been a major obstacle facing clinical islet transplantation.   The present study focused on the effects of activated protein C APC which plays a key role in crosstalk between coagulation and inflammation and determined whether APC has any beneficial effect on engraftments of islets transplanted into the liver of mice.   Streptozotocin STZ −induced diabetic mice n 8 receiving intrahepatic 200 syngeneic islets, the number of islets isolated from a single donor, remained hyperglycemic after transplantation.   In marked contrast, all of diabetic mice n 7 receiving 200 islets and treated with APC   40μ g, i.v. at 0, 2 and 4 hr   became normoglycemic. A histological examination revealed that APC prevented islet graft loss during the early post−transplant period and more of the islets were detected in the liver of the APC treated mice than in the controls.   Sixty days after transplantation, the APC treated mice showed better glucose tolerance than the control mice.   A flow cytometry analysis disclosed that Gr− 1 CD11b cells neutrophils with a high production of proinflammatory cytokines had accumulated in the liver of control mice at a peak of 6 hr after transplantation.   In mice receiving islets and treated with APC, the production of proinflammatory cytokines in these cells was down−regulated without affecting their number.   These findings show that APC prevents early loss of transplanted islets by inhibiting the production of proinflammatory cytokines deleterious to islet grafts, enabling successful transplantation from one donor to one recipient in mice.   The present study indicates that APC may improve the efficiency of clinical islet transplantation when the effect of APC is also the case in human.

Key words Islet transplantation, Engraftment, Activated protein C APC , Proinflammatory cytokine

Introduction

Although, the remarkable success of islet transplantation for type 1 diabetes patients was first reported by the Edmonton group in 2000[1] and subsequently confirmed by other groups,[2 −6] sequential transplantations with the use of islets from 2 or 3 donors are still required to achieve insulin independence after transplantation.   Thus, the inability of producing successful islet transplantation from one donor to one recipient has been a ma-

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jor obstacle facing clinical islet transplantation. Moreover, patients who underwent islet transplantation had a −cell function of only 20-30 of those in healthy individuals even though they had received islets from more than one donor.[7] Therefore, new strategies to prevent islet graft loss during the posttransplant period are needed for successful islet transplantation.

Since Kemp *et al*.[8] reported that syngeneic islet transplantation into the liver reversed drug−induced diabetes in rats in 1973, the liver has been regarded as an appropriate site for islet transplantation. In clinical settings, isolated islets are transplanted into the liver of recipients via the portal vein[1 −6] and form an embolism in the small portal veins stimulating blood coagulation. Thus, coagulatory events may participate in inflammatory response deleterious to transplanted islet grafts.

Under physiologic conditions, the endothelial cells inhibit blood coagulation by expressing thrombomodulin TM . TM binds to thrombin and makes a complex on the surface of the endothelial cells. TM−thrombin complex activates protein C to generate the anticoagulant enzyme activated protein C APC , a vitamin K−dependent serine protease, and this activation is enhanced by the endothelial protein C receptor EPCR .[9] Therefore, APC makes a complex with protein S and inhibits coagulation by inactivating two critical regulatory proteins, factor a and a.[10] In animal models of thrombus formation, APC was revealed to play a very important role in preventing blood coagulation.[11 12] Recently, many investigators have reported that there is a crosstalk between coagulation and inflammation, and APC plays a central role in regulating this interaction.[13 −16] Bernard *et al*. demonstrated that recombinant human APC significantly reduces the mortality of patients with severe sepsis, which was defined as sepsis associated with acute organ dysfunction resulting from a generalized inflammatory and procoagulant response to an infection, in their randomized multi−center trial.[17] Moreover, APC has been reported to have cytoprotective effects on renal cells and neurons, and reduced organ damages in animal models of ischemic−reperfusion injury and stroke.[18 −20]

This study investigated whether APC prevents islet graft loss during the early posttransplant period to improve the engraftment of intrahepatic islet grafts, facilitating to produce successful islet transplantation from one donor to one recipient in mice.

Research Design and Methods

Mice

Male C57BL/6 mice were purchased from Charles River Inc., Japan Kanagawa, Japan . Diabetes was induced in the recipients by an intravenous injection of 180 mg/kg streptozotocin STZ Sigma −Aldrich Japan, Tokyo, Japan . Mice whose plasma glucose levels exceeded 400 mg/dl at 2 days after STZ injection were used as recipients, and they remained hyperglycemic until the time of islet transplantation.

Reagents

APC, purified from fresh frozen plasma by immunoaffinity chromatography using monoclonal antibody to protein C, was supplied from The Chemo−Sero− Therapeutic Research Institute Kumamoto, Japan . The dosage of 40μ g of APC was dissolved in 0.2 ml of sterile normal saline and injected 3 times just before islet transplantation, 2 and 4 hr after transplantation intravenously via the tail vein. The mice of the control group were injected equivalent volume of saline in the same manner. Anti−EPCR antibody was a monoclonal antibody to recombinant mouse EPCR, and was a kind gift from Dr. Masaru Taniguchi RIKEN Research Center for Allergy and Immunology, Yokohama, Japan . Anti−EPCR antibody 100μ g was administered intraperitoneally on the day before transplantation. All other reagents were purchased from Sigma−Aldrich Japan Tokyo, Japan .

Islet isolation and transplantation

Islets were isolated by the static digestion method using collagenase[21] and then they were separated by centrifugation on Ficoll−Conray gradients.[22] The islets were collected manually using a Pasteur pipette with the aid of a dissecting microscope. Only the islets measuring 150 to 250 μ m in diameter were hand−picked and used for the

experiments. The size of individual islets on each islet isolation procedure was confirmed using a phase-contrast microscope equipped with a scale in the eye piece. Hand-picked islets were transplanted into the liver via the portal vein of the recipients at 3 days after the induction of diabetes by STZ injection.

Monitoring plasma glucose and body weight

The nonfasting plasma glucose level and body weight were monitored three times a week in all the recipients after islet transplantation. The plasma glucose levels were measured using a Beckman glucose analyzer Beckman Japan, Tokyo, Japan. Normoglycemia was defined to have occurred when the two consecutive plasma glucose levels after transplantation were less than 200 mg/dl.

Morphological study

The livers bearing the islet grafts were examined morphologically at the appropriate time after transplantation. The recipient mice were sacrificed, and the liver bearing the grafts were removed. To compare the numbers of islet grafts in the group of mice treated with APC and those of the control group, the whole liver was cut into 2 mm thin slices, fixed with Bouin's solution and embedded in paraffin. The embedded specimen was sliced from the bottom of the case into fifty continuous 5μ m thick sections. The sections were stained with hematoxylin and eosin HE and for insulin DAKO Co., Carpinteria, CA immunohistochemically. The number of islets in the first and last sections were counted to avoid counting the same islet graft twice because the transplanted islets ranged from 150 to 250μ m in diameter.

Intraperitoneal glucose tolerance test IPGTT

IPGTT was performed on day 60 after islet transplantation. The mice were fasted for 15 hr prior to the examination. Blood samples were obtained at 0, 30, and 120 min after the intraperitoneal administration of glucose 1.0 g/kg body weight. The plasma glucose was measured as previously described.

Preparation of hepatic mononuclear cells

Hepatic mononuclear cells HMNCs were pre-pared as described previously.[23] In brief, an excised liver was pressed through a stainless steel mesh, then the resulting dissociated liver was suspended in Dulbecco's modified Eagle medium D-MEM/F-12 Life Technologies, Tokyo, Japan and washed in PBS. Next the mixture was resuspended in an isotonic 33 Percoll solution containing heparin 67 U/ml, and centrifuge 2,000× g at 4 for 15 min. The resulting pellet was resuspended in 0.83 ammonium chloride solution to lyse the erythrocytes. After counting, these HMNCs were then washed twice in PBS and used for further analyses.

Antibodies and a flow cytometry analysis

The antibodies Abs used for the flow cytometry analysis were Fc block anti-mouse FcR II/III mAb, 2.4G2, Phycoerythrin PE -conjugated anti-mouse-CD11b mAb clone M1/70, Integrin-M chain, Mac-1 chain, Rat IgG2b, , Peridinin chlorophyll protein PerCP -Cy5.5-conjugated Rat anti-mouse Ly-6G and Ly-6C Gr-1 mAb clone RB6-8C5, Rat IgG2b, , Allophycocyanin APC -conjugated anti-mouse-IFN- mAb clone XMG 1.2, Rat IgG1, anti-mouse-TNF- mAb clone MP6-XT22, RatvIgG1, and their isotype control clone R3-34, Rat IgG1 were purchased from PharMingen San Diego, CA. For intracellular staining, the cells were incubated with blocking FcR II/III mAb, fixed and permeabilized by Cytofix/Cytoperm solution PharMingen, and stained with anti-IFN-, anti-TNF-, and their isotype control according to the manufacturer's instruction. The stained cells were analyzed on a FACSCalibur Becton Dickinson, Mountain View, CA and the data were processed by the CELLQuest software program Becton Dickinson. Ten thousands viable HMNCs were collected for Dot-Plot to analyze function of each cell population.

Statistical analysis

Data are presented as the mean± SE. Differences between the groups were analyzed by Mann-Whitney's U-test and Fisher's exact probability test. The differences were considered significant when the p values were less than 0.05.

Results

The beneficial effects of APC on amelioration of hyperglycemia in STZ-diabetic mice receiving 200 syngenic islets into the liver from a single donor

As shown previously,[24] hyperglycemica of STZ-diabetic mice was ameliorated by transplantation of 400 but not 200 syngenic islets isolated from a single mouse pancreas into the liver. Thus, all mice n 8 receiving intrahepatic 200 islets and treated with saline remained hyperglycemic at 60 days after transplantation Figure 1A . In marked contrast, all of the recipient mice treated with three 40μ g APC injections 0, 2, and 4 hr after transplantation became normoglycemic at 5.4 ± 1.4 days n 7 after transplantation Figure 1B . To determine whether this effect of APC was mediated by EPCR, the mice receiving islet grafts

were injected 100μ g of anti-EPCR antibody intraperitoneally on the day before transplantation and treated with 3 APC injections. Anti-EPCR antibody completely abolished the effect of APC, and all mice n 3 remained hyperglycemic on day 60 after transplantation Figure 1C . The administration of control antibody rat IgG2a did not affect the effect of APC treatment, and all mice n 4 treated with APC combined with control antibody became normoglycemic at 7.0± 2.4 days after transplantation Figure 1D .

Morphology at 6 and 24 hr after transplantation

The livers bearing the islet grafts were examined at 6 or 24 hr after transplantation to analyze the effect of APC. In the liver of the mice in the control group, the hepatocytes surrounding the islet grafts degenerated in a wide area because of an inflammatory reaction caused by the islet grafts.
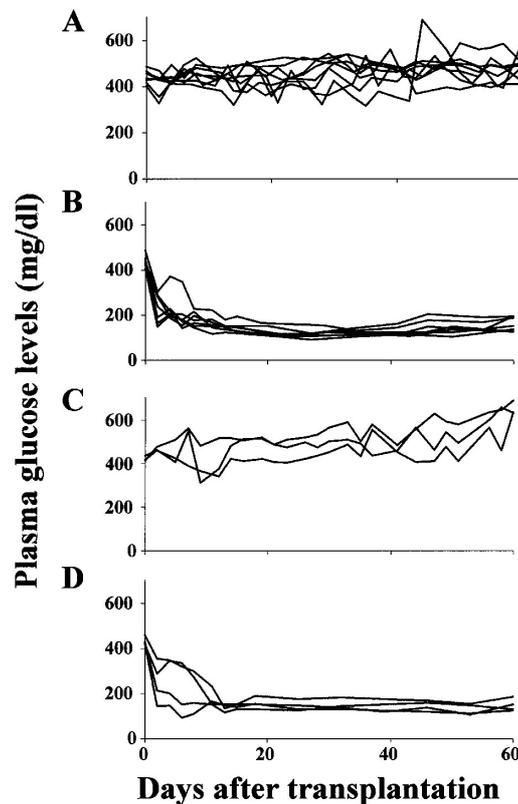

Figure 1. The plasma glucose levels after the transplantation of 200 syngeneic islets
A Saline, B APC, C APC Anti-EPCR antibody, D APC Control antibody
The individual line represents the plasma glucose levels of each animal.

Most of the nuclei of the islet cells also degenerated and showed picnosis. In the APC treated group, the degeneration of hepatocytes was prevented, although more islets were observed in the livers than in the control group. In addition, most of the islet cells nuclei were intact in the APC treated group Figure 2A . At both 6 and 24 hr after transplantation, more islet grafts were detected in the livers of the APC group than the control group 13.3± 4.7 islets/preparation vs. 7.7± 2.4 islets/preparation at 6 hr, 10.0± 1.5 vs. 3.8± 0.4 at 24 hr n 6 . The difference was statistically sig-

nificant at 24 hr after transplantation Figure 2B .

IPGTT on day 60 after transplantation

To evaluate the function of islet grafts in the liver of recipient mice, IPGTT was performed on day 60 after transplantation. The results are summarized in Figure 3. The plasma glucose levels of naive untreated C57BL/6 mice n 4 were 62.0± 1.1, 464.0± 15.3 and 178.0± 7.5 mg/dl at 0, 30 and 120 minutes, respectively, after the intraperitoneally injection of 1.0 g/kg glucose white circle , and those of the STZ-induced diabetic mice without
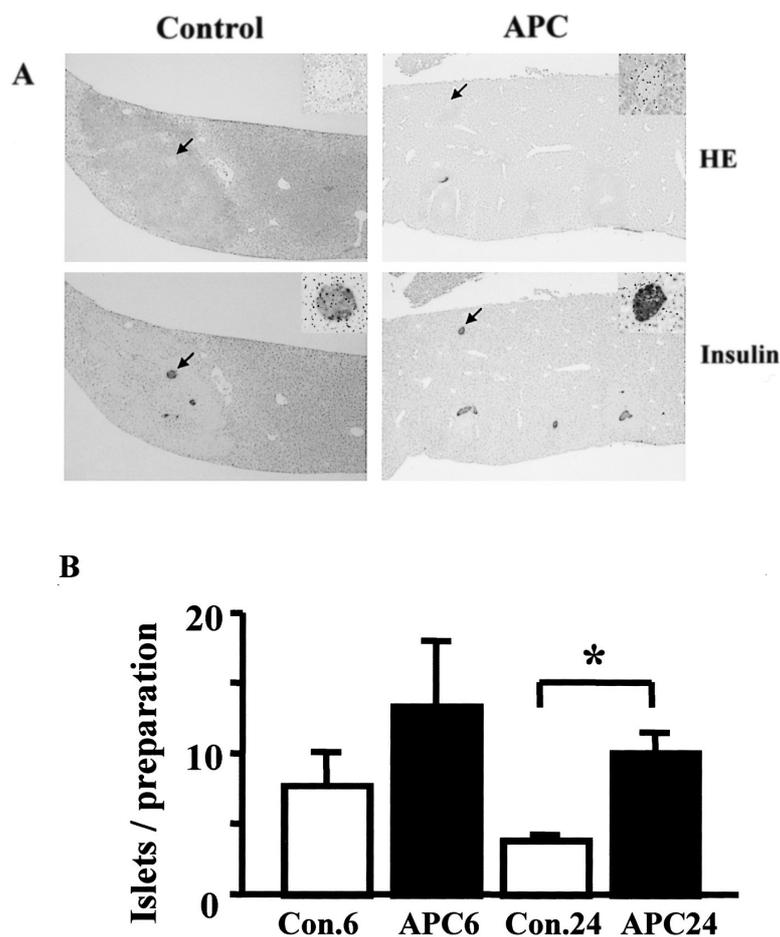

Figure 2. Beneficial effects of APC on engraftment of islets in the liver
A Photomicrography of the mouse liver receiving islets and treated with saline left column or APC right column .
Islet grafts indicated by arrows are magnified in right upper part. The sections were stained with hematoxylin and eosin upper panel and immunohitochemically for insulin lower panel . Original magnification × 40
B The number of islet grafts detected in the liver of mice after transplantation.
At both 6 and 24 hr after transplantation, more islet grafts were detected in the liver of mice treated with APC in comparison to control mice. The difference is statistically significant at 24 hr after transplantation n 6, p 0.01, Mann-Whitney's U-test .
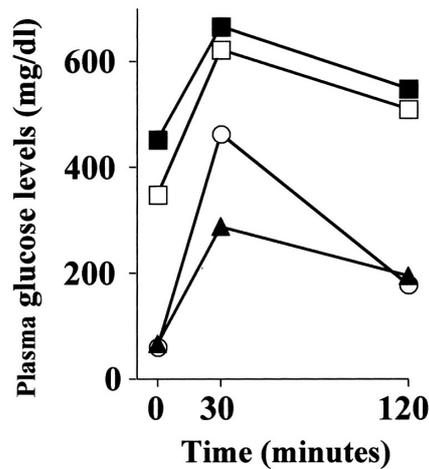
Figure 3. Intraperitoneal glucose tolerance test IPGTT
Intraperitoneal glucose tolerance test IPGTT in STZ-induced dia-
betic mice was performed at 60 days after islet transplantation.
Mice were fasted for 15 hours prior to IPGTT and glucose 1.0g/kg
was injected intraperitoneally. Blood samples were taken from the
orbital sinuses at 0, 30 and 120 minutes after the glucose injection.
Experimental groups include diabetic mice without islet transplanta-
tion black square, n 5 , those receiving 200 islets and treated with
saline white square, n 8 or APC black triangle, n 7 . Age-
matched naive untreated mice white circle, n 4 served as controls.

islet transplantation n 5 on day 60 after the
injection of STZ were 454.4± 19.6, 667.6± 7.9 and
549.0± 7.0mg/dl, respectively black square . The
plasma glucose levels of diabetic mice n 8 that
received 200 islets and were treated with saline
were 349.1± 23.3, 622.8± 31.5 and 509.6± 32.7mg/
dl, respectively white square . The difference in
the plasma glucose levels at 0 minute between the
diabetic mice without islet transplantation and the
mice the received 200 islets and were treated with
saline was statistically significant, but the differ-
ences at 30 and 120 minutes were not statistically
significant. On the other hand, the plasma glu-
cose levels of the normoglycemic mice n 7 that
received 200 islets and were treated with 40μ g APC
 0, 2, and 4 hr after transplantation were 65.1±
10.6, 289.0± 13.1 and 196.3± 13.2mg/dl black tri-
angle . The differences in the values at each time
point between the mice receiving 200 syngeneic is-
lets and treated with saline white square and
the mice treated with APC after transplantation
 black triangle were statistically significant.

**APC down-regulates proinflammatory cyto-
kine productions of HMNCs in mice receiving 200
islet grafts**

A flow cytometry analysis was performed to ex-
amine the effects of APC. This analysis disclosed
that increased numbers of Gr-1 CD11b cells accu-
mulated in the liver of both the control and APC
treated mice with a peak at 6 hr after islet trans-
plantation in comparison to naive mice 21.3
naive mice vs. 81.6 in control mice and 80.5 in
APC treated mice . The production of IFN- and
TNF- in Gr-1 or CD11b cells in the mice of the
APC treated group was found to be remarkably
down-regulated without affecting the accumula-
tion of these cells in the liver. Representative
data of 2 to 3 experiments are shown Figure 4 .

Discussion

Franklin *et al.*[25] described the sequential morpho-
logical changes of intrahepatic islet grafts in ro-
dent models over both brief and longer periods.
Islets and non-islet tissues were found lodged in
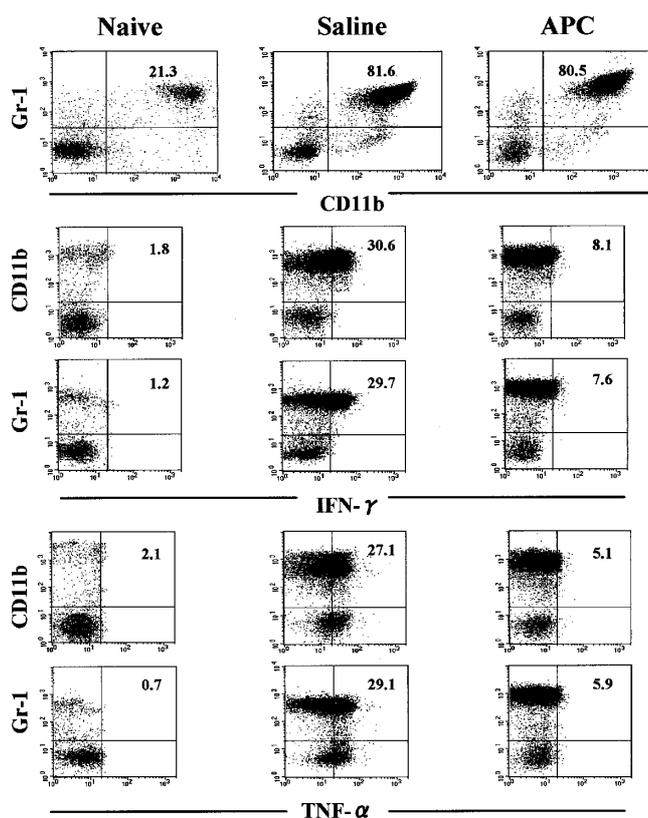the peripheries of the portal system and associated

Figure 4 A flow cytometry analysis of the hepatic mononuclear cells HMNCs in mice receiving 200 syngenic islets

Mononuclear cells in the liver of naive mice left panel were isolated and examined by flow cytometry. Mononuclear cells in the liver of mice receiving islets and treated with saline middle panel or APC right panel were isolated at 6 hours after transplantation and examined by flow cytometry. The figures show the percentage of the cells in the corresponding area. Representative data of 2 to 3 experiments are shown.

with thrombus formation and necrosis of adjacent hepatocytes one day after transplantation. Korsgren and coworkers noted that a thrombotic reaction occurred when purified human islets were exposed to non-anticoagulated ABO-compatible blood in surface-heparinized polyvinyl chloride tubing loops.[26][27] The same reaction occurred when porcine allogeneic islets were transplanted to the liver of another pig. They termed this thrombus formation after intrahepatic islet transplantation an instant blood-mediated inflammatory reaction IBMIR . The effects of IBMIR caused a disruption of islet graft morphology entrapped within a thrombus. On the other hand, in the first step of blood coagulation, tissue factor TF is expressed on endothelial cells and monocytes triggered by various stimulations such as endotoxin and the in-

flammatory cytokines.[28][29] In addition, isolated islets produced TF and this production triggered IBMIR and enhanced the destruction of islet grafts.[30][31] In a preliminary study, TF was expressed in islets transplanted in the liver, and APC treatment prevented this TF expression. From their nuclear shape and insulin negative character, most of the TF expressing cells in the transplanted islets were MNCs infiltrating into grafted islets data not shown . Gr-1 CD11b cells generated by transplantation and their IFN- production triggered by V 14 NKT cells are an essential component and a major cause of early graft loss following islet transplantation.[32] In this study, APC remarkably down-regulated the inflammatory cytokine production, including IFN- and TNF- , by Gr-1 CD11b HMNCs after intrahe-

patic islet transplantation. The fact that APC has both anti-inflammatory and anti-coagulation effects is very important in preventing the loss of intrahepatic islet grafts. In clinical settings, heparin is used to inhibit portal vein thrombosis after islet transplantation.[1-6] Heparin improves the engraftment of intrahepatic islet grafts.[26] However, in the current model, the beneficial effect of heparin was unclear and inferior to that of APC data not shown . APC is now used clinically in the treatment of deep vein thrombosis and acute pulmonary embolism in patients with congenital protein C or S deficiency. APC also shows significant therapeutic effects in the treatment of disseminated intravascular coagulation DIC , with less risk of bleeding than heparin.[33] For the application of APC in clinical islet transplantation, it is very important that the use of APC is associated with a low risk of bleeding because bleeding is one of the major complications in clinical islet transplantation.[1-6]

Interestingly, APC remarkably inhibited the production of IFN- and TNF- in Gr-1 CD11b MNCs without affecting the accumulation of these cells in the liver. Migration of MNCs into the liver and inflammatory cytokine production by these cells may be controlled by different mechanisms. Another possibility is that some populations of Gr-1 CD11b MNCs in the livers of APC treated mice had an anti-inflammatory effect instead of producing inflammatory cytokines. In fact, Gr- CD11b MNCs are recognized as myeloid suppressor cells in tumor metastasis models.[34 35] They produce TGF- and inhibit the cytotoxic T lymphocyte activity. Further studies are required to fully elucidate the function and role of Gr-1 CD11b MNCs in transplant models.

In this study, the hepatocytes surrounding islet grafts in a wide area and most of the islet cells were degenerated in the livers of the control mice at 24 hr after islet transplantation. In contrast, most of islet cells were intact and the regions of degenerated hepatocytes were relatively small in the livers of the APC treated mice. The cytoprotective effects of APC may contribute this result. Contreras et al. also has reported that recombinant murine APC inhibits the apoptosis of syngeneic islet grafts transplanted into the liver in rodent models.[36] In their paper, APC also reduced IL-1 and TNF- mRNA expression in the liver of the recipient mice.

In summary, this study demonstrated that APC remarkably down-regulated the production of inflammatory cytokines of accumulating Gr-1 CD11b cells in the liver of mice receiving islets facilitating to prevent early loss of transplanted islets. APC may play a key role in improvement of the efficiency of islet transplantation, restoring insulin independence after islet transplantation from one donor to one recipient in a clinical setting.

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