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Maintenance of Viability and Function of Rat Islets With the Use of ROCK Inhibitor Y-27632

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The number of patients with diabetes is on an increasing trend, thus leading to the belief that diabetes will be the largest medical problem of the 21st century. Islet transplantation can improve glycometabolic control in patients with type 1 diabetes. We studied the viability of Rho-associated protein kinase (ROCK) inhibitor Y-27632 in a culture system in vitro on freshly isolated rat islets. Islet isolation was conducted on a Lewis rat, and studies of culture solutions were split into two groups, one group using ROCK inhibitor Y-27632, and another without. On the seventh day of culture, we evaluated the differences for the cell morphology, viability, and insulin secretion. The Y-27632 group maintained form better than the group without Y-27632. With strong expression of Bcl-2 observed with the Y-27632 group, and expression suppressed with Bax, inhibition of apoptosis by Y-27632 was confirmed. The Y-27632 group predominantly secreted insulin. For islet transplantation, Y-27632 inhibited cell apoptosis in a graft and was also effective in promoting insulin secretion. We were able to confirm effective morphological and functional culture maintenance by separating islets from a rat and adding ROCK inhibitor Y-27632 to the medium.

Key words: Islet transplantation; Apoptosis; Embryonic stem cells; Induced pluripotent stem cells; Rho-associated protein kinase (ROCK) inhibitor; Y-27632

INTRODUCTION

The number of patients with diabetes is on an increasing trend, and the International Diabetes Federation (IDF) conducted a survey of 212 countries and regions. By October 2009, the number of diabetes patients exceeded 285,000,000, of which 4,870,000 had type 1 diabetes (40% of type 2 diabetes patients are believed to require insulin), and this is expected to increase 1.5 times to 435,000,000 by 2030, thus leading to the belief that diabetes will be the largest medical problem of the 21st century (11). Therefore, measures to fight diabetes are an immediate problem in the medical world. The standard treatment so far for type 1 diabetes has been to conduct intensive insulin therapy, but this has had limited effectiveness. For cases with glycemic control problems or acute hypoglycemia due to side effects and cases associated with chronic renal failure, transplantation procedures such as pancreas or islet transplants have been developed, and their effectiveness has been recognized (11,17–21,23). However, there is an inadequate number of donors for such transplant procedures, and even if the transplant procedure were to be successful, the patients must continue to take medication as part of the immunosuppressive therapy, and various problems occur due to the side effects of the medication, such as carcinogenic effects and compromised immune systems. As an alternative to transplant surgery, we are working on bioartificial pancreas (BAP) development as an innovative, next-generation therapy. Not only clinical medicine but also cell biology and the help of medical engineering are essential for the development of BAP as a practical application. From the results (12,26) of the bioartificial liver development, a whole-blood perfusion type BAP was developed by combining poly(amino acid)-urethane (PAU)-coated high-density polyethylene (HDPE) and ethylene vinyl alcohol (EVAL) hollow fiber, and its usefulness on a pig

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model pancreatectomy resection has been reported (9). Although effective in intensive care unit (ICU) therapy in the acute phase, the development of an implanted, wearable-type BAP rather than an external type is needed when considering the quality of life (QOL) of chronic patients. At present, a bag-type device has been created with a PAU-coated HDPE and an EVAL flat membrane, and satisfactory biocompatibility from implant experiments in the acute phase has been confirmed with animal experiments using rats (32). The device has an immune isolation effect and therefore not only eliminates the need for immunosuppression but could also allow for the use of pancreatic islets from other animals and thus could supplement the shortage of human islet donors. Compared to the operation of transplanting a pancreatic islet to the liver of a living body, the operation of filling the device with living cells will lower the function as well as threaten the lifetime of the cells. In fact, although islets experience various stresses from the separation procedure, there are additional stresses within the device's bag such as the cessation of nutritional factors, oxidative stress, agitation death, and low oxygen, resulting in apoptosis several days after transplantation.

The creation of an environment within the device similar to the living body's natural environment is therefore essential. In order to accomplish this, the integration of extracellular matrix engineering will also be required. So far, we have obtained satisfactory results by applying a certain puramatrix with a self-assembling gel to the islet culture. It is generally known that when cells are stressed during separation and culturing, the activation of Rho is triggered, and the activated Rho causes Rho-associated protein kinase (ROCK) activation. The activated ROCK will phosphorylate the myosin light chain (MLC) and cause cell contraction, thus leading to apoptosis by membrane blebbing (13). To prevent such cell death, and as a basic study on lengthening the lifetime of the cells in BAP devices, we studied the viability of ROCK inhibitor Y-27632 in a culture system using PAU-coated HDPE in vitro on freshly isolated rat islets.

MATERIALS AND METHODS

Isolation and Culture of Rat Islets

Islet isolation was conducted on three 300- to 400-g male Lewis rats (8–15 weeks old, Japan SLC Inc., Sizuoka, Japan). All animal studies were approved by the Institutional Animal Care and Use Committee of Okayama University. All surgical techniques were conducted under inhaled anesthesia of diethyl ether (100 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). An abdominal midline incision was made to the rat's abdomen, the duodenal papilla was clamped, and a 24-gauge plastic cannula (Terumo, Tokyo, Japan) was affixed to the ductus hepaticus communis. Ten milliliters of pre-iced Liberase RI (Roche Diagnostics, Tokyo, Japan) working enzyme solution (a collagenase) was injected, and the pancreas was allowed to swell. After the rat was euthanized by exsanguination, the swollen pancreas was completely removed and immersed in 5 ml of working enzyme solution and placed on ice. Islet isolation was conducted as described previously (18). The dithizone (Sigma-Aldrich)-stained islets were observed, and the number of islets was measured.

The viability of the separated islets was immediately measured. Measurement was conducted according to the instructions of the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies, Carlsbad, CA, USA). Observation was conducted by fluorescence microscope (Olympus, Tokyo, Japan) with green calcein-AM staining depicting living cells and red ethidium homodimer-1 depicting dead cells. Viability was determined by analyzing the area ratio of live/dead cells using the Image J area calculation software (NIH, Bethesda, MD, USA).

Culture Conditions

PAU-coated HDPE (Kuraray, Tokyo, Japan) that serves as a foundation for cells within the bioartificial pancreas was used, and studies of culture solutions were split into two groups, a control group (group 1) and a test group (group 2) using the ROCK inhibitor Y-27632. Glucose (5.5 mM), 10% fetal bovine serum (FBS) (Thermo Scientific, Kanagawa, Japan), and 1% penicillin/streptomycin (Sigma-Aldrich) were added to Roswell Park Memorial Institute medium (RPMI; Life Technologies), and 10 μM Y-27632 (Millipore, Billerica, MA, USA) was added as appropriate; after 24 h, the medium was exchanged for a medium not containing Y-27632. Using a six-well plate (Corning Japan, Tokyo, Japan), each well was seeded with 200 islet equivalents (IEQs) of islets and cultured for 7 days at 37°C and 5% CO_2.

Western Blot

The presence of ROCK proteins in the rat islets was confirmed using a Western blot method. It is believed that ROCK1 exists in all tissue except the prostate gland, and ROCK2 exists in the brain and skeletal muscle (16,24). After three washings with PBS, cells were sonicated (Tomys Seiko Co., LTD, Tokyo, Japan). Then, micrograms of cell extracts were fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Sigma-Aldrich) and transferred to polyvinylidenefluoride membranes (Immun-Blot PVDF Membrane; Bio-Rad, Hercules, CA) using transfer buffer containing 20% methanol, 25 mmol/L Tris base, and 192 mmol/L glycine (300 mA, 2 h; all from Sigma-Aldrich). After blocking at room temperature for 1 h in 50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween-20 [Tris-buffered saline with Tween (TBST); all from Sigma-Aldrich] with 5% nonfat dry milk (Sigma-Aldrich), the membranes were

incubated overnight at 4°C in TBST using 5% nonfat dry milk containing primary antibodies (BD Biosciences, San Jose, CA, USA) of ROCK1 and ROCK2, and then for 1 h at room temperature in TBST with 5% nonfat dry milk containing secondary antibody coupled to horseradish peroxidase (HRP; BD Biosciences). Coomassie Brilliant Blue (CBB) staining (Sigma-Aldrich) was performed to evaluate protein expression.

MTT Assay

MTT assay was used for determination of cell growth rate. Vybrant® MTT Cell Proliferation Assay Kit (Life Technologies Japan, Tokyo, Japan), which provides a simple method for determination of cell number using standard microplate absorbance readers (Bio-Rad) was used in this study. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay involves the conversion of the water-soluble MTT to an insoluble formazan. The formazan is then solubilized, and the concentration is determined by optical density at 570 nm. The result was a sensitive assay with excellent linearity up to approximately 10^6 cells per well.

Assessment of Islet Morphology

On the seventh day of culture, the suspended cells and cells from the nonwoven fabric were extracted as much as possible, and the cell form was evaluated using a phase-contrast microscope (Olympus). In addition, the ultrafine structure was evaluated in the same manner using a scanning electron microscope (SEM) (Hitachi, Tokyo, Japan). For scanning electron microscopy, the samples were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (all from Sigma-Aldrich). The samples were embedded in LR White (London Resin Company, London, UK).

Insulin Secretion

Islet function was assessed by monitoring the insulin secretory response of the islets during glucose stimulation. Briefly, 1,200 IEQs were incubated with either 2.8 or 25 mM glucose in RPMI 1640 for 2 h at 37°C and 5% CO_2. The supernatants were collected and insulin levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). The stimulation index was calculated by determining the ratio of insulin released from islets in high glucose to the insulin released in low glucose. The data were expressed as mean ± SE.

RT-PCR

The evaluation of apoptosis was conducted using RT-PCR methods. B-cell CLL/lymphoma 2 (Bcl-2) and Bcl2-associated X protein (Bax) gene expressions were observed with the RT-PCR method. Bax is a proapoptotic protein, and Bcl-2 is an antiapoptotic protein. The primers used were Bcl-2 sense 5′-AGGATTGTGGCCTTCTTTGAGT, antisense 5′-GCCGGTTCAGGTACTCAGTCAT; Bax sense 5′-CAGCTCTGAACAGATCATGA, antisense 5′-AGCTGCCACACGGAAGAAGA; and GAPDH sense 5′-GGAGTCTACTGGCGTCTTCA, antisense 5′-ATGAGCCCTTCCACGAT.

Transplantation of Islets and Immunostaining

As a transplant experiment, the resected rat islets were cultured with or without Y-27632, and after 24 h, 300 samples were randomly selected and extracted from each group and were transplanted to the renal subcapsule of syngeneic rats (8–15 weeks old, 300–400 g, male Lewis rat, $n=3$). Seven days later the kidneys were extracted and observed. Hematoxylin–eosin (H&E) staining (Sigma-Aldrich) and insulin immunostaining were conducted. For immunostaining, the tissues were blocked with 10% serum and 0.2% Triton X-100 in PBS (all from Sigma-Aldrich) and then incubated with primary antibody to mouse insulin (guinea pig polyclonal antibody to mouse insulin, 1:100, Abcam, Cambridge, UK) overnight at 4°C. Cells were further incubated with secondary antibody (goat polyclonal antibody to guinea pig IgG-H&L, 1:100, Abcam).

Statistics

The two groups were compared by Student's t test. The differences between each group were considered significant if $p<0.05$.

RESULTS

ROCK Expression in Islets and Effect of a ROCK Inhibitor, Y-27632

Islet isolation was conducted on three rats with an average weight of 370 g, and an average of 4,441 IEQs was isolated from each rat with a viability of 97%. ROCK1 and ROCK2 expression was confirmed without regard to the use of Y-27632 (Fig. 1A). We used a 10 μM concentration of Y-27632 for the rat islet culture. Although the optimal concentration of 10 μM is also noted in other articles (6,13), a confirmation that 10 μM is adequate was obtained from an MTT assay that was conducted with a dose–response curve for Y-27632, which achieved a plateau above 10 μM (Fig. 1B).

Scanning Electronic Microscopy

The ultrafine structure was evaluated in the same manner using an SEM. The islet control group showed variance and membrane blebbing (foaming) immediately after culture, and by the seventh day, cells morphed by stretching to the sides, indicating an irregularity in the margins. This membrane blebbing was inhibited in the Y-27632 combined group, and the normal cell form was maintained (Fig. 2).

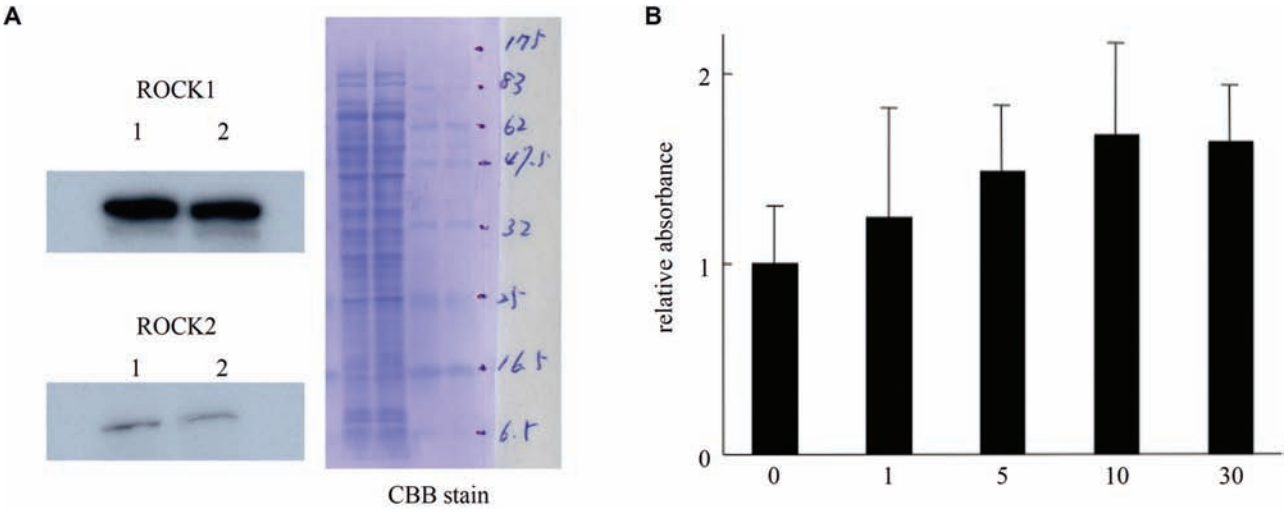


Figure 1. Rho-associated protein kinase (ROCK) expression in islets and effect of a ROCK inhibitor, Y-27632. (A) ROCK expression in islets. ROCK1 and ROCK2 expression was confirmed irrespective of Y-27632. Coomassie Brilliant Blue (CBB) stain showed similar protein expressions between the two groups. (B) Effect of ROCK inhibitor Y-27632. A dose–response curve of different concentrations of Y-27632 confirmed a plateau above 10 μM, suggesting that this concentration is adequate using an MTT assay.

Viability

On the seventh day of each culture group, fluorescence staining was conducted using a live/dead viability cytotoxicity kit, and viability was determined by the area ratio of green areas indicating live cells and red areas indicating dead cells. The viability of the control group was on average 19.9% compared with 68.7% for the Y-27632 group, indicating a significantly better survival rate following Y-27632 treatment (Fig. 3).

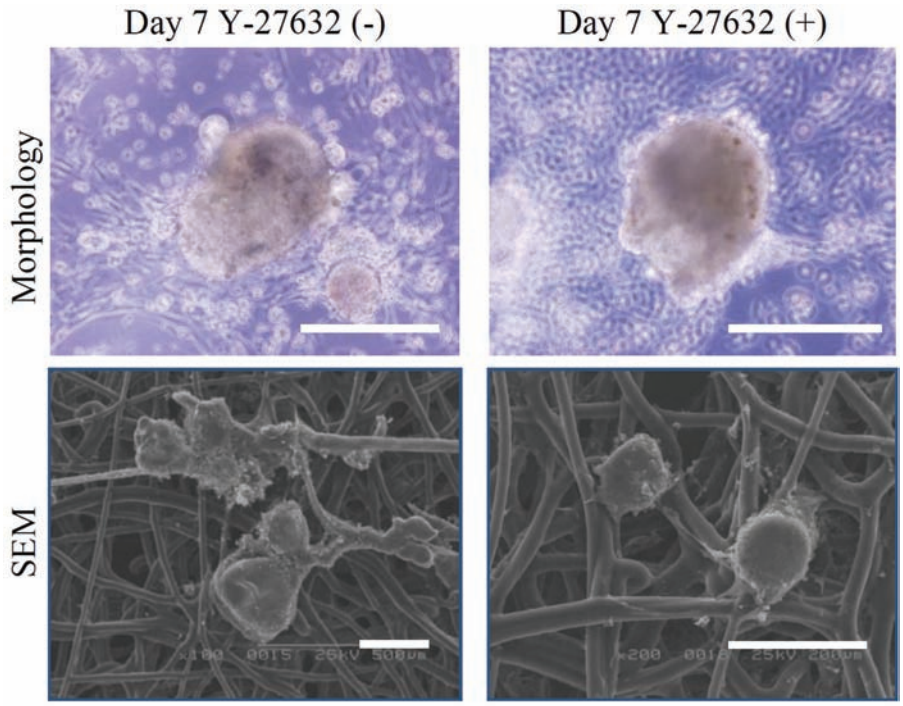


Figure 2. Morphology and electronic microscopy of islets. Morphology and the ultrafine structure was evaluated using a phase-contrast microscope and a scanning electron microscope (SEM). The Y-27632 group maintained form better than the control group with respect to spherical cell morphology, margin, boundary, and size. Scale bars: 200 μm.

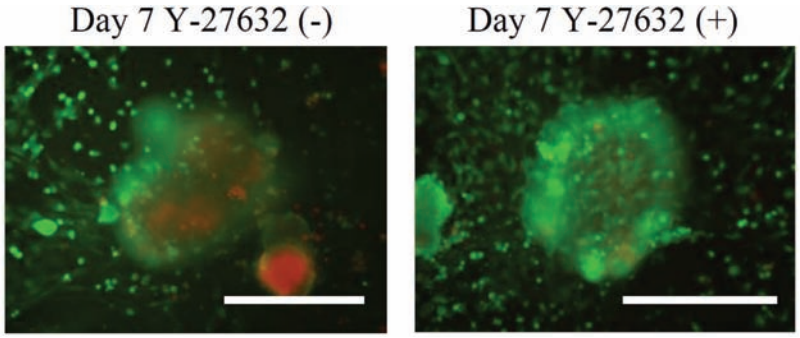


Figure 3. Viability of islets after 7 days in culture. A live/dead viability cytotoxicity kit was used to test the viability of the islets in the absence or presence of Y-27632. Green calcein-AM staining demonstrates living cells, whereas red ethidium homodimer-1 staining indicates dead cells. This demonstrated the survival of cells even after 7 days of culture. Scale bars: 200 μm.

Glucose-Responding Insulin Secretion

On the third day of culture, the 1-h use of low- and high-glucose media allowed glucose stimulation to be assayed using an ELISA kit. The stimulation index was significantly higher in the Y-27632 group than in the control group (control: 2.23 ± 0.30, $n=5$; Y-27632: 3.45 ± 0.17, $n=5$) (Fig. 4). Not only was apoptosis inhibited and cell form maintained, but it was found that Y-27632 was also useful in maintaining insulin secretion function.

Bcl-2 and Bax Expression

The expressions of proapoptotic Bax and antiapoptotic Bcl-2 were studied using RT-PCR as indicator of apoptosis. Bcl-2 was strongly expressed by the Y-27632 group, whereas Bax expression was reduced, suggesting apoptosis inhibition by Y-27632 (Fig. 5). On the other hand, Bax was strongly expressed in the control group, and Bcl-2 expression was inhibited.

Islet Transplantation

Seven days after transplantation of the resected rat islets, the kidney was extracted and the average cell adhesion area of the Y-27632 group was shown to be 8.85%, whereas that of the control group was 1.57%, indicating a predominantly large adhesion area following Y-27632. H&E staining showed that the graft of the nonuse group had sparse and random adhesion compared with consistent thickness and regular adhesion of cells observed after Y-27632. It can be surmised that the transplanted islets of the nonuse group could not maintain a consistent thickness due to apoptosis. Insulin expression was uniform in the Y-27632 group, but was only observed in

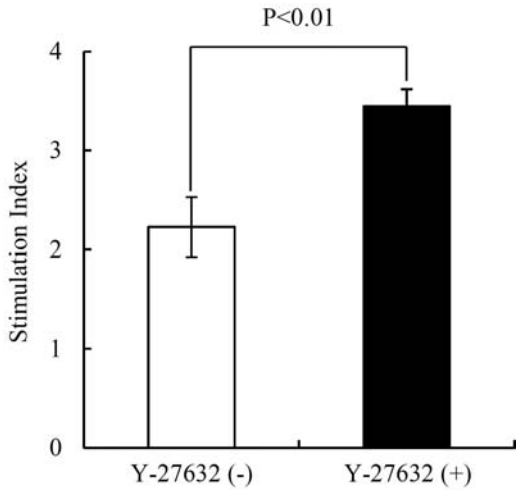


Figure 4. Glucose-responding insulin secretion of islets. The Y-27632 group was shown to significantly secrete more insulin than the control group ($p<0.01$). Y-27632 group: $n=5$. Control group: $n=5$. Data are expressed as the mean ± SE.

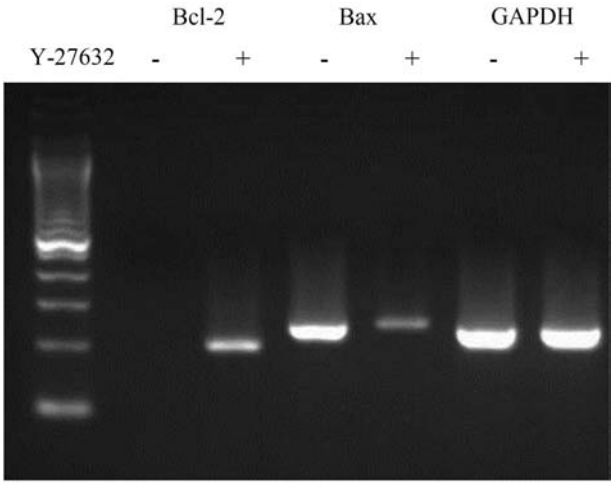


Figure 5. RT-PCR of islets. The strong expression of B-cell CLL/lymphoma 2 (Bcl-2) and lower expression of Bcl2-associated X protein (Bax). Bax observed with Y-27632 demonstrates inhibition of apoptosis compared to the absent Bcl-2 and higher Bax levels seen in controls.

a portion of the transplanted islets in the control group (Fig. 6). Y-27632 inhibited cell apoptosis in a graft and was also indicated as effective in insulin secretion. In the Y-27632-treated islets, uniform and satisfactory function of the islets following transplantation was apparent, and this matches the effective use of Y-27632 in vitro.

DISCUSSION

Cell transplantation has been receiving attention as a treatment for patients with neurodegenerative diseases such as stroke, spinal cord injury, and Parkinson's disease, and extensive transplant research has been conducted in an attempt to restore neurological function, which has

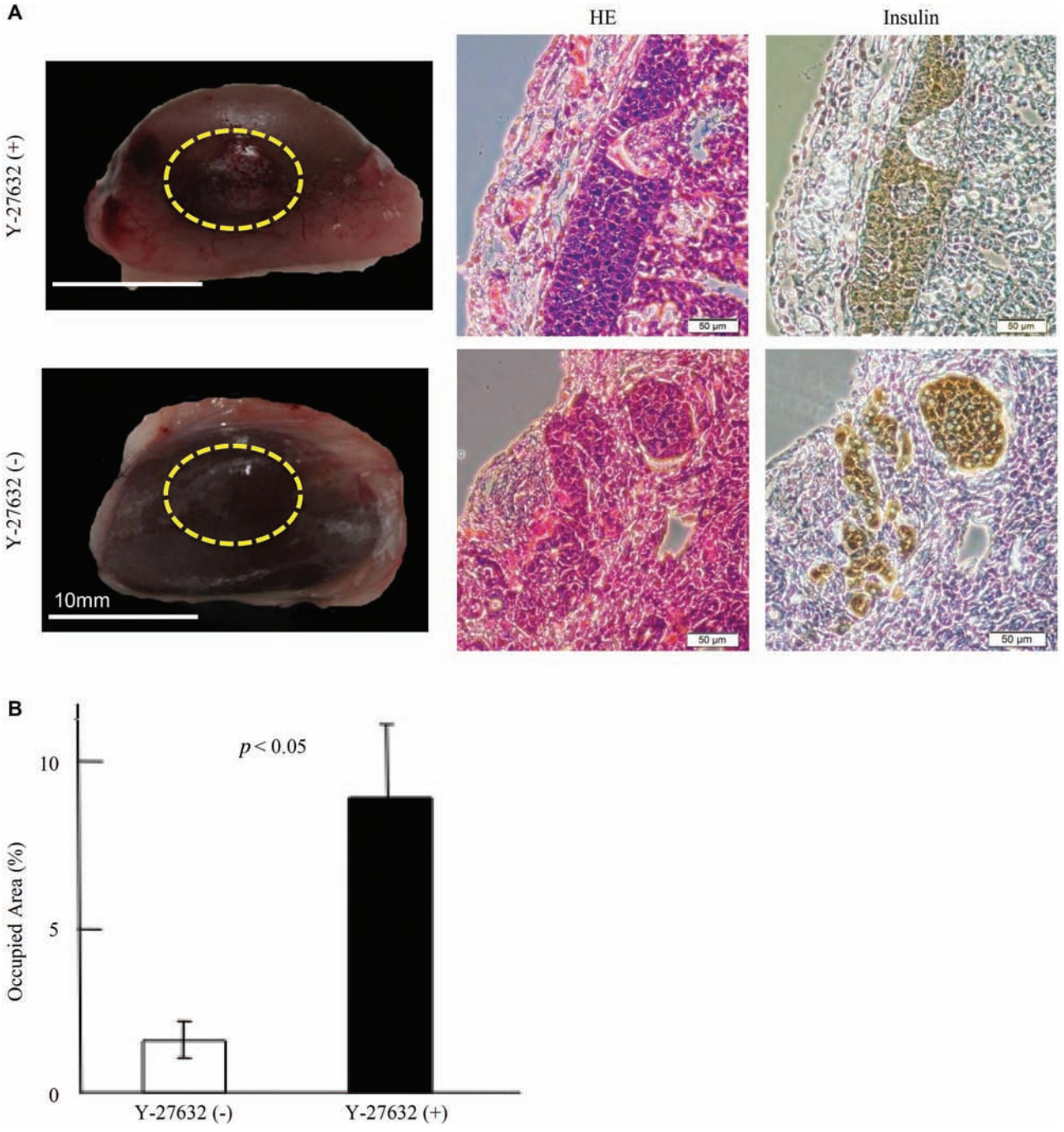


Figure 6. Transplantation of islets. Hematoxylin–eosin (H&E) staining demonstrated that the graft in the Y-27632 group was of a consistent thickness (A) and regular adhesion (B) compared with the sparse and random adhesion of the control group. Uniform insulin staining was observed with the Y-27632 group compared to the control group, insulin expression only being seen in a segregated portion of the transplanted islets in the control group. Scale bars: 10 mm or 50 μm as depicted.

been lost in these patients (3,8,10,29). However, many cell grafts suffer apoptosis several days after transplantation as a result of cell separation, cessation of nutrition, oxidative stress, agitation death, and low oxygen (7,25,33). With respect to human embryonic stem (ES) cells during separation and subculture, ROCK inhibitor Y-27632 has been reported to inhibit apoptosis (programmed cell death) due to variance (31). In human ES cell-derived neurons during cell separation and subculture, when the selective inhibitor Y-27632 of ROCK was used, cell death by human ES cell variance was strongly inhibited, and the colony creation rate from one cell was reported to accelerate by 30%. The idea that the factors within cells that govern apoptosis in a dispersion culture of human ES cells are not generally related to cell death due to ROCK has been verified for the first time. In addition, Y-27632 is known to possess the effect of inhibiting cancer cell infiltration and control of cell differentiation, and contraction of the vascular smooth muscle by cascade signaling of ROCK. With Y-27632 already having drug approval as a vasodilator, there is a large advantage in using Y-27632 for the practical application of BAP from a regulatory point of view. Also, Y-27632 is known to inhibit cytotoxicity in brain cells and possess tissue regeneration effects (6). However, there have been no reports related to the usefulness of Y-27632 in islet cultures. Therefore, we studied the usefulness of Y-27632 using rat islets and its morphological assessment, insulin secretion function assessment, and the comparative evaluation of Bax and Bcl-2 expressions that are apoptosis-related molecules.

Morphologically, on the third day of culture, cell membrane blebbing was observed in the islets from the control group, and the cell membrane border took on an irregular shape, with particle massing of the islets starting to occur. However, with the Y-27632 use group, such blebbing was inhibited and the cell form was satisfactorily maintained even after 7 days of culture. Bax expression was suppressed in the group using Y-27632, and Bcl-2 was strongly expressed. In addition, with the use of ROCK inhibitor Y-27632, inhibiting effects of apoptosis not only were observed, but the possibility of increasing the glucose-responsive insulin secretory function was determined. Currently, this mechanism is being studied. With the islets of the Y-27632 use group, it was suggested that the islet function was therefore uniform and satisfactory after transplantation. The performance of this transplant experiment matches the results of Y-27632 use in vitro.

Until now, there have been no reports of the usefulness of Y-27632 related to islet culture. To summarize our study results with the studies related to the effectiveness of Y-27632 on other cell types, we would like to propose a mechanism of effectiveness with respect to the islet culture of Y-27632, as shown in Figure 7. ROCK is a protein kinase that is activated by the Rho kinase that exists in the cytoplasm, and when ROCK is activated, MLC is directly phosphorylated, or the deactivation of myosin phosphatase causes an indirect increase of MLC phosphorylation, and membrane blebbing occurs. This causes contraction of the cells and is known to trigger apoptosis (13). Generally, apoptosis is regulated by the endogenous system (depending on the mitochondria) and the extrinsic (depending on the death receptor) channels (1,27,30). In the endogenous system pathway, cytochrome c is released from mitochondria by oxidants or DNA damage, toxins, anoxia, and pressurization, and the activation of caspase 3, 6, and 7 leads to apoptosis. On the other hand, with extrinsic pathways, caspase 8 is activated through death receptors such as tumor necrosis factor (TNF) or Fas ligand (FasL), and caspase 3, 6, and 7 are activated, leading to apoptosis (2,15,24). Although a clear report has not yet been reported regarding the mechanism of ROCK inhibitors, by using a ROCK inhibitor before Rho is activated, blebbing of the cell membrane is prevented, and it is known from recent research that apoptosis can be inhibited (5,14). Once ROCK is activated, cell contraction, dramatic membrane blebbing, nucleus decay, and cell fragmentation occur (22,24). However, the ROCK inhibitor causes various conditions that may reduce the apoptosis level in certain cells and tissue types, or lead (28) to apoptosis, with reports that in certain states the activation of ROCK or the inhibition of ROCK has no involvement with apoptosis (24), and thus, the clarification of the functions related to the signal pathway of ROCK is needed.

In addition, when BAP use in a human is considered, the cell source to be placed into the bag will be a problem. Problems such as ethical issues related to heterologous cell transplantation, problems with cell amounts to produce a sufficient effect, and the functional maintenance of sufficient cell survival within the bag must be considered. Although cells originating from humans are obviously preferred when considering ethical issues, transplant offers from brain-dead donors must currently be solely relied upon, with the current status of one recipient requiring numerous donors, resulting in a serious problem with the lack of donors. Human ES cells and induced pluripotent stem (iPS) cells may eliminate this problem. With regard to human ES cells, Watanabe et al. announced that by combining with Y-27632, apoptosis could be inhibited during cell separation and subculture, and its effectiveness was verified (31). In addition, with regard to human iPS cells, there have been reports of satisfactory cryopreservation and increased cell survival and colony amount by combining such cells with Y-27632 (4).

In addition, in order to ensure adequate cell amounts, the establishment of human insulin-secreting cell stock will likely be needed. In order to achieve that during subculture and cryopreservation, Y-27632 will be effectively used for the long-term maintenance of cell survival and

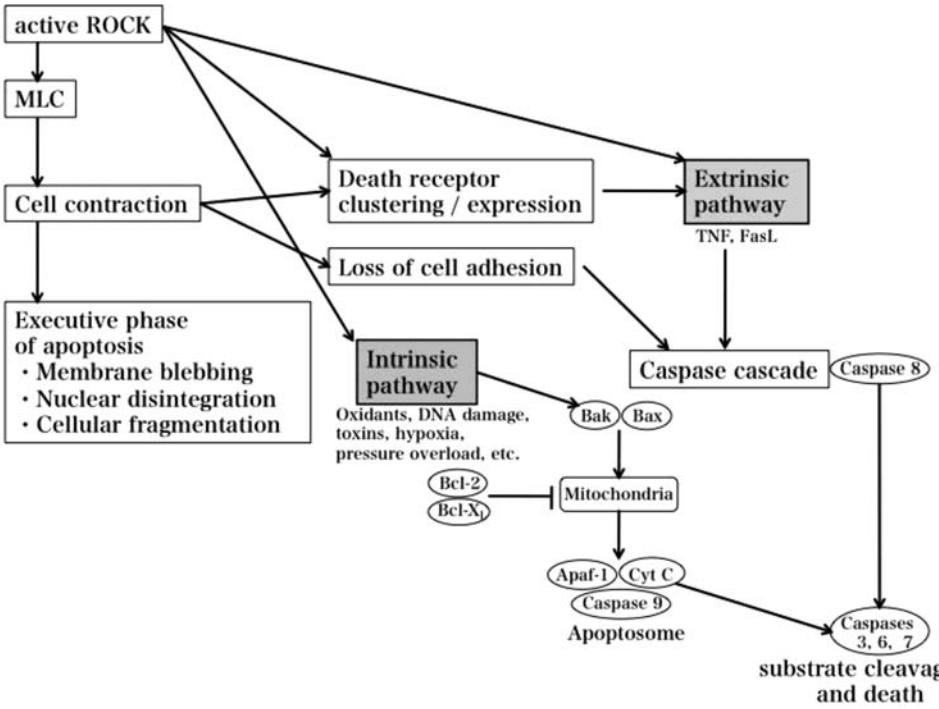


Figure 7. The role of ROCK. When ROCK is activated, the myosin light chain (MLC) is directly phosphorylated, or the deactivation of myosin phosphatase causes an indirect increase of MLC phosphorylation, and membrane blebbing occurs. This causes contraction of the cells and is known to trigger apoptosis. TNF, tumor necrosis factor; FasL, Fas ligand; Bcl-X_L, Bcl2-like 1; Bak, Bcl2-antagonist/killer 1; Apaf-1, apoptotic peptidase activating factor 1; Cyt C, cytochrome c.

function and is anticipated in helping islet transplantation therapy. Once the cell source has been resolved, a system will be developed to culture large amounts of insulin-secreting cells, and its use in various treatments in various circles is anticipated. In order to achieve this, the development of technology will be required to effectively cryopreserve large amounts of cells, and a system constructed to deliver those cells to all regions will be needed. In this study, the effect of apoptosis inhibition in an islet culture was observed with a ROCK inhibitor Y-27632. ROCK inhibitors are already being clinically used in brain/vascular patients and in the ophthalmologic field as a vasodilator, and a future clinical application of the BAP will have the benefit of not having to overcome any regulatory hurdles. Although further studies are needed related to the administered pathway of ROCK inhibitors before donor separation or immediately after separation of the cells, the effective use of ROCK inhibitors as apoptosis inhibitors is anticipated for the future.

In this study, we were able to confirm effective morphological and functional culture maintenance by separating islets from a rat and adding ROCK inhibitor Y-27632 to the medium. Based on rat islet separation and a functional culture, it is believed that it will be helpful in the clinical use of the BAP.

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