Effect of Aminated Gelatin on the Nasal Absorption of Insulin in Rats

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Absorption enhancers, which increase the permeability of drugs through epithelial membranes without damaging them, are especially useful for intranasal administration of peptide drugs. In this study, aminated gelatins, candidate enhancers, having different numbers of amino groups were prepared from gelatin (H-gelatin, isoelectric point=9.0, MW 100 kDa) and a partial gelatin hydrolysate (L-gelatin, isoelectric point=8.0, MW 5 kDa), and the enhancing effects on the nasal absorption of insulin, used as a model peptide drug, and 5(6)-carboxyfluorescein (CF), a paracellular marker, were examined in rats. The enhancing effect on insulin and CF depends on the MW and number of amino groups. A high correlation between the enhancing effects on insulin and CF was observed and this suggests that an increase in the paracellular permeability is the mechanism governing the nasal absorption-enhancement of aminated gelatins, at least as far as insulin and CF are concerned. The enhancing mechanism might be shared with other cationic polymers having absorption-enhancing effects.

Key words nasal absorption; aminated gelatin; absorption enhancer; insulin

Peptide and protein drugs are usually used as parenteral formulations but this method of administration is sometimes associated with tissue invasion and infection. To avoid such problems and improve patient compliance, non-invasive administration routes are needed for the delivery of peptide and protein drugs. Nasal drug delivery is an excellent method for the delivery of such drugs because of the relatively high permeability of the nasal epithelial membrane and avoidance of first-pass metabolism in the liver.¹⁾ The use of absorption enhancers²⁾ and proteolytic enzyme inhibitors³⁾ and suitably designed formulations⁴) are needed to develop nasal delivery systems for peptide and protein drugs exhibiting higher bioavailability. The absorption enhancers, which increase the permeability of drugs through the epithelial membranes without causing any damage to them, are especially useful in designing new formulations.¹⁾ Although many chemicals, including surfactants, bile salts and fatty acids, have been evaluated as absorption enhancers, most of them produce some form of membrane damage.^{5,6)}

It has recently been reported that cationic polymers including chitosan^{1,7,8)} and poly-L-arginine^{9,10)} are able to improve the nasal absorption of peptide and protein drugs while causing negligible damage to the nasal mucosal membrane. Such higher MW compounds are possible candidates as nasal absorption enhancers for peptide and protein drugs. The cationic polymers could interact with the luminal surface of mucus membranes directly by an ion–ion interaction and then induce signals that would open routes resulting in intercellular permeation.^{7,9)} The inability of polymers to pass through membranes might be related to the lower degree of damage caused to the membranes. Since the enhancing effects of polymers are reduced when they are degraded, the polymer itself and also the degradation products or the monomer will need to be biocompatible.

In our previously report, a cationized gelatin, an aminated gelatin, was prepared and its absorption-enhancing effect on insulin after nasal application was examined.¹¹⁾ An acid processed gelatin from porcine skin was chosen as the back-

bone protein because gelatin is widely used as an additive in many medicines. Ethylenediamine was made to react with the gelatin to form a cation and the resulting aminated gelatin exhibited an absorption-enhancing effect on insulin without any marked leaching of lactate dehydrogenase into the nasal cavity.

In this study, gelatin (H-gelatin) and a partially hydrolyzed gelatin (L-gelatin) were used for the preparation of the aminated gelatins. In the addition, the amination conditions were modified to obtain different aminated gelatins with a range of charge densities. The resulting aminated gelatins were examined to evaluate the effect of the molecular weight and charge density on the absorption-enhancing effect after nasal application of insulin.

MATERIALS AND METHODS

Materials H-gelatin (isoelectric point=9.0, MW 100 kDa) and L-gelatin (isoelectric point=8.0, MW 5 kDa) were kindly supplied by Nitta Gelatin Co. Ltd. (Osaka, Japan). Recombinant human insulin (28.7 IU/mg) and 1ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were purchased from Sigma Chemical Co. (St Louis, MA, U.S.A.). Glucose B-test kit and 1,2-ethylenediamine were purchased from Wako Pure Chemical Industries (Osaka) and Kanto Chemical Co. (Tokyo, Japan), respectively. 5(6)-Carboxyfluorescein (CF) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Acros Organics (NJ, U.S.A.) and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals were of reagent grade and used as received.

Synthesis of Aminated Gelatins H-gelatin or L-gelatin was reacted with 1,2-ethylenediamine to obtain aminated gelatins in the presence of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride as previously reported.¹¹) In brief, H-gelatin or L-gelatin (10 g) was dissolved in 0.1 M phosphate buffer (pH 5.0, 250 ml). 1,2-Ethylenediamine (0.28, 5.6 or 28 g) was added to the solution and then the pH

of the solution was adjusted to 5.0 with hydrochloric acid. The resulting solution was mixed with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (5.35 g) and the total volume was adjusted to 500 ml by addition of phosphate buffer (pH 5.0). The reaction was allowed to take place at 37 °C for 1 h. The generated aminated gelatins were purified by dialysis for 48 h. Eventually, the aminated gelatin powder was obtained by lyophilization.

Determination of Amino Group Content of Aminated Gelatins One milliliter of gelatin or aminated gelatin solution (0.50 mg/ml) in phosphate buffered saline (PBS, pH 7.4) was mixed with 1.0 ml of sodium bicarbonate solution (4.0%) and 1.0 ml of TNBS 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⁾ A calibration curve was prepared for β -alanine. The amino group content was expressed as amount of TNBS-reactive amino groups in 1 g of gelatin or aminated gelatin.

Preparation of Insulin Solution for Intranasal Administration Insulin (1.0 mg) was dissolved in 0.01 M hydrochloric acid (100 μ l) and then the solution was mixed with PBS (43.5 μ l) to give 200 IU/ml insulin solution. The solution was mixed with the same volume of gelatin or aminated gelatin solution (0.40%) in PBS. The pH value of the resulting solution for intranasal administration ranged from 6.9 to 7.2.

Intranasal Administration of Insulin Animal experiments were carried out in accordance with the Guiding Principles for the Care and Use of Experimental Animals, Hokkaido College of Pharmacy (1998). Male Wistar rats (Sankyo LaboService Co.), weighing 210-260 g, were fasted for 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, with an additional dose given intraperitoneally to maintain the general anesthesia as required. Surgery was performed according to the method of Hirai et al.^{12,13} Briefly, the rats were placed in the supine position, a tracheal cannulation was performed to maintain respiration, and the trachea leading to the nasal cavity was ligated to prevent the transuding liquid oozing from the surgical incision. Another polyethylene tube, with a closed top, was inserted through the oesophagus to the posterior part of the nasal cavity to prevent drainage of the applied drug solution into the nasopharynx. The insulin solutions were administered to the nasal cavity through the nostrils using a pipette (50 μ l/kg for each cavity, total 100 μ l/kg). The dose of insulin was 10 IU/kg. PBS was used as a blank control. Blood samples of 0.2 ml were withdrawn from the femoral vein 10 min before administration and at predetermined times after dosing for up to 5 h. After centrifugation of the blood samples at 10000 rpm for 5 min, the plasma was isolated and kept at -20 °C until analysis. The plasma glucose concentration was determined using a glucose B-test kit (glucose oxidase method) according to the manufacturer's instructions.

The plasma glucose levels were expressed as values relative to the glucose concentration before administration. The pharmacological effect of insulin was evaluated using the D% value defined by the following equation;¹³⁾

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

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

Intranasal Administration of CF CF solution (0.20% in PBS) was administered (0.40 mg/kg) to rats in the same way as in the case of insulin administration. Blood samples of 0.25 ml were withdrawn from the femoral vein at predetermined times after dosing for up to 8 h. After centrifugation of the blood samples at 10000 rpm for 5 min, plasma samples of 100 μ l were diluted 10-fold with PBS and the CF concentration in the solution was determined using a fluorescence spectrophotometer (*ex.* 495 nm and em. 515 nm, F-2000, Hitachi, Tokyo). The area under the CF concentration—time profile from 0 to 8 h (*AUC*_{CF}) was used as a parameter to evaluate CF absorption.

Statistical Analysis The mean values and the standard error of the mean (S.E.) were calculated in each experiment. The D% values after nasal application of insulin with aminated gelatins were compared with that of insulin alone. Statistical significance was evaluated by the Dunnett test. The effect of the MW of aminated gelatin on the enhancing effects was evaluated by analysis of covariance (ANCOVA). StatView software (Ver. 5.0, SAS Institute Inc.) was used for the calculation.

RESULTS

Preparation of Aminated Gelatins Two different gelatins, H-gelatin and L-gelatin, were used for the preparation of aminated gelatin. Different amounts of ethylenediamine were used to obtain aminated gelatins with different charge densities, and amino group contents. Table 1 shows the amino group content of the gelatins and aminated gelatins used in this study. The amino group content is the number of TNBS reactive primary amino groups and is expressed as mmol/g gelatin. The difference in amino group

Table 1. Amino Group Content of Aminated Gelatins Used

	H-gelatin			 L-gelatin		
	Ethylenediamine ^{a)} (g)	Amino group content ^{b)} (mmol/g gelatin)		Ethylenediamine ^{a)} (g)	Amino group content ^{b)} (mmol/g gelatin)	
H-gelatin	0	0.27	L-gelatin	0	0.47	
H-0.028	0.28	0.40	L-0.028	0.28	0.60	
H-0.56	5.6	0.63		_		
H-2.8	28	0.81	L-2.8	28	1.02	

a) Amount of ethylenediamine added to 10 g of H- or L-gelatin during the preparation. b) Amount of TNBS-reactive amino group in 1 g of gelatin or aminated gelatin.

content between native H-gelatin and L-gelatin is related to the number of N-terminals per unit weight.

Effect of Aminated Gelatin on the Nasal Absorption of The effects of aminated gelatins on the nasal ab-Insulin sorption of insulin were examined in rats. The absorption of insulin was detected by measuring its hypoglycemic effect, *i.e.* the change in plasma glucose concentration. Figure 1 shows the effects of aminated gelatins having different numbers of amino groups on the absorption of insulin. In the case of insulin solution without gelatins and with native H-gelatin, the plasma glucose profiles are similar to that following PBS application without insulin. The glucose concentration profiles showing a transient decrease and subsequent continuous increase might be due to surgical damage and the effect of the anesthesia on the rats. Unusual secretion of endogenous insulin caused by such stress might be related to the continuous increase of glucose level. In the case of aminated Hgelatins, however, the plasma glucose concentration fell markedly and the effects depended on the amino group content. The minimum glucose concentration was observed at 0.75 or 1 h in each rat given aminated gelatin and the maximum reduction (the lowest glucose concentration of 65.7±1.2% relative to baseline) was observed at 1 h in the rats treated with the aminated H-gelatin with the highest number of amino groups (H-2.8). These results suggest that amination is effective in producing a nasal absorption-enhancing effect.

In order to examine the effect of the MW of aminated gelatins on the enhancing effect, L-gelatin prepared by partial hydrolysis of H-gelatin was used for the preparation of aminated gelatins. Figure 2 shows the effect of aminated L-gelatins on insulin absorption. The enhancing effect of aminated L-gelatins is relatively lower than that of H-gelatins.

The contribution of the amino group content and MW of aminated gelatins to the enhancing effects on insulin absorption were evaluated from the D% values. Figure 3 shows the relationship between the amino group content of the gelatins applied and the D% after nasal administration of insulin with



Fig. 1. Effects of Aminated H-Gelatin on the Change in Plasma Glucose Levels after Intranasal Administration of Insulin to Rats

Each solution was applied to the nasal cavity of rats $(100 \,\mu l/kg)$. \blacklozenge , Phosphate buffered saline (pH 7.4, PBS); \blacksquare , insulin (100 IU/ml, 10 IU/kg) in PBS; \blacklozenge , insulin (100 IU/ml, 10 IU/kg) and H-gelatin (0.20%) in PBS; \bigcirc , insulin (100 IU/ml, 10 IU/kg) and H-0.028 (0.20%) in PBS; \bigtriangleup , insulin (100 IU/ml, 10 IU/kg) and H-0.56 (0.20%) in PBS; \square , insulin (100 IU/ml, 10 IU/kg) and H-0.56 (0.20%) in PBS; \square , insulin (100 IU/ml, 10 IU/kg) and H-0.56 (0.20%) in PBS; \square , insulin (100 IU/ml, 10 IU/kg) and H-2.8 (0.20%) in PBS. Each data set is the mean \pm S.E. (n=3—7).

the gelatins. Since the D% values after administration of insulin with H-0.56 (18.2±1.5%), H-2.8 (25.8±2.2%) and L-2.8 (10.5±2.2%) were significantly higher (p<0.05) than that of insulin alone (1.80±1.92%), not only aminated Hgelatins but also aminated L-gelatins enhance insulin absorption. However, the effect of aminated L-gelatins is low compared with that of H-gelatins. The ANCOVA results suggest that the D% depends directly on the amino group content of the aminated gelatins (p<0.001) but the slope of the relationship for H-gelatins is significantly higher than that for Lgelatins (p<0.005). These results suggest that gelatins require both a higher MW and a greater amino group content to have absorption-enhancing effects as far as the nasal absorption of insulin is concerned.

Effect of Aminated Gelatin on the Nasal Absorption of CF The effects of aminated gelatins on the nasal absorption of CF were examined in an attempt to discover the enhancing mechanism of the aminated gelatins. CF was used as a paracellular marker. Both suppression of the permeation



Fig. 2. Effects of Aminated L-Gelatin on the Change in Plasma Glucose Levels after Intranasal Administration of Insulin to Rats

Each solution was applied to the nasal cavity of rats $(100 \,\mu l/kg)$. \blacklozenge , PBS; \blacksquare , insulin $(100 \, IU/m1, 10 \, IU/kg)$ in PBS; \blacklozenge , insulin $(100 \, IU/m1, 10 \, IU/kg)$ and L-gelatin (0.20%) in PBS; \bigcirc , insulin $(100 \, IU/m1, 10 \, IU/kg)$ and L-0.028 (0.20%) in PBS; \square , insulin $(100 \, IU/m1, 10 \, IU/kg)$ and L-2.8 (0.20%) in PBS. Each data set is the mean \pm S.E. (n=3-7).



Fig. 3. Relationship between the Amino Group Content of Aminated Gelatins and the D% after Intranasal Administration of Insulin to Rats

•, H-gelatin; \bigcirc , Aminated H-gelatin; \blacktriangle , L-gelatin; \triangle , Aminated L-gelatin. Amino group contents (AGC) are expressed in mmol/g gelatin. Lines: $D\% = -5.09 + 37.6 \times \text{AGC}$ ($R^2 = 0.814$) for H-gelatins; $D\% = -3.93 + 18.6 \times \text{AGC}$ ($R^2 = 0.698$) for L-gelatins (p < 0.001). Each data set of D% is the mean \pm S.E. (n = 3—7).



Fig. 4. Effects of Aminated H-Gelatin on the Plasma Concentration of CF after Intranasal Administration of CF to Rats

Each solution was applied to the nasal cavity of rats $(100 \,\mu l/kg)$. \bigcirc , CF (4.0 mg/ml, 0.40 mg/kg) and H-gelatin (0.20%) in PBS; \bigcirc , CF (4.0 mg/ml, 0.40 mg/kg) and H-0.028 (0.20%) in PBS; \triangle , CF (4.0 mg/ml, 0.40 mg/kg) and H-0.56 (0.20%) in PBS; \Box , CF (4.0 mg/ml, 0.40 mg/kg) and H-0.56 (0.20%) in PBS; \Box , CF (4.0 mg/ml, 0.40 mg/kg) and H-2.8 (0.20%) in PBS. Each data set is the mean ± S.E. (n=3—7).



Fig. 5. Effects of Aminated L-Gelatin on the Plasma Concentration of CF after Intranasal Administration of CF to Rats

Each solution was applied to the nasal cavity of rats $(100 \,\mu l/kg)$. \bullet , CF (4.0 mg/ml, 0.40 mg/kg) and L-gelatin (0.20%) in PBS; \bigcirc , CF (4.0 mg/ml, 0.40 mg/kg) and L-0.028 (0.20%) in PBS; \Box , CF (4.0 mg/ml, 0.40 mg/kg) and L-2.8 (0.20%) in PBS. Each data set is the mean±S.E. (*n*=3—7).

barrier functions of the nasal mucous membranes and inhibition of proteolysis play a role in the enhancing mechanism of the aminated gelatins as far as insulin absorption is concerned. Since CF is not metabolized during the permeation process, the increase in CF permeation reflects suppression of the barrier function of the membranes. Figures 4 and 5 show the plasma concentration-time profiles of CF after intranasal administration of H-gelatins and L-gelatins, respectively. Since the CF profiles were irregular in some cases, the AUC_{CF} was used as a parameter to evaluate the enhancing effect of the aminated gelatins on CF absorption. Figure 6 shows the relationship between the amino group content of the aminated gelatins and the AUC_{CF} values after nasal administration of gelatins. The profiles in Fig. 6 are similar to those in Fig. 3. The ANCOVA results suggest that the absorption-enhancing effects depend on the amino group content (p < 0.001) and the effects of aminated H-gelatins are



Fig. 6. Relationship between the Amino Group Content of Aminated Gelatins and the AUC_{CF} after Intranasal Administration of CF to Rats

●, H-gelatin; ○, Aminated H-gelatin; ▲, L-gelatin; △, Aminated L-gelatin. Amino group contents (AGC) are expressed in mmol/g gelatin. Lines: AUC_{CF} =0.819+21.2×AGC (R^2 =0.490) for H-gelatins; AUC_{CF} =2.79+7.21×AGC (R^2 =0.318) for L-gelatins (p<0.001). Each data set of AUC_{CF} is the mean±S.E. (n=3—7).

different from those of aminated L-gelatins (p < 0.001). A higher MW and amino group content of gelatins are also needed to enhance the nasal absorption of CF.

DISCUSSION

The absorption-enhancing effects of cationic polymers depend on their chemical composition, MW and charge density. Schipper et al. reported that chitosans having a higher MW and a lower degree of acetylation (higher positive charge) were more effective in increasing the permeation of mannitol through Caco-2 cell monolayers.^{14,15)} Natsume *et al.* also examined the effect of MW and charge density on the enhancing effect of cationic enhancers.^{9,10)} They found that the charge density was the most important parameter for the enhancement of the nasal absorption of FITC-dextran (MW= 4 kDa), used as a paracellular marker. In this study, aminated gelatins with different amino group contents were prepared from H-gelatin and L-gelatin, and the absorption enhancing effects on the nasal absorption of insulin and CF were examined. In the case of insulin absorption, inhibition of proteolysis and modification of the aggregation state of insulin were involved in the mechanism of enhanced absorption.^{3,16)} Therefore, the effect of aminated gelatins on insulin absorption was compared with that on CF absorption. Figure 7 shows the relationship between the AUC_{CF} after nasal administration of CF and the D% after administration of insulin with various gelatins. The high correlation in Fig. 7 suggests that the increased paracellular permeability of the mucous membrane is the major mechanism of nasal absorption-enhancement not only for CF but also insulin. The enhancing effects of the aminated gelatins were higher if they had a higher MW and amino group content, both for insulin and CF. This is similar to the results obtained with chitosans and poly-L-arginine and. So, the cationic polymers might act by the same mechanism on the mucous membranes.¹⁷⁾ The binding of the cationic polymers to epithelial cells could be mediated through their positive charges.¹⁴⁾ The interaction might trigger the translocation of tight junction components to increase the paracellular permeability.¹⁸⁾ Since the L-gelatins with more N-terminals per unit weight were not effective as far as enhancement was concerned, the amino groups in the



Fig. 7. Relationship between the AUC_{CF} after Intranasal Administration of CF and the D% after Intranasal Administration of Insulin to Rats

●, H-gelatin; ○, Aminated H-gelatin; ▲, L-gelatin; △, Aminated L-gelatin. Line: $D\% = -4.78 + 1.68 \times AUC_{CF}$ (r=0.970, p<0.001). Each data set is the mean±S.E. (n=3-7).

N-terminals could not be involved in the interaction with epithelial cells. A number of positive charges, which are located at some distance apart on a polymer, might be needed to act as the trigger. Another significant enhancing mechanism could be a direct interaction between insulin and aminated gelatins. Indeed, in our preliminary experiment the diffusion coefficient of insulin slightly decreased following the addition of aminated gelatins, suggesting partial formation of a complex. However, since no interaction between CF and aminated gelatins was observed, the contribution of such an interaction for the enhanced absorption should be low.

When the aminated gelatins are degraded in the nasal cavity, the enhancing effects are reduced. The irregular concentration profiles of CF might be associated with such degradation. The time-profile of the enhancing effect of aminated gelatins should be evaluated in detail in the next step. Diabetic animals will be useful in the experiments to avoid the effect of endogenous insulin.

There are two types of formulations for intranasal delivery of peptide drugs: one is a solution and the other is a dry powder. The dry powders have the advantage that the drugs accumulate on the surface of the mucous membranes. The aminated gelatins are also useful as matrices for micro- and nano-particles.¹⁹⁾ Drug carriers which have bioadhesive properties and absorption-enhancing effects are suitable for the intranasal delivery of peptide drugs.^{4,20)} The aminated gelatins can be used as multifunctional delivery systems for peptide drugs.

In summary, aminated gelatins with different numbers of amino groups were prepared from H-gelatin and L-gelatin, and the absorption enhancing effects on the nasal absorption of insulin and CF were examined. The enhancing effects on insulin and CF depended on the MW and amino group content. This enhancing mechanism might be shared with other cationic polymers having absorption-enhancing effects. Although the bioavailability of insulin was not determined in this study, such quantitative evaluation would be needed to develop a nasal delivery system of insulin-containing aminated gelatins.

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