

Transplantation of insulin-secreting multicellular spheroids for the treatment of type 1 diabetes in mice

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Abstract

The efficacy of cell-based therapy depends on the function and survival of transplanted cells, which have been suggested to be enhanced by spheroid formation. However, few attempts at spheroid generation from insulin-secreting cells, which may be used to treat type 1 diabetes, have been reported. We therefore developed spheroids from the mouse insulinoma cell line NIT-1 by using polydimethylsiloxane (PDMS)-based microwells with a coating of poly(N-isopropylacrylamide) (PNIPAAm). The prepared NIT-1 spheroids or dissociated NIT-1 cells were transplanted into the subrenal capsule in streptozotocin-induced diabetic mice. NIT-1 spheroids prepared using the PNIPAAm-coated PDMS-based microwells had a uniformly sized spherical structure with a diameter of 200–300 μm . The PNIPAAm coating increased cell survival in the spheroids and the recovery of the spheroids from the microwells. In diabetic mice, the transplanted NIT-1 spheroids reduced blood glucose levels to normal values faster than dissociated NIT-1 cells did. Additionally, survival was higher among NIT-1 cells in spheroids than among dissociated NIT-1 cells 24 h after transplantation. These results indicate that insulin-secreting NIT-1 spheroids prepared using PNIPAAm-coated PDMS-based microwells are more effective for the treatment of type 1 diabetes than dissociated cells in suspension are.

Keywords: Allogeneic cell, Cell viability, Diabetes, Multicellular spheroid, Silicone elastomer, Transplantation

1. Introduction

Type 1 diabetes is also known as insulin-dependent diabetes mellitus and is caused by autoimmune destruction of insulin-secreting beta cells in the islets of the pancreas [1]. Almost all type 1 diabetics require daily insulin injections for the maintenance of their blood glucose levels. Because in patients with diabetes without beta cells, a decrease in blood glucose levels by using insulin injections alone is not possible, such patients require pancreatic transplantation depending on their clinical condition [2]. Such transplantations would provide freedom from injected insulin and lead to stable blood glucose levels. However, pancreas donors are rare and the surgical risk of the transplantation procedure is high [3]. Therefore, alternative therapies are necessary.

Islet transplantation is a reasonable method for the treatment of type 1 diabetes for the following 2 reasons: (1) insulin-secreting islets are able to reduce blood glucose levels; and (2) islet transplantation is much easier and safer than pancreas transplantation [4]. The Edmonton protocol, developed by Shapiro and Lakey of the University of Alberta, has greatly increased the success rate of islet transplantation [5, 6]. However, the limited number of islet donors still prevents the widespread clinical application of islet transplantation [7].

Recent progress in techniques for cell differentiation and proliferation has greatly increased the possibility of production of large numbers of insulin-secreting cells from donor cells or patients' own cells [8-10]. Although the transplantation of insulin-secreting cells has shown some promise, the therapeutic effects are short lived because of the rapid clearance or death of transplanted cells [11]. Therefore, approaches to increase the survival of insulin-secreting cells are necessary to enhance the potency of the treatment [12, 13].

Three-dimensional cell culture systems have recently been developed to culture cells under simulated *in vivo* conditions to facilitate extensive cell-cell interactions [14].

Three-dimensionally cultured spheroidal cells, i.e., multicellular spheroids, have been used to evaluate the characteristics and functions of various cells, including cancer cells, hepatocytes, and pancreatic cells [15-18]. Simple and reproducible methods have been developed for the preparation of multicellular spheroids [19], and the micromolding technique has attracted great attention because of its simplicity and versatility [20]. This technique enables preparation of multicellular spheroids with controlled size, geometry, and cell organization. In our previous study, we demonstrated that polydimethylsiloxane (PDMS)-based microwells were useful to obtain size-controlled multicellular spheroids of fibroblasts, adenocarcinoma cells, endothelial cells, and hepatocytes [21]. In addition, we also found that the coating of the microwells with poly(N-isopropylacrylamide) (PNIPAAm), a thermoresponsive polymer, was useful to increase the quality of the multicellular spheroids, because the coating effectively prevented the cells from adhering to the PDMS-based microwells.

Based on these findings, in the present study, we used PDMS-based microwells to prepare spheroids of insulin-secreting NIT-1 cells, which are a mouse insulinoma cell line. Some functional properties of insulinoma spheroids, such as insulin secreting ability and gene expression, were evaluated in previous studies, but little has been examined about the therapeutic potential of such spheroids *in vivo*. Therefore, we first examined the effects of the PNIPAAm coating on the properties of NIT-1 spheroids and then evaluated the therapeutic potential of the insulin-secreting multicellular spheroids in a mouse model of type 1 diabetes.

2. Materials and methods

2.1. Chemicals

Hank's balanced salt solution and phosphate-buffered saline were obtained from Nissui Pharmaceutical (Tokyo, Japan). PNIPAAm was obtained from Polysciences (Warrington, PA, USA). Ham's F-12K (Kaighn's Modification) medium and STZ were obtained from Wako Pure Chemicals Industry (Osaka, Japan). Fetal bovine serum (FBS) was obtained from GIBCO-Invitrogen (Carlsbad, CA, USA). A PDMS prepolymer and the curing agent (Silpot 184) were purchased from Toray-Dow Corning Co. (Tokyo, Japan). Trypan blue was obtained from Nacalai Tesque (Kyoto, Japan). Non-enzymatic cell dissociation buffer was obtained from Sigma Chemical Company (St. Louis, MO, USA). Trypsin was obtained from Becton Dickinson (Mansfield, MA, USA). CFSE was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of the highest grade commercially available.

2.2. Animals

Male BALB/c mice (8–10 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan), and maintained on standard food and water under conventional housing conditions. The protocols for the animal experiments were approved by the Institutional Animal Experimentation Committee.

2.3. Cell culture

The mouse insulinoma cell line NIT-1 was purchased from ATCC (Rockville, MD, USA) and grown in Ham's F-12K medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in humidified air

containing 5% CO₂.

2.4. Preparation of PDMS-based microwells

Micropillar arrays were fabricated as previously described [21]. A 10:1 mixture of PDMS prepolymer and the curing agent was poured onto micropillar arrays placed in a cell culture dish. The mixture was cured by 2 h of incubation at 60 °C after degassing for 30 min in a vacuum chamber. Subsequently, the PDMS-based microwell sheet was peeled off from the micropillar arrays and trimmed to fit a well in 6-well culture plates. The structure of the microwells was observed under a Biozero microscope (Biozero BZ-8000, Keyence, Osaka, Japan).

2.5. PNIPAAm coating of PDMS-based microwells

The PDMS-based microwell sheet in a well of a 6-well culture plate was immersed in 5% PNIPAAm solution in anhydrous ethanol. Air bubbles in the solution were removed under vacuum, and the excess PNIPAAm solution was removed by pipetting. Subsequently, the PNIPAAm-coated culture plates were dried in a 60 °C chamber for 2 h.

2.6. Adhesion of NIT-1 cells to culture plates

PNIPAAm solution in anhydrous ethanol or a solution containing the precursor of PDMS and the catalyst was added to wells of 12-well culture plates. The culture plates were dried by incubation at 60 °C for 2 h. Thereafter, 1×10^5 NIT-1 cells suspended in Ham's F-12K medium were added to each well and incubated for 24 h at 37 °C in a humidified 5% CO₂ incubator. Culture plates were incubated statically or shaken at a

speed of 65 rpm. At 24 h after incubation, floating NIT-1 cells were removed, and the number of NIT-1 cells adhering to the culture plates was measured by an MTT assay as described previously [22].

2.7. Preparation of NIT-1 spheroids

NIT-1 cells (5×10^6 cells) suspended in Ham's F-12K medium were added to PNIPAAm-coated PDMS-based microwells placed in 6-well culture plates and incubated for 48 h at 37 °C in a humidified 5% CO₂ incubator. The culture plates were shaken at a speed of 65 rpm. To maintain the PNIPAAm coating on the microwells, the coated microwells were kept at 37 °C during the experiments. After incubation, the temperature of the plates was allowed to decrease spontaneously to room temperature, at which point the PNIPAAm coating was dissolved. Then, NIT-1 spheroids were recovered from PDMS-based microwells and washed twice with PBS to remove possibly remaining PNIPAAm. Thereafter, NIT-1 spheroids were picked up using micropipettes. The diameter of the NIT-1 spheroids was measured under the Biozero microscope. The number and viability of NIT-1 cells in 1 spheroid were measured by staining the cells with trypan blue solution after dispersion of the spheroid by using a trypsin-EDTA solution (0.25% w/v).

2.8. Recovery of NIT-1 spheroids from PDMS-based microwell sheet

The PDMS-based microwell sheet with NIT-1 spheroids was detached from the culture plates, soaked in PBS on ice or at 37 °C, and shaken back and forth using forceps at a rate of 3 shakes per second as previously reported [21]. The remaining cell spheroids in the PDMS-based microwell sheet were counted.

2.9. Insulin secretion from NIT-1 cells

Ten NIT-1 spheroids that contained a total of approximately 73,000 were added to a 6-well culture plate and pre-incubated in KRB buffer (140 mM NaCl, 2 mM CaCl₂, 0.15 mM Na₂HPO₄, 5 mM NaHCO₃, 1 mM MgSO₄, 0.05% BSA, 4.6 mM KCl, and 30 mM HEPES, pH 7.4) containing 3 mM glucose for 30 min. Subsequently, the solution was replaced with fresh KRB buffer containing 3 or 20 mM glucose. After 30 min of incubation, the supernatants were collected and centrifuged at 4,000 rpm for 5 min. The amount of insulin secreted from the NIT-1 cells was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). Monolayered NIT-1 cells treated as described above were used as a control.

2.10. Transplantation of NIT-1 spheroids into STZ-induced diabetic mice

BALB/c mice received an intravenous injection of STZ dissolved in citrate buffer (pH 4.5) at a dose of 120 mg/kg of body weight. The non-fasting blood glucose level of the mice was measured, and mice with glucose levels of 350 mg/dl or higher were used as diabetic mice. Transplantation of NIT-1 spheroids or suspended NIT-1 cells was conducted according to the method reported by Zmuda et al. [23]. In brief, under pentobarbital sodium anesthesia, 150 spheroids (approximately 1.1×10^6 cells) suspended in Hanks' balanced salt solution were transplanted into the subrenal capsule of diabetic mice by using silicon tubing. The same number of suspended NIT-1 cells was transplanted to another group of diabetic mice. The blood glucose level was repeatedly measured using an ACCU-CHECK Active meter (Roche Diagnostics, Tokyo, Japan) or a G-checker (Sanko Junyaku Co., Ltd., Japan).

2.11. Intraperitoneal glucose tolerance test

At 28 days after transplantation, the glucose tolerance test was conducted using an intraperitoneal injection of D-(+)-glucose (Wako Pure Chemical Industries, Ltd., Japan) at a dose of 1 g/kg body weight in 16 h-fasted mice. The blood glucose level was measured as described above.

2.12. Imaging of NIT-1/CFSE spheroids or NIT-1/CFSE suspension in subrenal capsule

NIT-1 cells were labeled with 10 μ M CFSE for 30 min at 37 °C to obtain NIT-1/CSFE cells. In total, 300 NIT-1/CSFE spheroids (approximately 2.2×10^6 cells) or isolated NIT-1/CSFE cells suspended in Hanks' balanced salt solution were transplanted into the subrenal capsule by using silicon tubing as described above. Images of NIT-1/CFSE spheroids or dissociated NIT-1/CFSE cells in the subrenal capsule were visualized by fluorescence using a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

2.13. Statistical analysis

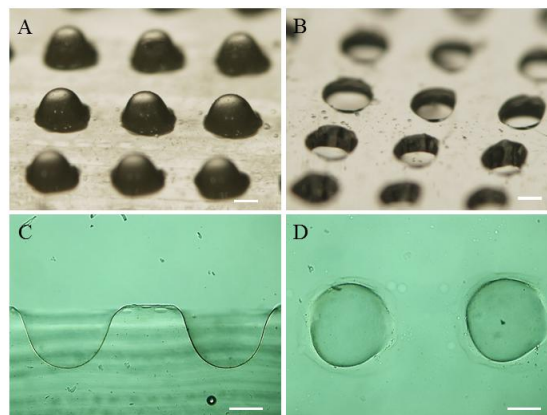
Differences were statistically evaluated using Student's t-test or a one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Preparation of PDMS-based microwells

Fig. 1 shows microscopic images of the micropillar arrays (Fig. 1A) and a PDMS-based microwell sheet (Fig. 1B–D). The images indicate that the PDMS-based microwell sheets have uniformly sized microwells. To prepare uniformly sized multicellular spheroids with a diameter of approximately 300 μm , microwells with a height, intermediate diameter, and maximum diameter of 310 ± 5 , 310 ± 3 , and 432 ± 20 μm , respectively, were used.

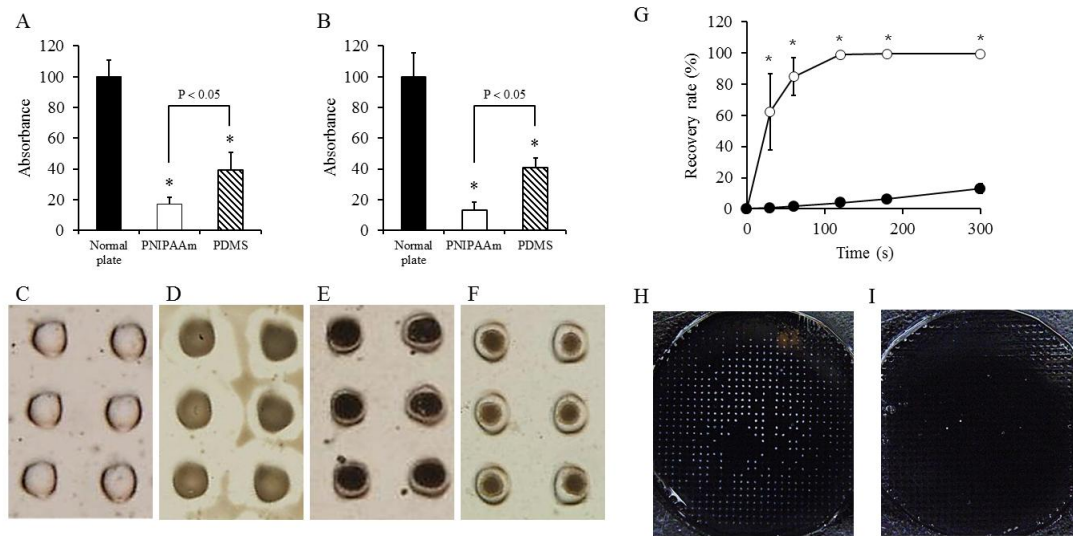
Fig. 1



3.2. Adhesion of NIT-1 cells to culture plates

The effect of PDMS or PNIPAAm coating on the adhesion of NIT-1 cells to polystyrene culture plates was examined. Fig. 2A and 2B show the number of NIT-1 cells remaining on the plate after washing. Under static conditions, PDMS or PNIPAAm coating reduced cell adhesion to 39.4% or 16.9% of the level observed on the non-coated plate, respectively. Similar results were obtained under shaking conditions.

Fig. 2



3.3. Coating of PDMS-based microwells with PNIPAAm

Fig. 2C and 2D show microscopic images of PDMS-based microwells before and after PNIPAAm coating, respectively. The apparently transparent PDMS-based microwells were almost uniformly colored by the coating (Fig. 2D), which suggests that the coating with PNIPAAm was even. Fig. 2E shows the PNIPAAm-coated PDMS-based microwells after seeding with NIT-1 cells. After 48 h of incubation, the microwells containing the NIT-1 cells were incubated below the transition temperature of 32 °C. This treatment dissolved PNIPAAm, and the NIT-1 spheroids were clearly observed in the microwells (Fig. 2F).

3.4. Recovery of NIT-1 spheroids from PDMS-based microwells

Fig. 2G shows the time course of the recovery of NIT-1 spheroids from PDMS-based microwells with or without PNIPAAm coating. Gentle shaking for 300 s resulted in the recovery of only approximately 13% of NIT-1 spheroids from the non-coated microwells. In contrast, over 90% of the NIT-1 spheroids were recovered from the

PNIPAAm-coated PDMS-based microwells in only 60 s. Fig. 2H and 2I show typical images of PDMS-based microwells, in which the remaining spheroids appear as white dots. These results demonstrate that the PNIPAAm coating is useful for efficient recovery of NIT-1 spheroids.

3.5. Size of NIT-1 spheroids and viability of NIT-1 cells in the spheroids

Fig. 3 shows histograms for the diameter of NIT-1 spheroids measured under microscopic observation. Irrespective of the PNIPAAm coating, the apparent diameter of the NIT-1 spheroids was 200–300 μm (Fig. 3A, 3B). The average diameter was approximately 260 μm in both the cases (Table 1). The number of NIT-1 cells per spheroid was approximately 7,300 and 7,600 for the PNIPAAm-coated and non-coated groups, respectively. The cell viability of the PNIPAAm-coated group (98.8%) was significantly higher than that of the non-coated group (91.7%).

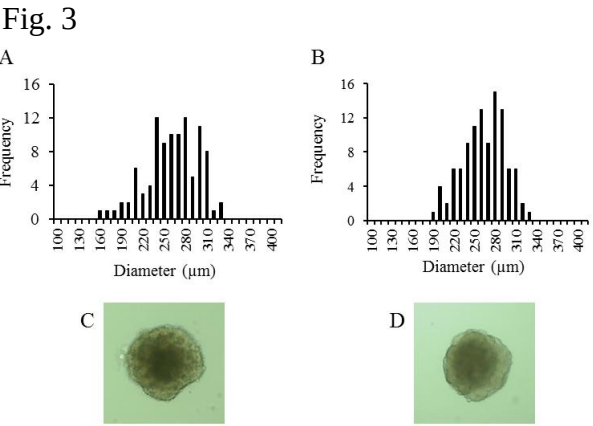


Table 1. Characteristics of NIT-1 spheroids.

	Diameter (μm)	Number of cells	Viability (%)
Non-coated	330 ± 46	$7,630 \pm 990$	91.7 ± 0.5
PNIPAAm-coated	331 ± 39	$7,310 \pm 640$	98.8 ± 0.2

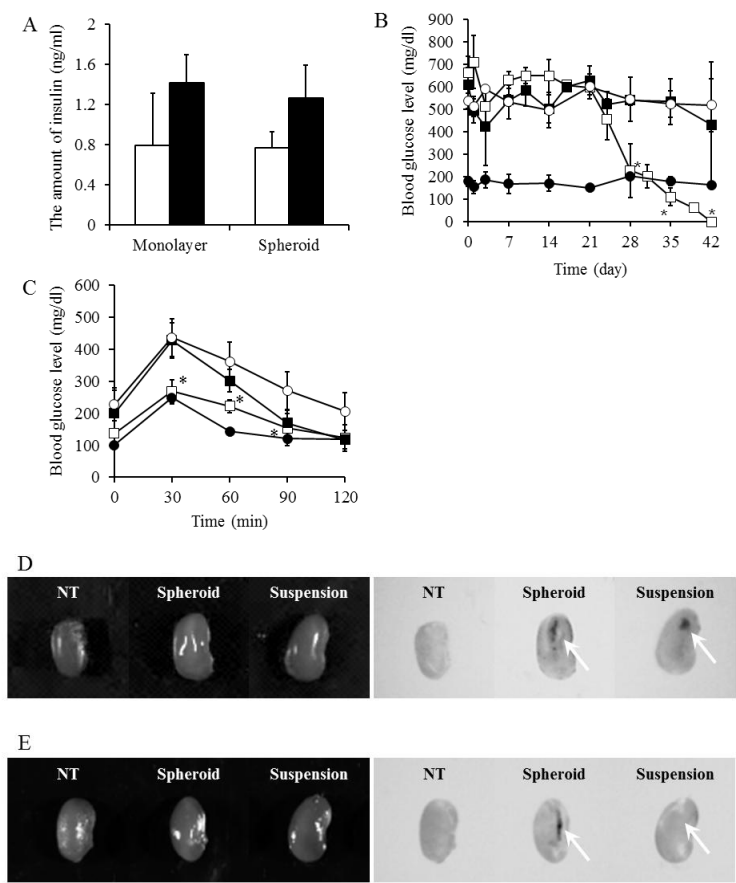
Results are expressed as the mean $\pm$ SD ($n = 5$).

* $P < 0.05$, statistically significant difference relative to the non-coated group.

3.6. Insulin secretion from monolayered NIT-1 cells and NIT-1 spheroids

NIT-1 spheroids prepared using the PNIPAAm-coated PDMS-based microwells were used in the following experiments. Fig. 4A shows the amount of insulin secreted from monolayered NIT-1 cells or NIT-1 spheroids. Insulin secretion from monolayered cells was 0.79 or 1.42 ng/ml in Krebs-Ringer bicarbonate (KRB) buffer containing 3 or 20 mM glucose, respectively. There was no significant difference in release between monolayered NIT-1 cells and NIT-1 spheroids at both the glucose concentrations.

Fig. 4



3.7. Treatment of streptozotocin-induced diabetic mice with NIT-1 spheroids

Suspended NIT-1 cells or NIT-1 spheroids were transplanted to streptozotocin (STZ)-induced diabetic mice at a dose of 1.1×10^6 cells/mouse. Fig. 4B shows the time course of the blood glucose levels of the mice after transplantation of NIT-1 cells. STZ treatment significantly increased the blood glucose level to approximately 600 mg/dl. The high blood glucose level in the STZ-treated, NIT-1 spheroid-transplanted mice started to decrease around 24 days after transplantation and was maintained in the normal range from days 28 to 35. In contrast, the blood glucose level in the STZ-treated, suspended NIT-1 cells-transplanted mice hardly changed over the experimental period of 42 days. To confirm the ability of the mice to dispose of a glucose load, an intraperitoneal glucose tolerance test was conducted at 28 days after transplantation. Fig. 4C shows the blood glucose levels of mice after intraperitoneal injection with 1 g/kg of D-glucose. The blood glucose level of the NIT-1 spheroid-transplanted mice was comparable to that of naïve mice, and much lower than that of the mice transplanted with suspended NIT-1 cells.

3.8. Distribution of NIT-1 cells after transplantation

NIT-1 cells were fluorescently labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), transplanted into mice, and visualized using a lumino-image analyzer. At 10 min after transplantation, strong fluorescent signals were detected in mice receiving either NIT-1 spheroids or suspended NIT-1 cells (Fig. 4D). However, the signals were only observed in mice receiving the NIT-1 spheroids at 24 h after transplantation (Fig. 4E).

4. Discussion

PDMS is one of the most frequently used materials in micromolding-based fabrication of three-dimensional cell culture devices. Some groups have developed PDMS-based devices whose surfaces were modified with hydrophilic polyethylene glycol to suppress cell adhesion [24, 25]. Although such a modification would be effective for suppressing cell adhesion, it requires several steps for preparation, including the introduction of functional groups and the conjugation of polymers. The PNIPAAm-coating method that we developed in our previous study [21] is much easier and may be more effective in obtaining reproducible results. We demonstrated that the recovery of NIT-1 spheroids was greatly improved by the coating (Fig. 2F–H) relative to other substrates. Mechanical stress would be a major factor to reduce the viability of cells in spheroids. PNIPAAm coating allowed us to use gentle pipetting for the recovery of spheroids, and this could be a reason for the high viability of cells in spheroids prepared using PNIPAAm-coated microwells. In addition, the high viability of the cells suggests that PNIPAAm is not toxic to cells [26, 27].

Insulin secretion from beta cells in islets is predominantly regulated in response to glucose concentration in the circulating blood. However, how transplanted insulin-secreting cells control the blood glucose level remains unclear. Kinoshita et al. reported that spheroid formation hardly improved glucose-responsive insulin secretion from mouse insulinoma MIN6 cells [28]. In contrast, Yang et al. reported that MIN6 spheroids showed higher glucose-responsive insulin secretion than monolayered MIN6 cells did [29]. The present study showed that glucose-responsive insulin secretion from NIT-1 cells was hardly changed by spheroid formation (Fig. 4A). One reason for this effect may be that NIT-1 cells are not as sensitive to glucose as pancreatic beta cells or MIN6 cells are [30]. Therefore, changes in insulin release are not responsible for the better *in vivo* results of NIT-1 spheroids relative to suspended NIT-1 cells.

In this study, the subrenal capsule was selected as the site for transplantation because subrenal capsule transplantation has been most widely used in rodent experiments [31]. The significant reduction in blood glucose levels in STZ-induced diabetic mice indicates that insulin was secreted from the transplanted NIT-1 spheroids. The decrease in blood glucose levels and the imaging of CFSE-labeled NIT-1 cells suggest that survival of NIT-1 cells after *in vivo* transplantation was greatly improved by spheroid formation (Fig. 4B, 4D, and 4E).

It has been reported that multicellular spheroids have several advantages over cell suspensions for cell-based therapy. Bhang et al. reported that spheroid formation increased the expression of anti-apoptotic factors in adipose-derived stem cells after transplantation [32]. Cell suspensions have generally been used as a conventional method of cell transplantation. However, cells in suspension are prone to undergo cell death by anoikis, a form of apoptosis induced by the loss of cell–matrix interactions. It was reported that spheroid formation prevented rat intestinal epithelial cells or hepatocytes from anoikis and improved their survival [33, 34]. The results of the present study using CFSE-labeled NIT-1 cells support these previous findings and indicate that spheroid formation prolongs cell survival after transplantation.

However, the mice transplanted with NIT-1 spheroids had died by the experimental end point (day 42), even though the blood glucose level was maintained at normal levels from day 28 to day 35. Hypoglycemia may have been the cause of death. In this study, we selected proliferating mouse insulinoma NIT-1 cells because of their ease of use in large-scale preparation. A previous study similarly used proliferating MIN6 cells in a hydrogel encapsulation model to restore normoglycemia in diabetic mice [35]. The relatively long lag time before the decrease in the blood glucose level would indicate that the NIT-1 cells need to proliferate to reduce the blood glucose level under the conditions examined. No significant changes in the mice receiving suspended NIT-1 cells strongly support the importance of spheroid formation on the therapeutic

activity of NIT-1 cells. Proliferative cells may not be desirable for clinical applications because of the potential for uncontrolled proliferation of the transplanted cells. A larger number of spheroids might be needed when non-proliferating insulin-secreting cells are used for transplantation. Thus, the use of non-proliferative insulin-secreting cells, such as pancreatic beta cells differentiated from induced pluripotent stem cells, may solve this problem in future preclinical studies.

5. Conclusions

We succeeded in preparing uniformly sized NIT-1 spheroids by using PNIPAAm-coated PDMS-based microwells. Spheroid formation greatly increased the therapeutic effect of NIT-1 cells in STZ-induced diabetic mice. The combination of this approach with pancreatic beta-like cells derived from induced pluripotent stem cells may provide an effective therapeutic modality to treat type 1 diabetics.

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Figure Legends

Fig. 1 Micropillar arrays and PDMS-based microwells. Images were taken under a microscope. (A) Micropillar arrays, (B) PDMS-based microwell sheet peeled from micropillar arrays, (C) Cross-sectional view of PDMS-based microwells, and (D) Top view of the PDMS-based microwells. Scale bar represents 200 μm in each image.

Fig. 2 Preparation of NIT-1 spheroids by using PNIPAAm-coated PDMS-based microwells.

(A) Number of NIT-1 cells attached to culture plates in (A) static culture or (B) shaking culture at 65 rpm. NIT-1 cells were added to culture plates with or without coating of PDMS or PNIPAAm and incubated for 24 h. The absorbance was measured using a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm. Results are expressed as mean $\pm$ SD (n = 4). * $P < 0.05$, statistically significant difference compared with the normal plate group. (C–F) Appearance of NIT-1 spheroids in the PDMS-based microwell sheet. (C) Untreated PDMS-based microwell sheet, (D) PNIPAAm-coated PDMS-based microwell sheet, (E) PNIPAAm-coated PDMS-based microwell sheet with NIT-1 cells, and (F) NIT-1 spheroids in the PDMS-based microwell sheet after dissolution of PNIPAAm at room temperature. (G–I) Recovery of NIT-1 spheroids from PDMS-based microwells with or without coating of PNIPAAm. (G) Time-course of NIT-1 spheroid recovery from non-coated (●) or PNIPAAm-coated (○) PDMS-based microwells. * $P < 0.05$, statistically significant difference compared with the non-coated group. Representative images of (H) the non-coated PDMS-based microwell sheet and (I) the PNIPAAm-coated PDMS-based microwells sheet are shown after shaking for 300 s. White dots indicate remaining NIT-1 spheroids in the microwells.

Fig. 3 Characteristics of NIT-1 spheroids. The diameter of the spheroids was measured under a microscope, and the histograms of the diameter are shown. One hundred spheroids were measured. (A) NIT-1 spheroids prepared using non-coated PDMS-based microwells and (B) NIT-1 spheroids prepared using PNIPAAm-coated PDMS-based microwells. Typical images of

(C) NIT-1 spheroids prepared using non-coated PDMS-based microwells and (D) NIT-1 spheroids prepared using PNIPAAm-coated PDMS-based microwells.

Fig. 4 Biological and therapeutic activities of NIT-1 spheroids. (A) Insulin secretion from monolayered NIT-1 cells or NIT-1 spheroids. The amount of insulin secreted from NIT-1 spheroids or NIT-1 suspension was measured in KRB buffer containing 3 mM glucose (white bar) or 20 mM glucose (black bar). Results are expressed as the mean $\pm$ SD ($n = 3$ or 4). (B) Non-fasting blood glucose levels in STZ-diabetes mice were measured after transplantation of 150 NIT-1 spheroids and the same number of suspended NIT-1 cells. (C) Intraperitoneal glucose tolerance tests were conducted in each mouse on day 28 after transplantation. STZ group ($\circ$); naïve group ($\bullet$); STZ-treated, NIT-1 spheroid-transplanted group ($\square$); and STZ-treated, suspended NIT-1-transplanted group ($\blacksquare$). The experiment was conducted in duplicate, and a representative experiment is shown. Results are expressed as the mean $\pm$ SD ($n = 4$). * $P < 0.05$, statistically significant difference relative to the STZ-mice group. (D, E) Typical images of NIT-1/CFSE spheroids and dissociated NIT-1/CFSE cells in the subrenal capsule. Images show non-treated renal tissue (NT) or renally transplanted NIT-1/CFSE spheroids (spheroid) or suspended NIT-1/CFSE cells (suspension) (D) 10 min and (E) 24 h after transplantation. Arrows show positions of NIT-1/CFSE cells in the subrenal capsule. Left, photographic images; right, fluorescent images.