Pretreatment of donor islets with papain improves allograft survival without systemic immunosuppression in mice

Kenjiro Kumano MD[1,2], Hitomi Nishinakamura PhD[1], Toshiyuki Mera MD, PhD[3],

Takeshi Itoh MD, PhD[1,4], Hiroyuki Takahashi MD[1,3], Toshiyoshi Fujiwara MD, PhD[2],

Shohta Kodama MD, PhD[1,4,#]

[1]Department of Regenerative Medicine and Transplantation, Faculty of Medicine, Fukuoka University, Fukuoka, Japan.

[2]Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

[3]Department of Gastroenterological Surgery, Faculty of Medicine, Fukuoka University, Fukuoka, Japan.

[4]Center for Regenerative Medicine, Fukuoka University Hospital, Fukuoka, Japan.

[#]**Correspondence information** Shohta Kodama, M.D., Ph.D.7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan Tel +81-92-801-1011, Ext 3631, Fax +81-92-801-1019 E-mail: skodama@fukuoka-u.ac.jp

Abstract

Although current immunosuppression protocols improve the efficacy of clinical allogenic islet transplantation, T cell-mediated allorejection remains unresolved, and major histocompatibility complexes (MHCs) play a crucial role in this process. Papain,

a cysteine protease, has the unique ability to cleave the extracellular domain of the MHC class I structure. We hypothesized that pretreatment of donor islets with papain would diminish the expression of MHC class I on islets, reducing allograft immunogenicity and contributing to prolongation of islet allograft survival. BALB/c islets pretreated with papain were transplanted into C57BL/6J mice as an acute allorejection model. Treatment with 1 mg/mL papain significantly prolonged islet allograft survival. *In vitro*, to determine the inhibitory effect on T cell-mediated alloreactions, we performed lymphocyte proliferation assays and mixed lymphocyte reactions. Host T cell activation against allogenic islet cells was remarkably suppressed by pretreatment of donor islet cells with 10 mg/mL papain. Flow cytometric analysis was also performed to investigate the effect of papain treatment on the expression of MHC class I on islets. One or 10 mg/mL papain treatment reduced MHC class I expression on the islet cell surface. Pretreatment of donor islets with papain suppresses MHC class I-mediated allograft rejection in mice and contributes to prolongation of islet allograft survival without administration of systemic immunosuppressants. These results suggest that pretreatment of human donor islets with papain may reduce the immunogenicity of the donor islets and minimize the dosage of systemic immunosuppressants required in a clinical setting.

Keywords

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Introduction

Clinical allogenic islet transplantation has become an accepted procedure for treatment of type 1 diabetes mellitus.[1-3] Furthermore, current combinatorial therapy by T cell depletion and anti-tumor necrosis factor-α antibody treatment has certainly enhanced graft survival.[4] However, functional decline and graft loss remain barriers for successful islet transplantation. Furthermore, the required doses of systemic immunosuppressive drugs should be decreased as low as possible for reducing side effects such as infection and oncogenesis.[5-7]

Allorejection caused by T cell-mediated immune reactions is one of the major problems that lead to islet graft loss. There is no sufficient protocol to control allorejection to date.[6] Alloreaction occurs through major histocompatibility complex (MHC) class I and II molecules that are both expressed on the cell surface. MHC class I molecules are expressed on whole nucleated cells of the body, whereas MHC class II molecules are only expressed on the surface of certain types of immune cells, including macrophages and dendritic cells, which are known as professional antigen-presenting cells. Host CD8^{+} T cells recognize allo-MHC class I-peptide complexes as alloantigens through T cell receptors, while host CD4^{+} T cells recognize allo-MHC class II similarly.

The mechanism of allorecognition is divided into three pathways including direct,

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indirect and semidirect.[8-11] Among the three pathways, the direct pathway, which involves both allo-MHC class I and II antigens, plays a crucial role in acute alloreactions.[12] Host CD8^{+} T cells, the main effector cells of this phase, recognize allo-MHC class I molecules on grafts and elicit a destructive immune response.[13] Makhlouf et al. showed that MHC class I-CD8^{+} T cell-mediated reactions are more involved in islet allograft rejection than MHC class II-CD4^{+} T cell reactions in C57BL/6 mice.[14] Markmann et al. demonstrated a significant improvement of allograft survival in mice with MHC class I-deficient islets induced by gene disruption.[15] Moreover, some donor MHC class I-removal strategies have shown successful outcomes in cellular transplantation such as insulin-secreting islets of Langerhans and fetal pig neuron transplants in animal models.[16-23] In the present study, we explored a more applicable method to reduce MHC class I expression on islets.

Papain is a sulfhydryl protease isolated from the surface of the green fruit of papaya, which is known for its unique ability to cleave the extracellular domain of the MHC class I heavy chain.[24-26] Galati et al. showed a papain dose-dependent removal effect on MHC class I from mouse lymphocytes *in vitro*.[27] Moreover, Mera et al. reported that papain cleaves human leukocyte antigen class I from human lymphocytes in a dose-dependent manner and inhibits mixed lymphocyte reactions (MLRs) by reducing immunogenicity *in vitro*.[28] From these back grounds, we hypothesized that

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pretreatment of donor islets with papain would diminish the expression of MHC class I on islets, reducing allograft immunogenicity and contributing to prolongation of islet allograft survival in mice.

Results

Papain pretreatment prolongs allograft survival of islet transplantation

We initially investigated the effects of papain pretreatment of islets on allograft survival in an acute rejection model by transplanting BALB/c islets into C57BL/6J mice. As shown in Figure 1A and 1B, the 1 mg/mL papain treatment group showed the longest engraftment among the four groups ($p<0.001$). The median survival times (MSTs) of 0 (n=10), 0.1 (n=10), 1 (n=11), and 10 mg/mL (n=5) papain groups were 19, 19, 26, and 12 days, respectively (Table 1). A significant difference in MST was observed between 0 and 1 mg/mL papain treatment groups ($p=0.0088$). Moreover, the mean graft survival of 0 and 1 mg/mL papain groups was 20.4±2.0 and 54.2±13.4 days (mean±SE), respectively ($p<0.05$). Two of 10 mice in the 0.1 mg/mL papain treatment group and 3 of 11 mice in the 1 mg/mL papain treatment group remained normoglycemic until 120 days after transplantation. The 0.1 mg/mL papain treatment showed a higher graft survival rate than the 0 mg/mL papain control, but the MST was not prolonged.

Nephrectomy was performed on the recipient mice at 120 days after transplantation.

After nephrectomy, all mice became hyperglycemic, indicating that the normoglycemia was maintained by the islet grafts (Figure 1A).

Histological examinations revealed intact islets in the 1 mg/mL papain treatment group at up to 120 days after transplantation. In contrast, severe infiltration of mononuclear cells and degenerated islets were observed in the 0 mg/mL papain group at 14 days after transplantation (Figure 1C). Few Foxp3-positive regulatory T cells (Tregs) were observed in both 0 and 1 mg/mL papain groups (Figure 1C).

Papain treatment of islet cells inhibits allogenic lymphocyte proliferation *in vitro*

To investigate the effects of papain pretreatment of BALB/c islets on C57BL/6J lymphocyte proliferation *in vitro*, we assessed lymphocyte proliferation by MTT assays. Single islet cells derived from BALB/c mice as stimulators were pretreated with papain. As shown in Figure 2, the proliferation of C57BL/6J lymphocytes against BALB/c islet cells pretreated with 10 mg/mL papain was significantly inhibited compared with 0 mg/mL papain at 24–120 h. However, there was no significant difference in lymphocyte proliferation between 0 and 1 mg/mL papain groups. These results indicated that treatment with 10 mg/mL papain inhibited lymphocyte activation against allogenic islet cells *in vitro*.

Papain treatment removes MHC class I molecules from islet cells

Previously, Mera et al. had reported that papain diminishes human leukocyte antigen class I molecules on the surface of human lymphocytes in a dose-dependent manner *in vitro.*[28] To investigate whether papain performs cleavage of MHC class I on BALB/c mouse islet cells *in vitro*, we analyzed the expression of MHC class I by flow cytometric analysis. As shown in Figure 3, the proportions of MHC class I-positive islet cells treated with 0, 0.1, 1, or 10 mg/mL papain were 53.0, 55.4 (data not shown), 43.5, and 39.9%, respectively. These results revealed a decrease in MHC class I expression on the surface of dispersed islets in a papain dose-dependent manner *in vitro*.

Papain treatment inhibits MLRs *in vitro*

To confirm the results of lymphocyte proliferation against allo-islets (Figure 2), we performed an MLR assay. BALB/c lymphocytes as stimulator cells were pretreated with papain to remove MHC class I before co-culture with responder T cells (C57BL/6J). The proliferation of responder CD3$^+$ T cell was measured by flow cytometric analysis of CFSE with the decay of CFSE reflecting T cell proliferation. Briefly, the parent population is represented by a single peak (black). The cells proliferating from the parent population, i.e. the daughter population, are represented by three other linear histograms. Thus, the number of daughter generations as well as

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the number of cells in each peak represent the proliferative activity. As shown in Figure 4A, the 0 mg/mL papain group showed a normal active proliferative response in the MLR. In contrast, the MLR response was remarkably inhibited by 10 mg/mL papain pretreatment of the stimulator cells between 24 and 96 h of culture. MTT assays were also performed at 72 h of culture to quantitatively evaluate the MLR. As a result, the proliferation of T cells was significantly inhibited by 10 mg/mL papain treatment compared with the 0 mg/mL papain group (Figure 4B), which corresponds to the results of flow cytometry. These results indicated that 10 mg/mL papain treatment suppressed the T cell alloresponse.

Papain pretreatment has no negative effects on islet viability *in vitro*

Finally, we analyzed the effect of papain treatment on islet viability. As shown in Figure 5A, there were few annexin V-positive islet cells treated with any papain concentration. Moreover, the PI-positive rate of islets treated with 0, 0.1, 1, or 10 mg/mL papain was 98±1.95, 99±0.47, 99±0.27, and 98±0.7%, respectively (Figure 5B). These results demonstrated that papain pretreatment has no negative effects on islet viability.

Discussion

Papain treatment has a long history as a useful analysis method of MHC class I structure and immunogenicity because of its well-known ability to cleave the MHC

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class I heavy chain.[26, 29-31] However, there has been no reports of allogenic cellular or organ transplantation using papain in animal models. Thus, it remains unclear how papain treatment to remove donor MHC class I on islet affects the outcome of allo-islet transplantation from the viewpoint of immunogenicity, especially alloreactions. In the present study, we demonstrated for the first time that papain treatment improves the outcomes of allo-islet transplantation, and revealed the inhibitory mechanism against the direct pathway involved in MHC class I-T cell-mediated alloreactions in mice.

The results of this study clearly showed that donor islets pretreated with 1 mg/mL papain had significantly prolonged allograft survival in mice. More than 35% of the 1 mg/mL papain-treated group showed graft survival for more 60 days. Furthermore, three of 11 mice in the 1 mg/mL papain-treated group showed remarkably longer allograft survival than 120 days after transplantation without immunosuppressive treatment. We assumed that these recipient mice might have completed immune tolerance at some point after transplantation. Generally, Tregs play an important role in controlling alloimmune tolerance, and some Treg-inducing strategies have achieved successful islet transplantation outcomes.[32-35] Hence, we investigated the proportions of Tregs to mononuclear cells infiltrating into the graft site at day 120 (1 mg/mL papain group) in comparison with grafts at day 14 of 0 and 1 mg/mL papain

groups by Foxp3 (Treg marker) staining. However, unexpectedly, there was no difference between the grafts at day 120 and 14. As a result, we concluded that papain treatment has little effect on Treg induction (Figure 1). However, our *in vivo* results are quite similar to those of Markmann et al. who reported preparation of MHC class I-deficient islets by gene disruption, although the recipient mice were BALB/c.[15] Thus, a strategy using papain may be comparable to a gene-targeting strategy.

In vitro, we revealed that both 1 and 10 mg/mL papain treatment reduced MHC class I on the islet cell surface by flow cytometric analysis (Figure 3). Fifty-three percent of dispersed islet cells were positive for MHC class I, and 1 or 10 mg/mL papain treatment decreased MHC class I expression by 9.5 and 13.1%, respectively, compared with the control. These results suggested that cleavage of MHC class I on the islet cell surface by papain treatment is partial and temporary, because MHC class I may be re-expressed. Thus, papain pretreatment with systemic immunosuppression is thought to be an attractive therapy to reduce the use of systemic immunosuppressants.

The lymphocyte proliferation assay to determine the effect of papain on alloreactions showed that host lymphocyte activation against allogenic islet cells was significantly suppressed by 10 mg/mL papain treatment (Figure 2). Furthermore, we confirmed that CD3^{+} T cell proliferation in MLRs was remarkably suppressed by 10 mg/mL

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papain treatment (Figure 4). These results indicate that the decrease in MHC class I expression by papain treatment leads to suppression of T cell-mediated allorejection in mice.

Interestingly, only pretreatment of donor islet cells with 10 mg/mL papain inhibited host T cell activation against allogenic islet cells *in vitro*. However, the 10 mg/mL papain group showed the shortest graft survival among the four groups. There are several possible reasons to explain this phenomenon. First, we designed *in vitro* experiments using single islet cells instead of islet clusters, because single cells facilitate analysis of the papain efficacy compared with islet clusters. On the other hand, islet clusters were used for *in vivo* experiments. A form of the islet cells, such as single cells or clusters may affect the effective papain dosage between *in vivo* and *in vitro* conditions. Therefore, both results are acceptable. Second, although 10 mg/mL papain treatment had no negative effects on islet viability *in vitro* (Figure 5), we found quite a few fragmented islets after 10 mg/mL papain treatment. Previously, Williams et al. reported that rat islets treated with papain became frangible and did not improve the outcome of islet transplantation in a syngenic model.[36, 37] Thus, their report supports our results. It is presumed that such a fragmented islet structure may accumulate damage over time and eventually affect islet viability and functions negatively *in vivo*, which would not support long-term engraftment. Third, papain is

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used to induce experimental pulmonary emphysema in animal models, because papain administration causes air space dimensions with destruction and thickening of lung tissue, which are involved in inflammation.[38] Moreover, it has been previously reported that cytokines secreted from transplanted islets cause local inflammation that triggers the innate response and graft failure.[39] Although we confirmed that the islets maintained their viability just after papain treatment *in vitro* (Figure 5), it is possible that islets pretreated with a higher papain concentration cause stronger inflammation by releasing high levels of cytokines, leading to early graft loss. Taking these ideas into consideration, we presume that 1 mg/mL papain is the optimal concentration to balance positive and negative effects of papain in islet transplantation.

To date, some strategies to remove donor MHC class I have had success in cellular transplants including insulin-secreting islet transplants in murine models and even primates.[17-20, 22] Markmann et al. revealed that MHC class I-deficient islets improve allograft survival in mice.[15] Moreover, CD8^{+} T cell-deficient recipients show prolonged survival because they lack MHC class I-CD8^{+} T cell-mediated alloreactions.[20] Additionally, Coffman et al. concluded that genetic manipulations to reduce MHC class I expression may be effective to overcome some effects of MHC incompatibility in kidney transplants.[40] These data strongly support our results and confirm that the

MHC class I system serves as an essential component of the allorejection that is common to both cellular and organ transplantations. Because it is a well-known fact that mammals, including humans, have similar MHC systems, the ultimate goal of this study is practical application to a clinical setting. Thus far, the elimination of MHC antigen expression by gene targeting is not practical as a clinical procedure for islet transplantation in terms of both technical and ethical issues. However, although the effect of papain is somewhat limited, this procedure is much more convenient and worth trialing at the clinical stage. Mera at el. showed that it is possible to maintain the activity of papain at 4 °C, suggesting that targeted enzymatic cleavage of donor MHC class I by papain has potential use in the whole organ transplant as well as tissue transplant setting.[28] We also expect that papain pretreatment of allogenic grafts has the potential to decrease the required doses of systemic immunosuppressants. From this viewpoint, we propose combinatorial therapy with papain pretreatment of islets and low-dose systemic immunosuppressive drugs. Recently, Brooks et al. reported that antibody-mediated allo-rejection was important and caused by antibodies against mismatched donor HLA-A2 (MHC class I) in the recipients after transplantation.[41] They revealed that MHC class I is a crucial antigen not only for cellular immunity but also humoral immunity. Thus, the MHC class I molecule as an immune antigen may have deeper implications going forward.

In conclusion, pretreatment of donor islets with papain suppresses MHC class I-mediated allograft rejection in mice and contributes to the prolongation of islet allograft survival without administrating systemic immunosuppressants. These results suggest that pretreatment of human donor islets with papain may reduce the immunogenicity of the donor islets and minimize the dosage of systemic immunosuppressants required in a clinical setting.

Materials and Methods

Animals

Male BALB/c (H-2^d) and C57BL/6J (H-2^b) mice were purchased from Charles River Japan and CLEA Japan Inc., respectively. All mice were maintained under specific pathogen-free conditions. Recipient C57BL/6J mice were applied to experiments at 10–11 weeks of age. Diabetes was induced in the recipients by intravenous injection of streptozotocin (180 mg/kg body weight) (Sigma-Aldrich Co. S0130). Mice with persistent nonfasting blood glucose levels above 400 mg/dL at 2–3 days after streptozotocin injection were considered diabetic and used as transplant recipients. The experiments were approved by our Institutional Animal Care and Use Committee.

Islet isolation

Donor islets from BALB/c mice were isolated by the static digestion method using Type V collagenase (Sigma-Aldrich Co. C9263) and then separated by centrifugation

on Ficoll-Conray gradients.[42] Islets of 150–250 μm in diameter were hand selected using a Pasteur pipette with the aid of a dissecting microscope. Isolated islets were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific 11885-084) supplemented with 10% fetal bovine serum (GE Healthcare 172012) and 1× Antibiotic-Antimycotic (Thermo Fisher Scientific 15240-062) overnight before transplantation.

Papain preparation

To display papain enzymatic activity from papaya latex (Sigma-Aldrich Co. P4762), two papain activating agents, L-cysteine (Sigma-Aldrich Co. 168149) and EDTA (Sigma-Aldrich Co. EDS) were required. Low glucose (1 g/L) DMEM supplemented with 0.05 M L-cysteine and 0.02 M EDTA, adjusted to pH 8.0 by 5 M NaOH was prepared freshly.[28] Papain has excellent solubility to produce a high concentration solution. Papain at 0 (control), 0.1, 1, and 10 mg/mL was used for islet treatment.

Papain pretreatment and islet transplantation

Recipient mice were divided into four groups: 0, 0.1, 1, and 10 mg/mL papain groups. Prior to islet transplantation, 200 hand-picked islets were pretreated with each papain concentration at 37 °C for 15 min and then transplanted under the left kidney capsule (KC) of recipient mice.

Nonfasting blood glucose levels and body weight were monitored three times a

week in all recipients until 120 days after islet transplantation. Blood glucose was measured using a GlucoCard DIAmeter (Arkray).

Normoglycemia after transplantation was defined as two consecutive blood glucose level readings below 200 mg/dL. Graft rejection was defined as two consecutive hyperglycemias over 400 mg/dL. The first day confirmed as hyperglycemia was defined as the day of rejection. Nephrectomy was performed in recipients with functional grafts at more than 120 days after transplantation to ensure a normal glucose level had been maintained by the islet allograft.

Immunohistochemistry

Islet grafts with the kidney were fixed in 10% formaldehyde, processed, and embedded in paraffin. The sections were cut into 3 µm-thick sections and stained with hematoxylin-eosin (HE) or a Guinea pig anti-mouse insulin antibody (DAKO A056401) and rabbit anti-guinea pig secondary antibody (abcam ab97152) with a Warp Red™ Chromogen Kit (BIOCARE MEDICAL BRR806AH) in the presence of alkaline phosphatase for histochemical detection of insulin localization.[43, 44]

Foxp3 staining was performed using an anti-Mouse/Rat Foxp3 antibody (Affymetrix eBioscience 14-5773-80) visualized by the chromogen diaminobenzidine and counterstaining with hematoxylin.

Lymphocyte proliferation assay

To analyze the activity of host lymphocytes against donor islet cells *in vitro*, BALB/c islet cells with or without papain pretreatment and C57BL/6J lymphocytes were co-cultured as a lymphocyte proliferation assay. For inducing immunological sensitization in recipient C57BL/6J mice, BALB/c islets were transplanted under the KC of the recipient C57BL/6J mice 10 days before harvesting splenocytes, and then the harvested splenocytes were used as responder cells. Donor islets were isolated from BALB/c mice, cultured at 24 °C for 24 h, and then dissociated into single cells. After incubation at 37 °C for 24 h, the dispersed islet cells were treated with 0, 1 or 10 mg/mL papain at 37 °C for 15 min and used as stimulator cells. Then, stimulator cells (1×10^4 cells) and responder cells (5×10^5 cells) were co-cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 55 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin in 96-well V-bottomed plates (total volume: 200 µl at 37 °C and a humidified atmosphere with 5% CO_2). Proliferation was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays as a quantitative analysis using a Cell Counting Kit-8 (DOJINDO MOLECULAR TECHNOLOGIES, INC 347-07621) at 24, 48, 72, 96, and 120 h.

Flow cytometric analysis

Isolated islets were cultured at 24 °C overnight and then dissociated into single cells

by incubation with 0.04% EDTA and dispase I (GODO SHUSEI CO. 386-02271) at 37 °C for 15 min.[45] Dispersed islet cells were incubated at 37 °C overnight and then stained with a phycoerythrin (PE)-anti mouse IgG2aκ antibody (isotype control; BioLegend 400211) or PE anti-mouse H-2K^d/H-2D^d antibody (BioLegend 114708). The cells were analyzed by a BD FACSVerse (BD Biosciences) and FlowJo software (Tree Star Inc.).

MLR

After inducing immunological sensitization, T cells were isolated from the spleens of recipient C57BL/6J mice using nylon wool fiber columns (Polysciences, Inc. 21759-1), incubated with carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen C34554) at 37 °C for 15 min, and then used as responder cells. Spleen cells obtained from naive BALB/c mice were pretreated with 25 µg/mL mitomycin C (Sigma-Aldrich Co. M4287) at 37 °C for 1 h. Then, BALB/c spleen cells were treated with 0, 1, or 10 mg/mL papain at 37 °C for 15 min and used as stimulator cells. The responder cells (C57BL/6J, 1×10^5 cells) were cultured in the presence of stimulator cells (BALB/c, 1×10^5 cells) in RPMI 1640 (Thermo Fisher Scientific 22400-089) supplemented with 10% fetal bovine serum, 55 µM 2-mercaptoethanol (Thermo Fisher Scientific 21985-023) and 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific 15140-122) in 96-well V-bottomed plates (total volume: 200 µl at 37 °C in a

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humidified atmosphere with 5% CO_2) and collected after 24, 48, 72 or 96 h of incubation. A PerCP/Cy5.5-conjugated anti-mouse CD3 antibody (BioLegend 100218) was used to determine CD3^{+} T cells, and then the CD3^{+}-gated cell population was assessed by flow cytometric analysis at 24, 48, 72, and 96 h to detect changes in CFSE fluorescence. MTT assays were also performed as a quantitative analysis of proliferation using the Cell Counting Kit-8 after incubation for 72 h.

Islet viability assay

After culture for 24 h, isolated islets were treated with 0, 0.1, 1 or 10 mg/mL papain at 37 °C for 15 min. The islets were then stained with annexin V-FITC and propidium iodide (PI; TREVIGEN, Inc. 4830-01) to evaluate the number of apoptotic and necrotic cells, together with 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co. D9564) for nuclear staining. Isolated islets treated with 0, 0.1, 1 or 10 mg/mL papain were also stained with Hoechst 33342 and PI, and then the percentage of viable islet cells (viability) at each papain concentration was calculated using an average of ten islets. Viability (%) was calculated by subtracting the PI-positive ratio from 100.

Statistical analysis

Survival data were analyzed using GraphPad Prism 6 statistic software and shown by Kaplan-Meier methods. Islet graft survival between experimental groups was compared using the Log-rank test. All data were statistically assessed by one-way

analysis of variance, followed by the Tukey-Kramer test to compare two groups.

P-values less than 0.05 was considered to be statistically significant.

Abbreviations

MHC   major histocompatibility complex.

MLR   mixed lymphocyte reaction.

MST   median survival time.

MTT   3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

Treg   regulatory T cell.

Acknowledgments

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TABLE1. Graft survival of BALB/c islets pretreated with papain in C57BL/6J

recipients

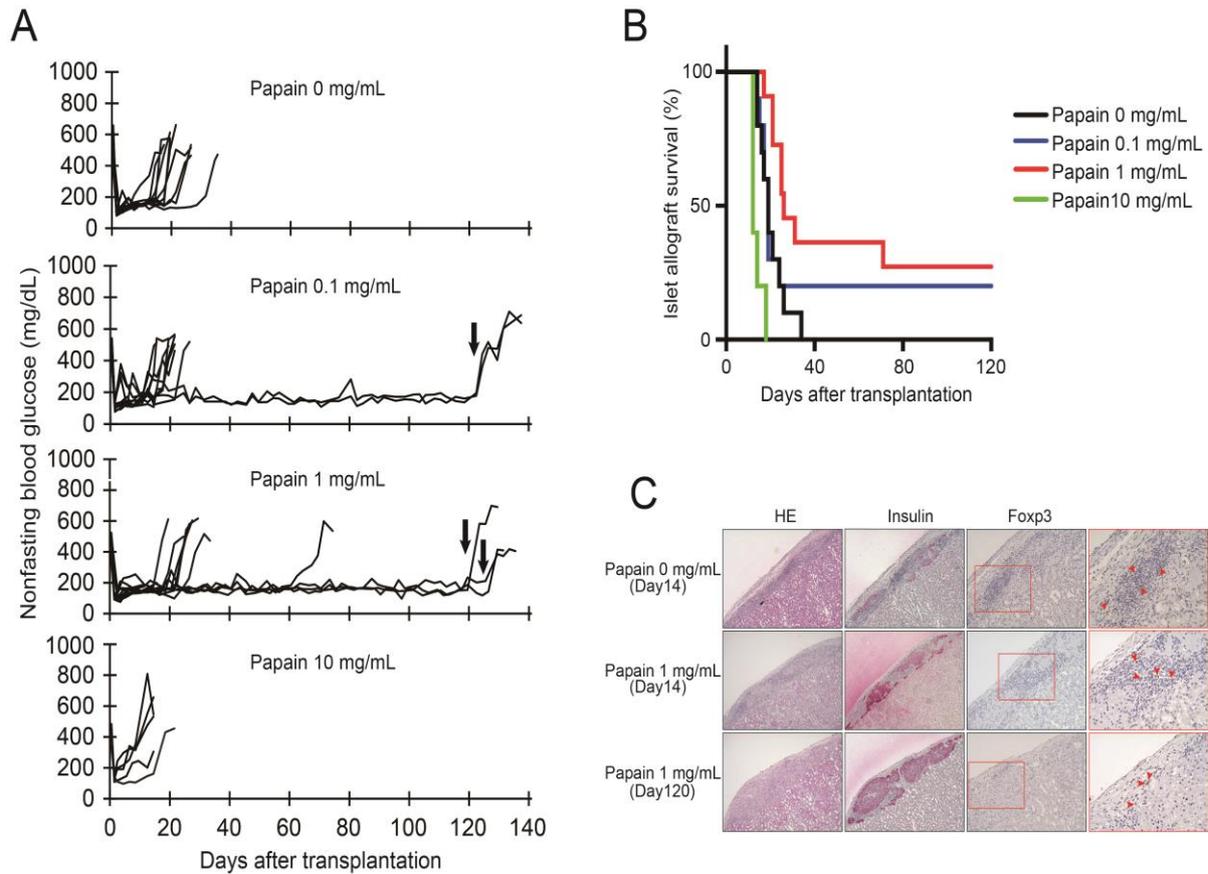| Group	n	Graft survival (days)	MST* (days)
Papain 0 mg/mL	10	14, 14, 16, 17, 19, 19, 21, 24, 26, 34	19
Papain 0.1 mg/mL	10	14, 15, 17, 17, 19, 19, 19, 24, 120<, 120<	19
Papain 1 mg/mL	11	17, 21, 21, 25, 25, 26, 31, 71, 120<, 120 <, 120<	26
Papain 10 mg/mL	5	12, 12, 12, 14, 18	12

*Median survival times

FIGURE 1. Effect of papain pretreatment on islet allograft survival Isolated

BALB/c islets were pretreated with various papain concentrations (0, 0.1, 1, and 10

mg/mL) prior to islet transplantation. Two hundred islets were transplanted under the

left KC. **A.** Individual blood glucose readings for a cohort of papain-pretreated

islet-transplanted C57BL/6J mice. As indicated by the arrow, nephrectomies were

performed on recipient mice that survived up to 120 days after receiving an islet

allograft. **B.** Survival of islet allografts (BALB/c to C57BL/6J) in recipient mice. The

MSTs were calculated and compared using the Kaplan-Meier method. **C.** Histological

analysis of the grafts of 0 and 1 mg/mL papain groups (upper panels: 0 mg/mL papain

(day 14); middle: 1 mg/mL papain (day 14); lower: 1 mg/mL papain (day 120)) Islet

30

grafts were harvested on days 14 or 120 after transplantation. Paraffin-embedded

sections were stained with HE, and anti-insulin and -Foxp3 antibodies. Arrowheads

indicate Foxp3-positive cells. (HE and insulin staining: original magnification, ×100;
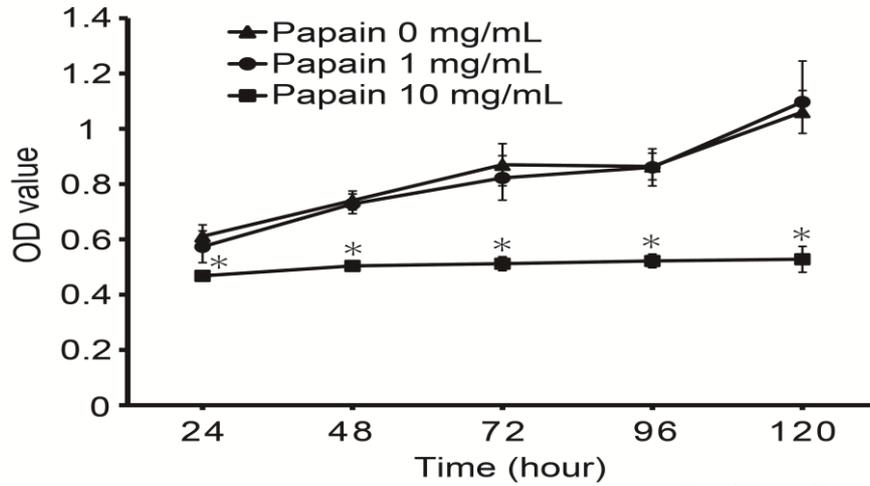
Foxp3 staining: left, ×200 and right, ×400)

FIGURE 2. Effects of papain pretreatment of isolated islets on prevention of lymphocyte proliferation against islet alloantigens Prior to the lymphocyte proliferation assay *in vitro*, BALB/c mouse islets were transplanted into C57BL/6J recipient mice to induce immunological sensitization. Ten days after transplantation, a lymphocyte proliferation assay was used to examine the proliferative response of the recipient spleen-derived lymphocytes to donor single islet cells (BALB/c). Proliferation of lymphocyte was measured by MTT assays after 24, 48, 72, 96, and 120 h of culture. The result of each well is presented as the mean±SD (n=7). The experiment was repeated three times. *p<0.001 compared with the corresponding 0 papain mg/mL group.
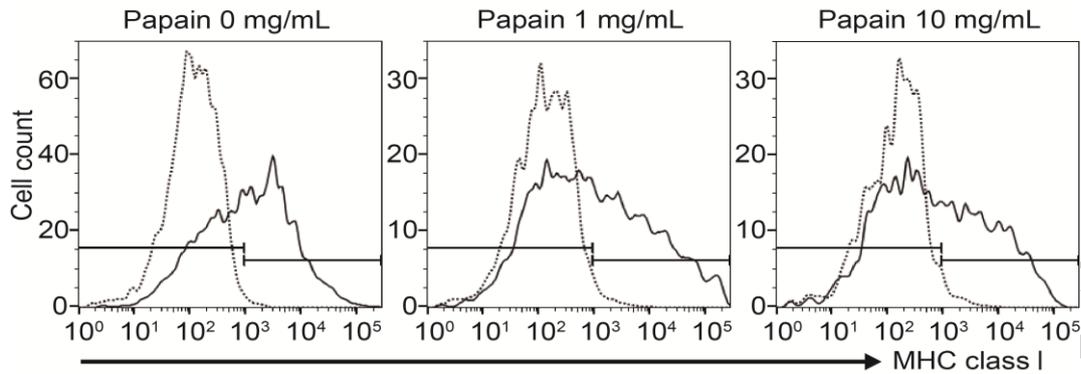
FIGURE 3. MHC class I expression on papain-pretreated islet cells Flow cytometric analysis of MHC class I on papain-treated islet cells. Donor islets (BALB/c) were dissociated into single cells and treated with 0, 1, 10 mg/mL papain at 37 °C for 15 min. Then, the dispersed islet cells were stained for MHC class I. The histograms are indicative of representative data of three separate experiments.
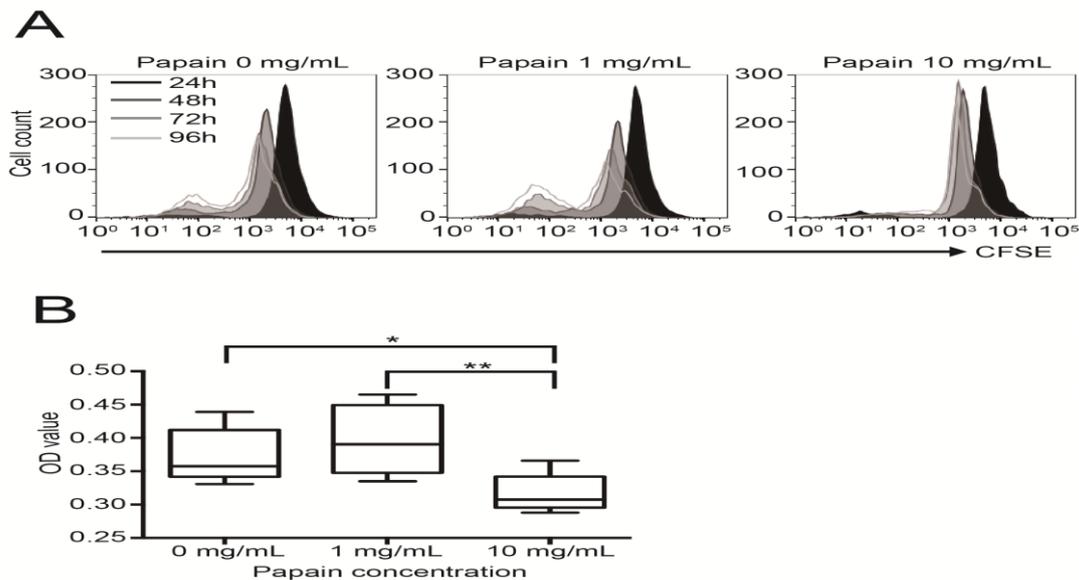
FIGURE 4. Effects of papain pretreatment on the reduction of an MLR Prior to T cell proliferation assays *in vitro*, BALB/c mouse islets were transplanted into C57BL/6J recipient mice to induce immunological sensitization. Ten days after transplantation, MLRs were performed to examine the proliferative response of the recipient spleen-derived T cells to donor-type spleen cells (BALB/c). **A.** Time-dependent change of CD3+ T cell proliferation detected by flow cytometric analysis of CFSE-stained cells. The black shadowed area represents CD3+ T cell proliferation at 24 h, while the three other linear histograms represent the cell proliferation at 48, 72, and 96 h. A representative histogram of three samples is shown. **B.** The MLR was also analyzed by an MTT assay after incubation for 72 h. The result of each well is presented as the mean±SD (n=8). The experiment was repeated three times. *$p<0.05$, **$p<0.01$.
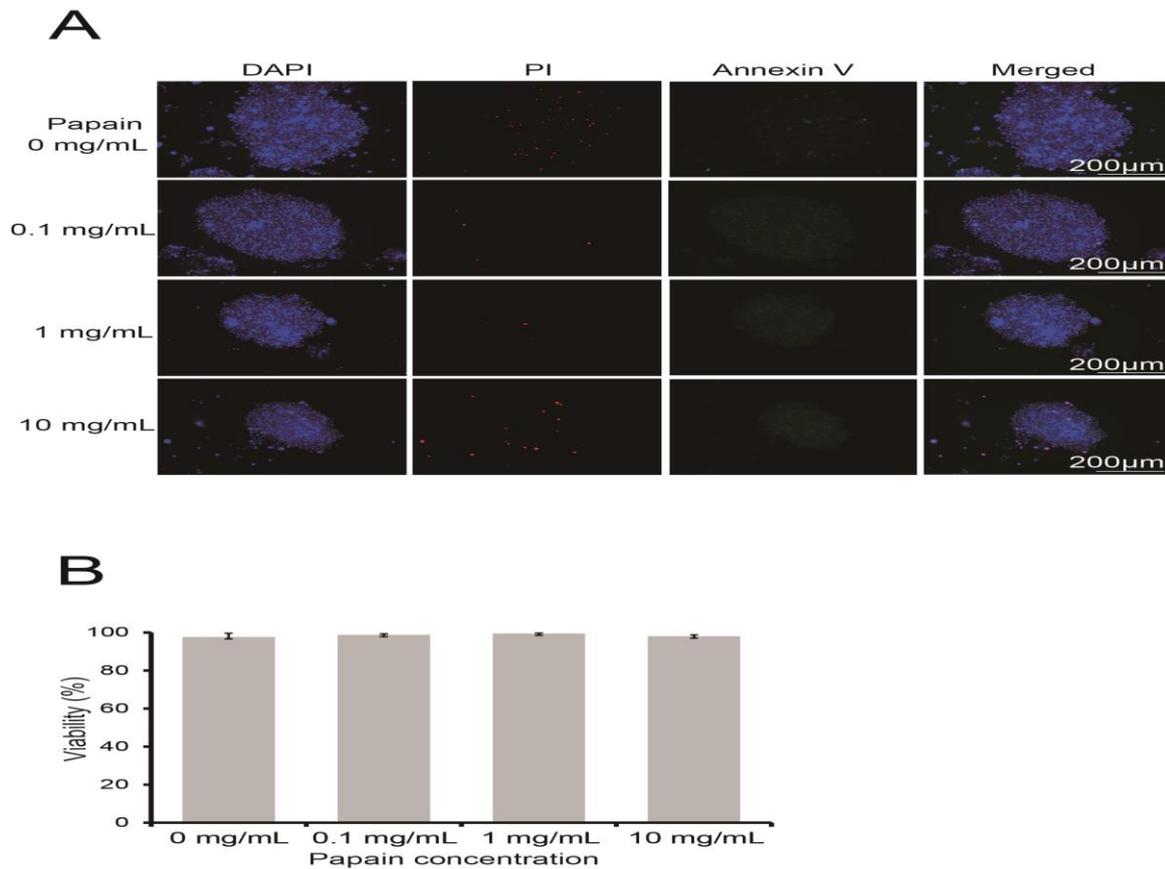
A

B

FIGURE 5. Effect of Papain on islet cell viability Fluorescence photomicrographs of isolated donor islets (BALB/c) treated with 0, 0.1, 1, and 10 mg/mL papain (37 °C, 15 min). **A.** Islets were stained with annexin V-FITC (green), PI (red), and DAPI (blue) to reveal the number of viable, necrotic, and apoptotic cells. **B.** Islets were stained with Hoechst 33342 and PI. The percentage of viable islet cells (viability) in each condition was calculated by the average of ten islets. Viability (%) =100-PI-positive ratio.