—Regular Article—

Effects of Sperminated Pullulans on the Pulmonary Absorption of Insulin

Toshinobu SEKI,*,[a] Nanako FUKUSHI,[b] Hiroto MARU,[a]
Soichiro KIMURA,[a] Sumio CHONO,[b] Yuya EGAWA,[a]
Kazuhiro MORIMOTO,[b] Hideo UEDA,[a] and Yasunori MORIMOTO[a]

[a]*Faculty of Pharmaceutical Sciences, Josai University, 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan and* [b]*Hokkaido Pharmaceutical University School of Pharmacy, 7–1 Katsuraoka-cho, Otaru, Hokkaido 047–0264, Japan*

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Sperminated pullulans (SP) having different molecular weights (MWs) were prepared, and the enhancing effect on the pulmonary absorption of insulin in rats was examined. SP acted as enhancers of insulin absorption when a 0.1% solution was applied with insulin simultaneously and their enhancing effects depended on the MW of the SP; the same solutions exhibited low toxicity in the *in vivo* LDH leaching test. In the *in vitro* experiments using Calu-3 cells, tight junction-opening effects and a toxic effect of SP in the MTT assay were observed at lower concentrations compared with the *in vivo* experiments. A mucus layer might interfere with the interaction between SP and the cell surface and might suppress both these effects and toxicity. SP having a high MW will be useful for preparing safe and efficient formulations of peptide and protein drugs. The change in the localization of the tight junction proteins may be related to the permeation-enhancing mechanism of SP.

Key words——sperminated pullulan; insulin; pulmonary absorption; absorption enhancer

INTRODUCTION

Since the pulmonary alveoli have a large surface area and a rich blood supply, pulmonary administration of peptide and protein drugs is one possible way to avoid the problems associated with parenteral formulations, such as tissue invasion, and also to improve patient compliance.[1] In order to increase the bioavailability of peptide and protein drugs in pulmonary delivery systems, absorption enhancers, which increase the permeability of drugs through the epithelial membranes without causing any tissue damage, are especially useful.[2,3] Although surfactants, bile salts and fatty acids have been evaluated as absorption enhancers, they produce membrane damage.[4,5] Therefore, safe absorption enhancers are needed for suitable pulmonary delivery systems for peptide and protein drugs.

It has recently been reported that cationic polymers, including chitosan and its derivatives, poly-L-arginine and aminated gelatins, are able to improve the absorption of peptide and protein drugs through mucosal membranes while causing negligible damage to these membranes.[6–8] The cationic polymers can interact with the luminal surface of mucus membranes directly by an ion-ion interaction, and then may increase the intercellular permeation of water-soluble drugs. In our previous reports, sperminated polymers having different numbers of amino groups were prepared and the absorption-enhancing effects on the pulmonary absorption of insulin were examined in rats.[9] The results obtained suggest that sperminated pullulans (SP) are potential candidates as additives for pulmonary delivery systems of insulin, exhibiting high absorption-enhancing effects with little irritation of local tissues.

In this study, SP having different molecular weights (MWs) were prepared and their absorption-enhancing effects on the pulmonary absorption of insulin were evaluated in rats. In addition, the enhancing mechanism of SP was examined using Calu-3 cell monolayers.

METHODS

Materials Pullulans (MW 6 kDa, 47 kDa, 112 kDa, 788 kDa) were purchased from Showa Denko (Tokyo, Japan). Recombinant human insulin (26 IU/mg), Glucose B-test kits, and N,N′-carbonyldiimidazole were purchased from Wako Pure Chemical Industries (Osaka, Japan). Spermine, glucosamine hydrochloride, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were

*e-mail: sekt1042@josai.ac.jp

purchased from Sigma Chemical Co. (St Louis, MA, USA). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of reagent grade and used as received.

Synthesis of SP Pullulan (50 mg) and N,N′-carbonyldiimidazole (225 mg) were dissolved in dimethyl sulfoxide (DMSO, 5.0 ml) and then the solution was mixed with a spermine solution in DMSO (1.87 g in 43 ml).[10] The resulting solution was kept for 20 h at 34°C and the SP was purified by dialysis for 72 h and then the powder was obtained by lyophilization. Nanosep® centrifugal devices (3K, MW cutoff 3,000, PALL Life Sciences, Ann Arbor, MI, USA) were used to check contamination by free spermine.

In order to determine the amino group content of SP, a 1 ml solution of SP (0.50 mg/ml) in phosphate buffered saline (PBS, pH 7.4) was mixed with 1.0 ml sodium bicarbonate solution (4.0%) and 1.0 ml TNBS aqueous solution (0.10%). The mixture was kept at 40°C for 2 h protected from light and then the absorbance of the solution at 415 nm was determined.[11] Calibration curves were prepared using glucosamine. The primary amino group content (PA) was expressed as the amount of TNBS-reactive amino groups in 1 g SP. Since the addition of spermine introduced not only primary amino groups but also secondary groups to the pullulan, the total amino group content (TA) of SP was calculated using Eq (1):[9]

$$TA = PA \times 3 \quad (1)$$

where PA is the primary amino group content of SP. Elemental analysis was used to confirm the amino group content and the results obtained suggested the validity of the TNBS method (data not shown).

Pulmonary Administration of Insulin to Rats Animal experiments were carried out in accordance with the Guiding Principles for the Care and Use of Experimental Animals, Hokkaido Pharmaceutical University (2006). Male Wistar rats (Sankyo Labo-Service Co.), weighing 200–240 g, were fasted for 18–24 h before the experiments, but had free access to water. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg. Insulin solution (40 IU/ml, in PBS), with or without 0.1% SP, was administered to the bronchus using a MicroSprayer® (Penn Century Inc., PA, USA) at a dose of 10 IU/250 μl/kg. Blood samples (0.1 ml each) were withdrawn from the jugular vein 10 min before administration and at predetermined times after dosing for up to 5 h. After centrifugation of the blood samples at 8,000$\times g$ for 5 min, the plasma was isolated and the plasma glucose concentration was determined using a Glucose B-test kit (glucose oxidase method). In order to quantify the enhancing effect on insulin absorption, we calculated the D% value defined by Eq (2):[8]

$$D\% = \frac{AUC_{G,\,PBS} - AUC_{G,\,Insulin}}{AUC_{G,\,PBS}} \times 100 \quad (2)$$

where $AUC_{G,\,PBS}$ and $AUC_{G,\,Insulin}$ are the area under the curves of the plasma glucose levels from 0 h to 5 h after pulmonary administration of PBS and insulin solution, respectively.

Toxicity of SP after Pulmonary Application The male Wistar rats were treated in the same way as in the insulin application experiments. SP or other enhancer solutions were administered to the bronchus using the MicroSprayer®. The trachea was cannulated 5 h after application and the lungs were lavaged three times with 5 ml ice-cold PBS. These solutions were centrifuged (650$\times g$, 5 min) to obtain the bronchoalveolar lavage fluid (BALF) as the supernatant.[12] The lactate dehydrogenase (LDH) activity in the BALF was measured using an LDH-Cytotoxic Test Wako (Wako Pure Chemical Industries).

MTT Assay with Calu-3 Cell Monolayers Calu-3 cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 40 μg/ml gentamicin and 1% nonessential amino acids, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells from passage number 43–63 were seeded (4.5×10^5 cell/cm^2) on polyester filter inserts (pore size 0.4 μm, area 0.33 cm^2, Transwell, Costar, NY, USA) and cultivated in the medium for 9–15 days before starting the experiments. SP-47 or SP-112 in Hank's balanced salt solution (HBSS) was added to the apical side of the monolayer, and the cells were kept in a CO_2 incubator at 37°C for 60 min. As a control, HBSS was added instead of SP-47 or SP-112 solution. The apical solution was replaced with MTT in culture medium (5 mg/ml), and the cells were kept in the CO_2 incubator for 4 h. After removal of the apical solution, a DMSO-2-propanol (1 : 1) mixture was added for extraction of the purple formazan product. The absorbance of the retrieved apical solution at 570 nm was then measured using a UV-VIS spectrophotometer (UV-1600, SHIMADZU, Kyoto, Japan).

Evaluation of Penetration-enhancing Effects of SP in Calu-3 Cell Monolayers Transepithelial electrical resistance (TEER) was measured to evaluate the penetration-enhancing effect in the Calu-3 cells monolayers using a Millicell®-ERS (Millipore, MASS, USA) and the monolayers exhibiting 330–660 $\Omega \cdot cm^2$ TEER were used for the evaluation. SP-47 or SP-112 solution in culture medium was applied to the apical side 30 min after the initialization with SP-free medium. The TEER time profiles were measured from the initialization to 90 min after SP application. The TEER values were expressed as a percent of the initial values.

Immunohistological Observations Calu-3 cells were cultured on a Chamber Slide® (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells were exposed to either SP-47 or SP-112 solution for 1 h, rinsed with HBSS 3 times, and fixed with 4% paraformaldehyde in PBS for 15 min. After rinsing 3 times in PBS, the fixed cells were permeabilized with 0.1% Triton® X-100 for 10 min, blocked with 1% bovine serum albumin in PBS for 1 h, and then incubated with mouse anti-occludin (Zymed Laboratories Inc., CA, USA) and rabbit anti-claudin-1 (Zymed Laboratories Inc.) overnight at 4℃. They were then rinsed 3 times with PBS and incubated with either FITC-conjugated goat anti-mouse IgG antibody (Zymed Laboratories Inc.) or anti-rabbit IgG antibody (Zymed Laboratories Inc.) for 1 h at room temperature. After rinsing 3 times with PBS and once with water, the samples were embedded in VECTASHIELD® (Vector Laboratories Inc., CA, USA) for observation under a phase contrast fluorescence microscope (OLYMPUS IX71, Tokyo, Japan).

RESULTS

Amino Group Content of SP Table 1 shows the amino group content of the prepared SP. The SP prepared did not contain unbound spermine. The amino group content was slightly reduced depending on the MW of the pullulans used for the synthesis. The content of SP-788 was 80% that of SP-6. The reaction efficiency might be associated with the mobility of the pullulans in the reaction medium. PA and TA are both shown in Table 1. Our previous report suggested that TA is a better parameter than PA with regard to the enhancing effect.[9]

Effect of SP on the Pulmonary Absorption of Insulin in Rats The effects of SP on the pulmonary absorption of insulin were examined in rats. The PS concentration (0.1%) was chosen after referring to our previous report.[9] The absorption of insulin after pulmonary application was evaluated by the change in plasma glucose. Figure 1(a) shows the effects of SP with different MWs on the time-profiles of plasma glucose after pulmonary administration of insulin. In the case of insulin solution without SP, the plasma glucose profiles are similar to those following PBS application without insulin (data not shown). The glucose profiles showing an initial fall and then a steady increase may be due to the effect of the anesthetic on the rats. In the case of SP treatments, the reduction in plasma glucose levels depended on the MW. Figure 1 (b) shows the D% values, used as indexes of the pharmacological effect of insulin, correlating with the MW of SP.

Table 1. Amino Group Content of SP Used

	MW[a] (kDa)	Primary amino group content[b] (NH_2–) (mmol/g polymer)	Total amino group content[c] (NH_2–, NH=) (mmol/g polymer)
SP-6	5.9	2.10±0.14	6.31
SP-47	47.3	2.07±0.09	6.21
SP-112	112	1.79±0.09	5.37
SP-788	788	1.69±0.14	5.06

[a] Molecular weight of pullulan used for the preparations of SP. [b] The primary amino group content (PA) was expressed as the amount of TNBS-reactive amino groups in 1 g SP (mean±S.E.M., $n=5$). [c] The total amino group content in SP (TA) was calculated using the following equation: TA=PA×3.

Toxicity of SP after Pulmonary Application The toxicity of SP was examined using the LDH-leaching test for BALF (Fig. 2). Benzalkonium chloride (0.1%) and Triton X100 (0.25%) were used as a positive control. The LDH-leaching of SP (0.1%) was similar to that of an insulin solution (40 IU/ml) or a native pullulan (MW= 47.3 kDa, 0.1%) and was lower than sodium taurocholate (2 μmol/l) and EDTA-2Na (20 μmol/l), used as other absorption enhancers. This result shows that SP is a safe absorption enhancer for pulmonary delivery of insulin.

Cell Toxicity of SP in Calu-3 Cells The effects of SP on the viability of the Calu-3 cells were examined using MTT assay. Figure 3 shows the viability after a 1 h application of SP solutions. The viability was about 80% less than that of 0.02% SP without any concentration-dependence. Above 0.05%, the viability was suppressed and that for 0.1% SP was about 60% after a 1 h treatment, although no LDH-

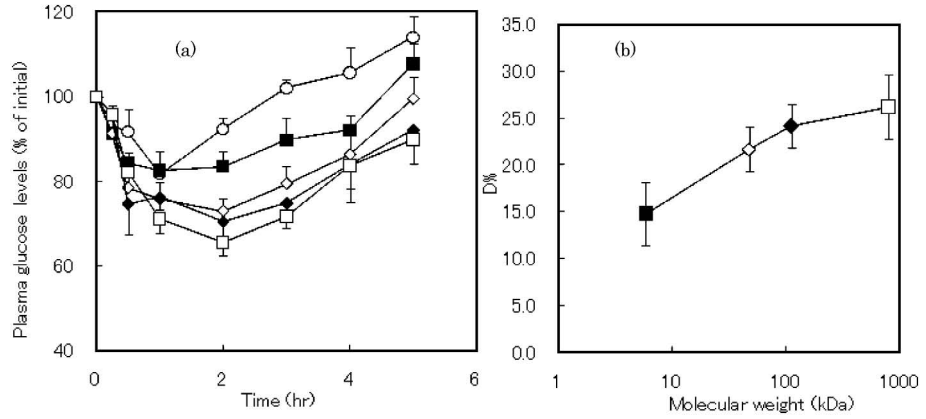

Fig. 1. Effect of SP on the Pulmonary Absorption of Insulin in Rats

(a) Glucose levels in plasma after pulmonary administration of insulin. (b) Relationship between the MW of SP and the D% values. Insulin (10 IU/kg) alone, ○; insulin (10 IU/kg) with SP-6 0.1%, ■; insulin (10 IU/kg) with SP-47 0.1%, ◇; insulin (10 IU/kg) with SP-112 0.1%, ◆; insulin (10 IU/kg) with SP-788 0.1%, □. Each data set is the mean+S.E.M. (n=4).

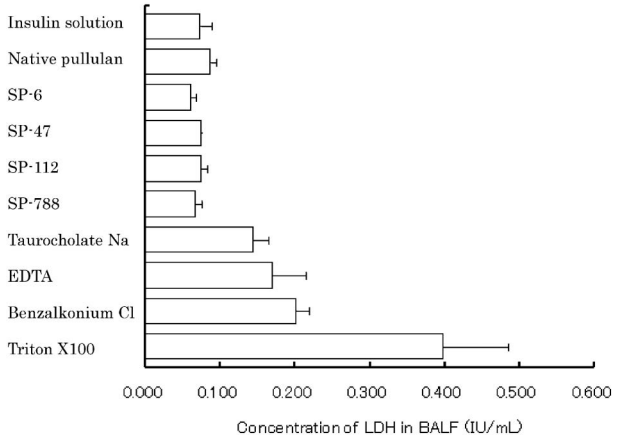

Fig. 2. Effect of SP and Absorption Enhancers on the Leaching of LDH into BALF 5 h after Pulmonary Administration of Each Solution

Insulin solution (40 IU/ml), native pullulan (0.1%), SP-6 (0.1%), SP-47 (0.1%), SP-112 (0.1%), SP-788 (0.1%), taurocholate Na (2 μmol/l), EDTA-2Na (20 μmol/l), benzalkonium Cl (0.1%), Triton X100 (0.25%). Each data set is the mean+S.E.M. (n=4).

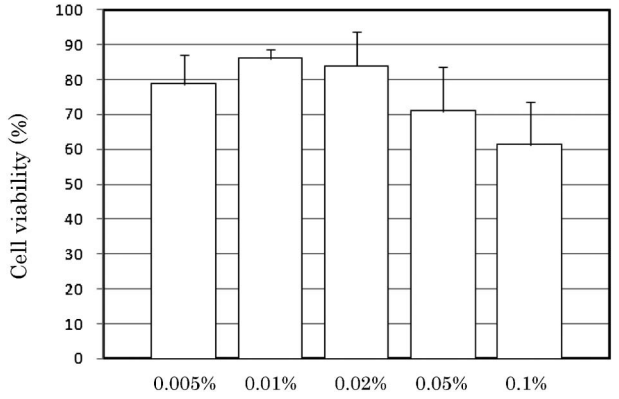

Fig. 3. Effect of SP-112 on the Viability of Calu-3 Cells

SP-112 solution (0.005–0.1%) was applied to the Calu-3 cell monolayer for 1 h and the cell viability was evaluated by MTT assay. Each data set is the mean+S.E.M. (n=3).

leaching was observed at the same concentration in the *in vivo* studies for 5 h.

Effects of SP on the TEER of Calu-3 Cell Monolayers The effects of SP on the TEER of the Calu-3 cell monolayers were evaluated to determine the tight junction-opening effects of SP under *in vitro* conditions. Figure 4 shows the TEER time-profiles of the Calu-3 cell monolayers following application of the SP solutions (SP-47=(a), SP-112=(b)). The values were reduced with time to a degree that depended on both the MW and concentration of SP. Figure 5 shows the reduced TEER values at 90 min ($\Delta TEER_{90}$) *versus* the concentration of SP. SP-112 had a stronger effect on the tight junction-opening than SP-47.

Effects of SP on the Tight Junction Proteins of Calu-3 Cell Monolayers Immunostaining was carried out for occludin and claudin-1 to evaluate the effects of SP on the location of the tight junction proteins in the Calu-3 cell monolayers (Fig. 6).[13] Under control conditions, occludin and claudin-1 were located at the periphery of the individual cells, suggesting their functions. In the cell monolayers treated with SP-112 solution (0.02 and 0.1%), the staining of occludin and claudin-1 was less intense at the periphery of the cells for both occludin and claudin-1.

DISCUSSION

In previous reports, we prepared cationized polymers with different numbers of amino groups using gelatin and pullulan and showed that their enhancing effects on the pulmonary absorption of insulin in rats depended on the amino group content.[9] In this study,

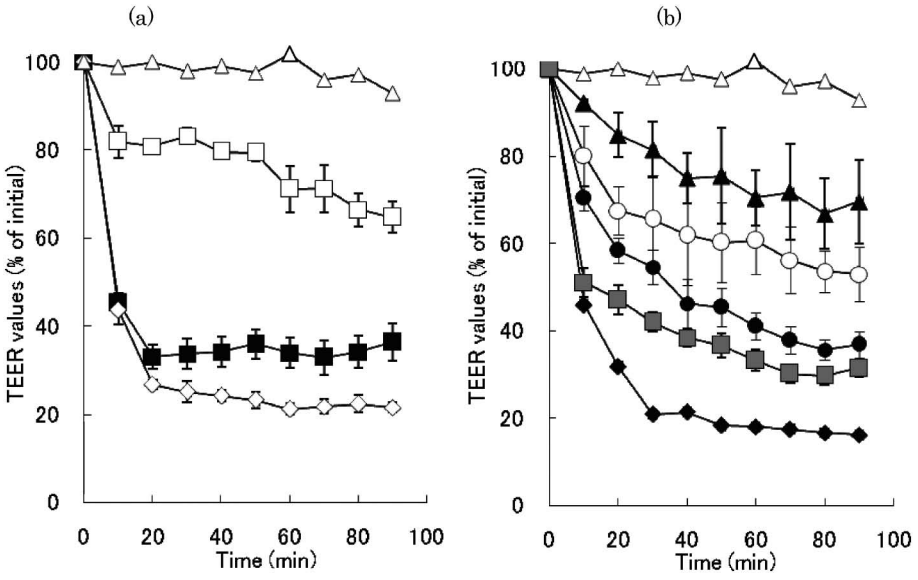

Fig. 4. Effect of SP on the TEER of Calu-3 Cell Monolayers

(a) SP-47: control, △; 0.02%, □; 0.05%, ■; 0.1%, ◇. (b) SP-112: control, △; 0.001%, ▲; 0.005%, ○; 0.01%, ●; 0.02%, ■; 0.1%, ◆. Each data set is the mean+S.E.M. (n=4).

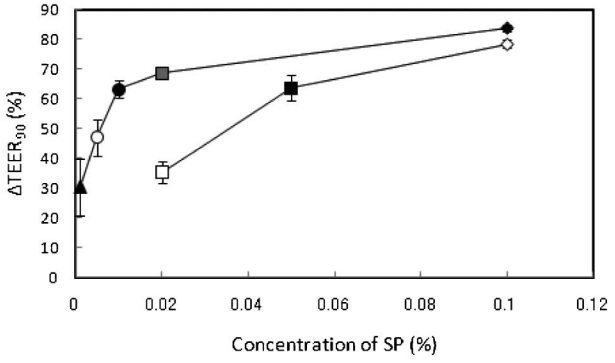

Fig. 5. Relationship between the Concentration of SP and the Reduction in TEER at 90 Min ($\Delta TEER_{90}$)

SP-47: 0.02%, □; 0.05%, ■; 0.1%, ◇. SP-112: 0.001%, ▲; 0.005%, ○; 0.01%, ●; 0.02%, ■; 0.1%, ◆. Each data set is the mean+S.E.M. (n=4).

we prepared SP having different MWs to examine the MW dependence on the absorption-enhancing effects. The amino group content of the SP prepared was slightly dependent on the MW. However, since the dependence was very weak and the values of the higher MWs were the lowest, the SPs prepared were suitable for our purpose.

The enhancing effects of SP were first evaluated in terms of the pulmonary absorption of insulin in rats. In the *in vivo* experimental system, SP acted as enhancers when the 0.1% solution was applied with insulin simultaneously and their enhancing effects depended on the MW (Fig. 1). The D% for SP-788 was 1.8 times higher than that of SP-6, although the amino group content was 0.8 times that of SP-6 and the number of molecules applied was only 0.75% when SP-788 was compared with SP-6. This MW dependence agrees with the findings of Natsume *et al.* for nasal absorption-enhancing effects of poly-L-arginines.[6,14] The larger molecules have a higher potential for absorption-enhancing effects. In such *in vivo* experiments, differences in the access to and retention at the absorption site, *e.g.*, the pulmonary alveolus, should be considered. The higher viscosity of larger SP might affect their local disposition as well as that of insulin in the lung. Spermine itself is known as a mucosal absorption-enhancer for water-soluble macromolecules.[15] However, the results in this report were related to the SP, not free spermine, because SP was stable in BALF for a few hours (data not shown).

In order to confirm the safety of SP, LDH leaching tests were carried out for BALF 5 h after pulmonary application of 0.1% SP solution. The toxicity of SP was low when compared with other absorption enhancers (taurocholate Na and EDTA-2Na), an additive for a topical product (benzalkonium Cl), and a well known positive control (Triton X100). This suggests that SP will be safe absorption-enhancers for pulmonary delivery systems of peptide and protein drugs.

The toxicity of SP was also examined using an

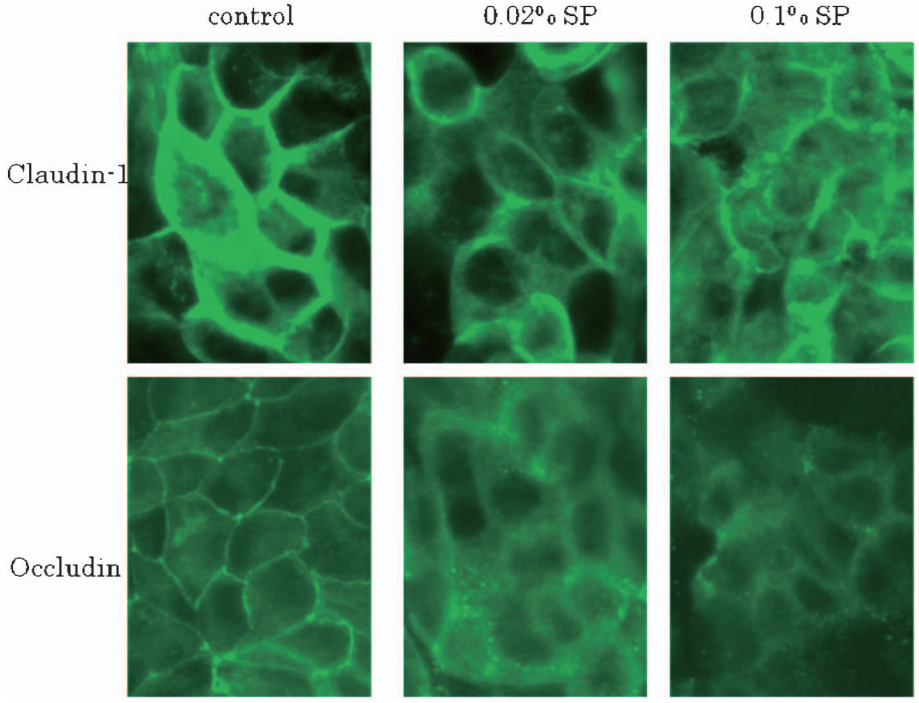

Fig. 6. Effect of SP-112 on the Distribution of Claudin-1 and Occluding in Calu-3 Cells

MTT assay for Calu-3 cells. Suppressed cell viability was observed after a 1 h treatment with a 0.1% solution of SP-112, while the *in vivo* toxicity was negligible for the 5 h treatments. The MTT assay was more sensitive than the LDH leaching test. The presence of a mucus layer on the epithelium could be related to the difference in toxicity between *in vitro* and *in vivo* findings. Since the cell surface has a negative charge, cationized SP can interact with it as a trigger for the enhancing effects. The mucus layer might interfere with the interaction between SP and the cell surface and might suppress the effects.

Since the MW and concentration-dependence of the penetration-enhancing effects of SP could be different *in vitro* and *in vivo*, these were also examined using Calu-3 cell monolayers. In the *in vitro* system, SP can easily interact with the cell surface. This binding can trigger both the enhancement and toxicity. A rapid reduction in TEER was observed in the Calu-3 cell monolayers treated with SP-47 and SP-112 within 1 h. The $\Delta TEER_{90}$ values were used to evaluate the tight junction-opening effects of SP-47 and SP-112 at various concentrations in the Calu-3 cell monolayers (Fig. 5). A concentration-dependence was observed both for SP-47 and SP-112, but SP-112 had a junction-opening effect at a lower concentration than SP-47. The MW-dependence was more marked compared with the *in vivo* enhancing effects for the pulmonary absorption of insulin. Since differences in the viscosity of the solution applied would have little effect on the access to and retention at the surface of the cell layers in the *in vitro* experimental system, the MW-dependence was simply due to a difference in the biological activity of the SP. The initial tight junction-opening effects of SP-112 within 1 h varied over the concentration range of 0.005%–0.02%, while the result of the MTT assay was independent of concentration over the same range. This means that the enhancing effect and toxicity can be controlled separately, suggesting the possibility of designing a safe and efficient formulation. While the existence of a mucus layer and other factors can affect the enhancing effects of SP in the *in vivo* experiments, the *in vitro* system using Calu-3 cells is better for examining the enhancing mechanism of SP. Long-term experiments using Calu-3 cells will be needed as the next step, considering cell toxicity during long-term application and the reversibility of the tight junction-opening.

In our previous report about the enhancing effects of cationized polymers in Caco-2 cell monolayers, the enhancing mechanism for insulin permeation involves an increase in the number of permeation pathways in the tight junctions.[16] The tight junctions, acting as a

permeation barrier, could be converted to permeation pathways by the opening-effect of the cationized polymers. Such tight junction-opening results in a reduction in the TEER of the cell layers. The positively charged SP could interact with the luminal surface of mucus membranes directly by an ion-ion interaction and then the surface could be given a positive charge. This interaction could be a trigger to induce signals that would open tight junctions resulting in intercellular permeation of water-soluble drugs.[17] In the addition, the positive surface might help allow the negatively charged insulin access to the permeation pathways.[18] The mechanism of signal transduction to open the tight junctions following the ion-ion interaction on the cell surface is still unknown. The pore pathways in the tight junctions might be physiologically regulated in different ways and the contribution of protein kinase A (PKA) and protein kinase C (PKC) to each process has been discussed.[19,20] While activation of PKA does not change the barrier function for large molecules, activation of PKC may increase the permeability of these large molecules.[20] This PKC pathway may be one of the possible mechanisms for the penetration enhancers for insulin allowing them to pass through epithelial cell layers.[21] A change in the F-actin cytoskeleton might involve this pathway. Ranaldi *et al*. reported that chitosan affected the F-actin cytoskeleton and localization of tight junction proteins, ZO-1 and occludin, resulting in a reduced TEER and increased inulin passage.[22] Similar changes in the F-actin cytoskeleton and tight junction proteins have also been reported for protamine and poly-L-lysine treatments.[23–25] In our preliminary experiment, SP-112 suppressed the intensity of immunostaining of F-actin at the periphery of the Calu-3 cells (data not shown). In this report, the immunostaining suggests that SP affects localization of occludin and claudin-1 at the periphery of the cells. Such changes in the location of tight junction proteins should be related to the formation of permeation pathways for peptide and protein drugs.

CONCLUSION

In this study, SP having different MWs were prepared, and their enhancing effects on the pulmonary absorption of insulin in rats and the tight junction-opening effects in Calu-3 cell monolayers were examined. In the addition, the toxicity of SP was examined by the *in vivo* LDH leaching test for BALF and the MTT assay in Calu-3 cells. Although the effects and toxicity depended on the MW and/or the concentration of SP in both the *in vivo* and *in vitro* experiments, these were more marked in the *in vitro* experiments. Since the enhancing effect of SP was observed at a low concentration without any toxicity, SP will be useful for the safe and efficient design of new formulations for peptide and protein drugs. The change in the localization of the tight junction proteins should be related to the permeation-enhancing mechanism of SP.

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