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Visualization of the Specific Interaction of Sulfonylurea-Incorporated Polymer with Insulinoma Cell Line MIN6

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A derivative of sulfonylurea (SU) that mimicks glibenclamide in chemical structure was synthesized and incorporated into a water-soluble polymeric backbone as a biospecific polymer for stimulating insulin secretion. In this study, a backbone polymer fluorescence-labeled with rodamine-B isothiocyanate was found to be strongly adsorbed onto MIN6 cells, probably due to its specific interaction mediated by SU receptors on the cell membrane. The intensity of fluorescence on the cells was significantly increased by increasing the incubation time and polymer concentration. To verify the specific interaction between the SU (K^+ channel closer)-incorporated copolymer and MIN6 cells, the cells were pretreated with diazoxide, an agonist of the ATP-sensitive K^+ channel (K^+ channel opener), before adding the polymer to the cell culture medium. This treatment suppressed the interaction between SU and MIN6 cells. A confocal laser microscopic study confirmed this effect. The results of this study provide evidence that SU-incorporated copolymer stimulates insulin secretion through the specific interactions of SU moieties in the polymer with MIN6 cells.

Key words: diazoxide, fluorescence, MIN6 cells, specific interaction, sulfonylurea.

For decades, considerable efforts have been devoted to developing a biohybrid artificial pancreas (BAP) for the treatment of insulin dependent diabetes mellitus (IDDM) (*1–3*). Major approaches have focused on islet macroencapsulation into a chamber with vascular grafts (*4–5*) and microencapsulation of islets within permselective membranes (*6–8*). Problems involved in these methods that is still unsolved, however, one of the most significant is how to increase cell functionality such as insulin secretion when the BAP is implanted.

To address the problem associated with large implant volume, an approach has been proposed to enhance insulin secretion by co-encapsulating islets of Langerhans with an insulinotropic agent, sulfonylurea (SU), which is grafted onto water-soluble polymers to prevent its diffusional loss from the capsule, with the expectation of reduced number (or volume) of cells required for normoglycaemia after implantation (*9–10*). In an *in vitro* experiment, the SU-incorporated polymers were able to stimulate insulin secretion from the islets by up to 30%, particularly at low glucose concentrations. It was thought that the conjugate specifically binds with β-cells in islets *via* SU-receptors, a part of the K^+ channel (SUR1), on the cell membrane (*11–12*). This binding closes ATP-sensitive K^+ channels, preventing K^+ outflux (*13*), and depolarizes the cell membrane potential, resulting in increasing calcium flux through the voltage-dependent Ca^{2+}channel. The increase in cytoplasmic Ca^{2+} concentration eventually triggers insulin secretion from pancreatic islets (*14–15*).

In a previous study, we reported a new concept of double ligands consisting of sugar-bearing polystyrene (PS) derivatives and an additional drug ligand of SU as the specific agent to islets (*16*). An SU derivative, as a specific ligand to islets, was copolymerized with sugar bearing PS derivatives that could specifically recognize SU-receptors on the cell surface.

In this study, MIN6 cells, a pancreatic β cell line, were used instead of primary islets to examine the effectiveness of SU-conjugated polymers in promoting insulin secretion and to provide more direct evidence of a specific interaction of the cell line with SU-conjugated polymer. The insulinoma cell line has a high potential for use in place of primary islets or β-cells in a BAP, because the use of primary islets involved such difficulties as limited human pancreas donation, the risk of zoonosis in the case of an animal source, low isolation yield, and preservation. Although cultured cell line may carry the risk of causing tumor formation if it escapes from the capsules it can be supplied for BAP without limitation and with minimal risk of contamination by an unidentified animal viral strain. MIN6 was derived from transgenic mice and secretes normal levels of insulin. The insulin secretion kinetics and dose response to glucose are characterized to be close to those of primary culture of β-cells. In addition, MIN6 cells express ATP-sensitive K^+ channel and subunit of SU receptors (*17–18*). Thses characteristics prompted us to investigate the interactions of this cell line with SU-conjugated polymers.

To visualize the interaction between the SU and its receptor, we attempted a new approach using a glibenclamide-mimetic SU-incorporated copolymer. For more detailed observation of the interaction of the synthetic polymer with the cell, a fluorescence-labeled polymer was

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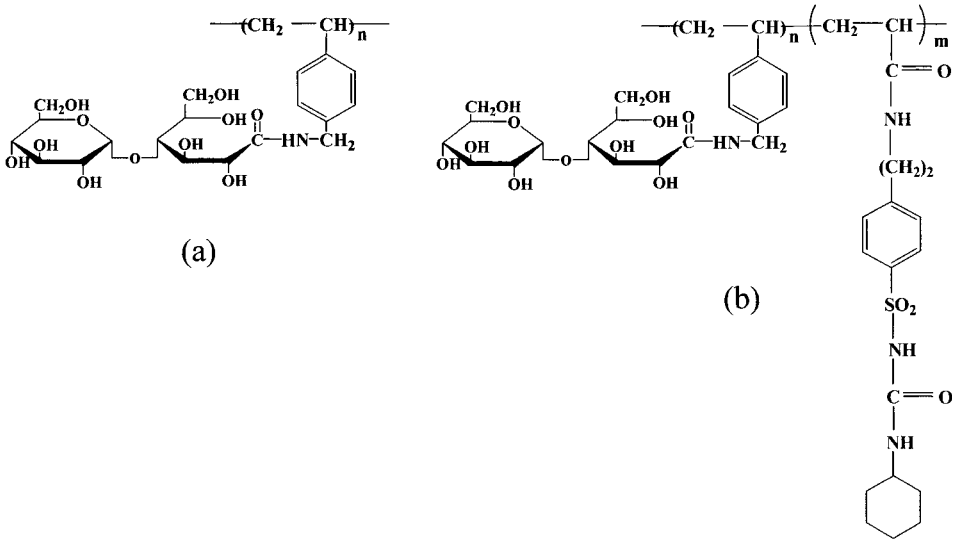

Fig. 1. **Chemical structures of polymers used in this study.** (a) PVMA and (b) p(VMA-co-SU).

used, and the interaction was monitored by fluorescence intensity measurement and confocal microscopic analysis.

MATERIALS AND MATHODS

Materials—MIN6 cells were kindly provided by Dr. Junichi Miyazaki's Lab. Diazoxide, ethylenediaminetetraacetic acid (EDTA), and rhodamine B isothiocyanate (RITC) were purchased from Sigma Chemical (St. Louis, MO). Bovine serum albumin (BSA), fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), streptomycin, and penicillin sulphate were purchased from GIBCO BRL (Grand Island, NY). 4-(2-Aminoethyl) benzene sulfonamide, cyclohexyl-isocyanate, and dimethyl sulfoxide (DMSO)-d_6 were purchased from Aldrich Chemical (Milwaukee, WI) and DMSO and methanol were purchased from Showa Chemical (Tokyo).

Methods—An antidiabetic SU analog was synthesized as follows. Briefly, *p*-aminoethyl benzene sulfonamide (1 g, 6 mmol) and acryloyl chloride (0.45 g, 6 mmol) dissolved in a mixture of 5 ml of acetone and 5 ml of 1 N NaOH aqueous solution was reacted for 2 h at room temperature, and the product was recrystallized twice in MeOH. The 4-[(2)′-acrylamido-ethyl] benzene sulfonylamide (AEBSA) (2.7 g, 7 mmol) was then swollen in acetone and poured into 7 ml of 1 N NaOH aqueous solution, and this solution was reacted with cyclohexylisocyanate (0.9 ml, 7 mmol) dissolved in 3.5 ml of acetone for 16 h at room temperature with stirring. The reaction mixture was then poured into 1 N HCl aqueous solution (7 ml), and the precipitate obtained was vacuum-dried.

Preparation of Copolymers Containing SU—A mixture of VMA, cyclohexyl 4-[(2)′-acrylamido-ethyl] benzene SU and AIBN as an initiator dissolved in DMSO was poured into a glass ampule. The sealed ampule was placed in a thermostat at 60°C for 6 h. After polymerization, the mixture solution was poured into methanol to obtain a precipitate. The product was reprecipitated into methanol and freeze-dried.

Fluorescent Labeling of Polymers—The polymer was labeled with RITC. To a solution of the polymer (500 mg) in 5 ml of DMSO were added 50 mg of RITC and 15 mg of dibutyltin dilaurate, and the mixture was allowed to stand for 2 h at 90°C. After cooling to room temperature, the mixture was poured into an excess of ethanol to precipitate fluorescence-labeled polymer. The polymer was dissolved in 30 mL water and dialyzed at 4°C against 1,000 ml of mild alkaline water (pH 8.0 with NaOH) for 1 d, and then against distilled water for 2 wk with daily chages of the water. The product of RITC-labeled polymer was obtained by freeze-drying.

Cell Culture—MIN6 cells were cultured in DMEM (25 mM glucose) equilibrated with 5% CO_2 and 95% air at 37°C. The medium was supplemented with 15% fetal calf serum, 50 mg/liter streptomycin and 75 mg/liter penicillin sulfate.

Diazoxide Treatment—MIN6 cells (5×10^5) suspended in DMEM containing 25 mM glucose were seeded onto a polystyrene culture dish and precultured for 3 d. Cells were then treated with 300 μM diazoxide for 20 min then washed with HEPES-balanced KRBB without glucose and BSA. Finally, the cells were incubated for 2 h in HEPES-balanced KRBB with 3.3 mM glucose, the synthesized PS derivative, and the SU-incorporated polymer. The media were then collected and stored at –20°C until insulin assay by use of a rat RIA kit.

Fluorescence Intensity Analysis—MIN6 cells (5×10^5) were plated in a 24-well tissue culture dish and cultured for 3 d. The solution of RITC-labeled polymers (0.0001, 0.001, 0.01, and 0.1 w%) in DMEM medium were added and incubated at 37°C for 1 h. After incubation, MIN6 cells were washed three times with HEPES-balanced KRBB, and the fluorescence intensity of cells in 1 ml of HEPES-balanced KRBB was measured with a fluorescence microplate reader (FL600, Bio-Tek, USA).

Confocal Laser Microscopic Analysis—MIN6 cells in confluent state were detached by treatment with phosphate-buffered saline (PBS) containing 0.5 mM EDTA for 5 min. The detached MIN6 cells were preincubated in HEPES-balanced KRBB (pH 7.4, 3.3 mM glucose) equilibrated with 5% CO_2 and 95% air at 37°C for 20 min, the incubated in HEPES-balanced KRBB containing RITC-labeled at 4°C or 37°C for 1 h. The cells were then washed three times with HEPES-balanced KRBB and placed on a

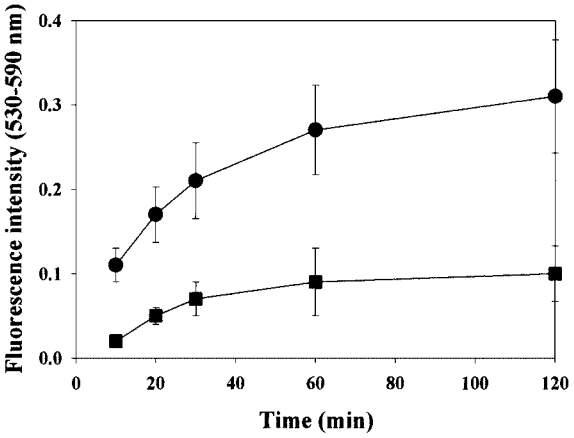

Fig. 2. **Time-course of the binding of RITC-labeled SU- incorporated copolymer with MIN6 cells.** Each sample was incubated in HEPES-balanced KRBB (without BSA) with low glucose concentration for 2 h with labeled polymer. The error bars represented as mean±SD. Circles: copolymer with SU ligand [p(VMA-co-SU)] and squares: polymer without SU ligand (PVMA).

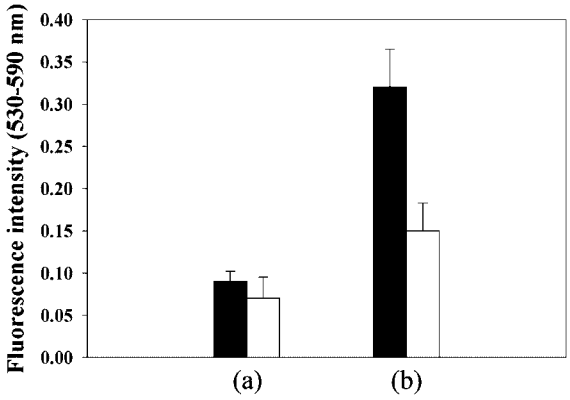

Fig. 4. **Effect of diazoxide on polymer binding affinity to MIN6 cells.** Cells were pretreatment with diazoxide (100 μM) for 20 min to inhibit the interaction between p(VMA-co-SU) and cells. (a) copolymer with SU ligand [p(VMA-co-SU)] and (b) polymer without SU ligand (PVMA) (black bars, diazoxide-free condition; white bars, pretreatment with diazoxide).

cover glass, which was gently placed on a slide glass. The lumination of MIN6 cells was observed using a confocal laser microscope (Leika, Heidelbulg, Germany).

RESULTS AND DISCUSSION

Observation of Interaction between SU and SU Receptor—A SU derivative mimicking glibenclamide in chemical structure was synthesized and incorporated into a water-soluble polymeric backbone. The bioactivity of SU-incorporated copolymer toward MIN6 cells was assessed in terms of insulin secretion, which increased approximately 2.2-fold at low glucose concentration and 1.8-fold more at high glucose concentration than unstimulated MIN6 cells in previous studies (*19–20*).

To confirm the bioactivity of SU-incorporated copolymer to MIN6 cells, in this study, we attempted to use the fluorescence labeled copolymer for the determination of fluorescence intensity on the cells. It is reported that SU receptors on the ATP-sensitive K^+ channels are reported to be located in the lipid phase of the cellular membrane, and thus SUs are likely to access their receptors in the lipid phase of the cell membrane via lateral diffusion after partition in the cell membrane bilayer (*21*). To qualify time-dependent and dose-dependent interactions of SU-incorporated copolymer with MIN6 cells, a labeled SU-incorporated polymer with RITC was synthesized, and 0.01 w/v% of the polymer was added to MIN6 cell culture. After incubation for a given time, MIN6 cells were washed with HEPES-balanced KRBB to remove unbound polymer and fluorescence intensity on the cells was determined. Figure 2 shows the time course of binding of SU-incorporated copolymer to MIN6 cell membrane over a period of 10–120 min. After 10 min, significant intensity was observed. This intensity increased with time and at 120 min reached more than 2-fold that at 10 min. This result suggested that the binding process of SU-incorporated copolymer to MIN6 cells is time-dependent. This can be explained by assuming that the SU-incorporated polymers located near the cells partitioned into the cell membrane in 10 min, resulting in the decrease in local SU concentration, which drives diffusion of the polymers from the bulk to the cell surface and accounts for more binding with time.

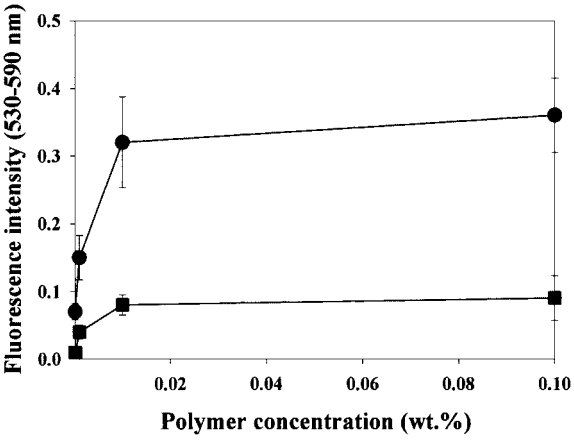

Fig. 3. **Dose-dependent interaction of SU- incorporated copolymer with MIN6 cells.** Each sample was incubated in HEPES-balanced KRBB (without BSA) with low glucose concentration for 2 h with labeled polymer. The error bars represented as mean ± SD. Circles: copolymer with SU ligand [p(VMA-co-SU)] and squares: polymer without SU ligand (PVMA).

The effect of polymer concentration on binding to MIN6 cells is presented in Fig. 3. With the higher polymer concentration, the higher fluorescence intensity was observed. The fluorescence intensity may not directly reflect the degree of binding of SU units with theirs receptors because of the macromolecular nature of the ligands. In other words, once a single SU unit in a polymer molecule has bound to the cell surface, all RITCs and unbound SU molecules in the same polymer molecule may locate adjacent to the cells, resulting in an apparent increase fluorescence intensity on the cell surface. However, it can be thought that a higher population of the SU-conjugate polymers adjacent to cell membrane may lead to a higher probability of SU binding to the cells.

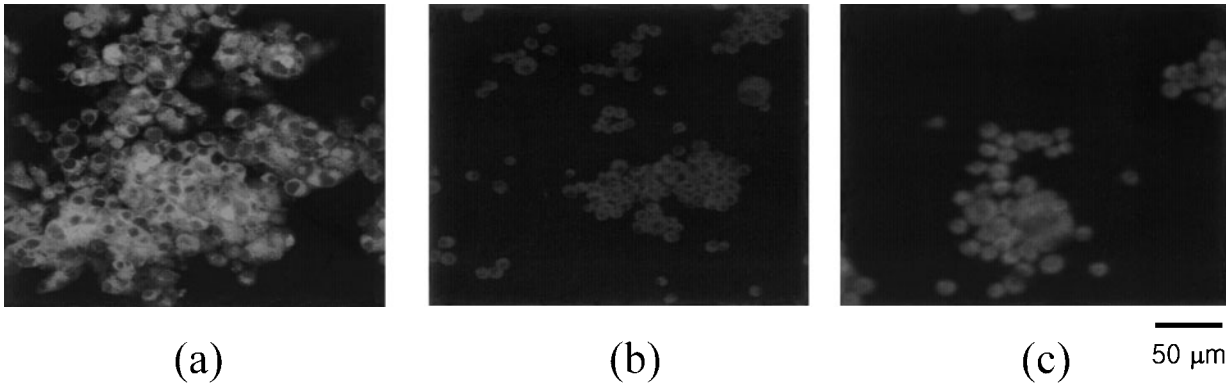

Fig. 5. **Confocal laser microscopic view of MIN6 cells incubated with RITC-labeled polymer in the presence or absence of SU ligand at 37°C.** Pretreated and normal MIN6 cells were incubated in HEPES-balanced KRBB (without BSA) with low glucose concentration for 2 h with labeled polymer. (a) copolymer with SU ligand [p(VMA-co-SU)], (b) polymer without SU ligand (PVMA), and (c) diazoxide pretreated cells with SU ligand copolymer [p(VMA-co-SU)].

Suppression of Binding Affinity by Diazoxide—The ATP-sensitive potassium channel plays a central role in regulating physiological insulin secretion from β-cells in the islets of Langerhans. SU is a K^+ channel closer (antagonist) and stimulates insulin secretion (*22*). Diazoxide behaves in the opposite way by opening the K^+ channel (agonist), which then inhibits insulin secretion (*23*). It is known that binding of diazoxide with its receptor can partially suppress the binding of SU onto SUR1 and disturbs the effects of SU on both electrical activity and ion fluxes (*24*). Diazoxide is also known to suppress ATP synthesis in the cells. When diazoxide was added 20 min after the start of the incubation, by which time ATP in the cells at a high glucose concentration is expected to be completely converted to ADP by hydrolysis, it reduced glibenclamide binding to the same extent as that found when it was present from the start of the incubation (*25*, *26*). For this reason, in this study, diazoxide was used for the inhibition of fluorescence intensity on the cells. Figure 4 illustrates that the suppression on fluorescence intensity was observed by pretreatment with diazoxide (100 μM) in the presence of low glucose concentration (25 mM). Diazoxide suppressed the effect of SU-incorporated copolymer on binding of SU to MIN6 cells to approximately 50% of diazoxide-free condition due to its inhibition of binding of SU moieties to the SU-receptor. Also, the monomer type of SU was used for a competition test. Commercial glibenclamide and SU-conjugated polymer were also tested for competition with each other. The ratios of polymer versus glibenclamide were 10:1, 1:1, and 1:10. As the concentration of glibenclamide increased (0.1 nM, 1 nM, and 10 nM), the binding affinity to the cells were significantly decreased. The binding affinities of glibenclamide and SU-conjugated polymer at a ratio of 1:10 were hihger than that at any other ratio (data not shown). This result suggested that the pretreated diazoxide abolished the responsiveness of MIN6 cells to SU moieties of copolymer.

Fluorescence microscopic observation of MIN6 cells was used to visualize the specific interaction. In Fig. 5a, MIN6 cells were strongly luminated by interaction with RITC-labeled SU-incorporated copolymer. However, in the control experiment, no lumination was observed on MIN6 cells incubated with PVMA homopolymer, which did not incorporate SU ligands (Fig. 5b). These results indicate that the SU-incorporated copolymer has negligible non-specific interactions with cells and support the notionthat SU moieties on the copolymer strongly associated with SU receptors on the cell membrane *via* receptor-mediated specific interaction. Figure 5c, illustrates that the specific interaction between RITC-labeled SU-incorporated copolymer and MIN6 cells was inhibited by pretreatment of the cells with diazoxide. Since diazoxide is known to have lower binding affinity than SU for cells, and these compounds influence each other's binding affinity, the MIN6 cells were allowed to interact with diazoxide before exposure to SU. Therefore, the lumination of MIN6 cells incubated with RITC-labeled SU-incorporated copolymer was significantly decreased when the cells were pretreated with diazoxide.

In conclusion, the specific interaction between a synthetic polymer and K_{ATP} channel was confirmed by confocal microscopy and by an inhibition test using diazoxide. Confocal microscopic images well agreed with the previous study. Based on the results of the present study, SU-incoporated polymer can be a useful tool for the study on physiological action of K_{ATP}.

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