

Hypoglycemic Effect of Insulin-Transferrin Conjugate in Streptozotocin-Induced Diabetic Rats¹

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ABSTRACT

Transferrin (Tf) receptor-mediated transcytosis of insulin-transferrin conjugate (In-Tf) has been demonstrated in cultured human enterocyte-like Caco-2 cells. In the present report, oral delivery of insulin as a Tf conjugate in streptozotocin (STZ)-induced diabetic rats was investigated. Human insulin was conjugated at a 1:1 molar ratio to iron-loaded human Tf by a disulfide linkage. The stability of In-Tf and the free insulin released from In-Tf was studied in the presence of rat liver slices by using radioimmunoassay. The release of free insulin involved a disulfide reduction reaction that was inhibited by the pretreatment of the liver slice with a sulfhydryl-reactive reagent *N*-ethylmaleimide. A protease inhibitor cocktail also showed a partial inhibition of insulin degradation. The biological activity of

the conjugate was tested in STZ-induced diabetic rats with s.c. administration, and the conjugate exhibited a slow but prolonged hypoglycemic effect compared with that of the native human insulin. In-Tf also displayed a slow but prolonged hypoglycemic effect after oral administration in fasted STZ-induced diabetic rats in a dose-dependent manner. Furthermore, In-Tf was detected in the serum of rats at 4 h after oral administration of the conjugate, indicating that In-Tf can overcome the barriers in the gastrointestinal tract and be absorbed as an intact conjugate. These results demonstrate that transepithelial transport via TfR-mediated transcytosis is a feasible approach for developing the oral delivery of insulin, as well as other peptide drugs.

Insulin has been the cornerstone of type I diabetes treatment since its initial administration to humans in 1922 (Best, 1956). There are nearly 20 million people in the United States who have diabetes, and approximately 10% of these diabetics are treated using insulin therapy (Anonymous, 1997). Conventional insulin treatment is basically a replacement therapy, in which exogenous insulin is administered s.c. to mimic, as close as possible, insulin secretion of a healthy pancreas. However, s.c. injection of insulin has risk factors, such as hyperinsulinemia, pain, and inconvenience, and localized deposits of insulin that lead to local hypertrophy and fat deposits at injection sites (Skyler, 1986). Researchers are trying to find various alternatives to deliver insulin via noninvasive routes, such as nasal (Chien and Banga, 1989), rectal (Ritschel et al., 1988), pulmonary (Adjei and Gupta, 1994), and ocular deliveries (Morgan and Huntzicker, 1996). However, among all alternative routes for the administration of insulin, the oral route is the most convenient. In addition, because orally administered insulin undergoes a first hepatic pass, it will produce a similar effect as

pancreas-secreted insulin by inhibiting the hepatic gluconeogenesis and suppressing the hepatic glucose production (Lewis et al., 1996).

Unfortunately, oral delivery of peptides or proteins such as insulin poses unique problems of instability, susceptibility to proteolysis, and inability to traverse membranes and biological barriers due to their large molecular size (Roberts and Sandra, 1992). As a result, the absolute amount of intact protein reaching the target site is too small to be of pharmacological benefit. To overcome these major problems, it was suggested to administer insulin with penetration enhancers (Shao et al., 1993) or enzyme inhibitors (Yamamoto et al., 1994). However, it is generally believed that penetration enhancers or enzyme inhibitors are not acceptable for chronic use because they have been shown to be associated with various adverse side effects (Lee et al., 1991; Morishita et al., 1993). Other approaches for increasing oral absorption of insulin are to circumvent the digestion of this polypeptide in the GI tract by entrapping insulin in polymeric microspheres (Uchida et al., 1996) or by coating with polymer films (Saffran et al., 1986). However, there are still several unsolved problems associated with these approaches (Saffran et al., 1986; Uchida et al., 1996).

Receptor-mediated transcytosis has been considered an ef-

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ABBREVIATIONS: GI, gastrointestinal; TfR, transferrin receptor; Tf, transferrin; In-Tf, insulin-transferrin conjugate; Caco-2, a human colon adenocarcinoma cell line; STZ, streptozotocin; SPDP, *N*-succinimidyl 3-(2-pyridyldithio) propionate; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PIs, proteinase inhibitor cocktail; RIA, radioimmunoassay.

fective approach for achieving specific delivery of proteins and peptides across cellular barriers such as endothelium and epithelium (Pardrige et al., 1987; Shen et al., 1992). Unlike penetration enhancers, a receptor-mediated transcytotic process does not change the structure of plasma membranes or the paracellular junctions and conceivably has fewer unwanted side effects. Among all receptors, transferrin receptor (TfR) appears to be a good candidate for designing an oral delivery system because TfR density is very high in human GI epithelium, and transferrin (Tf) is a natural transport protein for iron and is resistant to tryptic and chymotryptic digestions (Azari and Feeney, 1958; Banerjee et al., 1986; Crichton, 1990).

Our laboratory has reported previously that human insulin conjugated to Tf via a disulfide linkage (In-Tf) was transported across cultured epithelial cells via TfR-mediated transcytosis (Shah and Shen, 1996), and that a hypoglycemic effect in diabetic mice was observed after oral administration of In-Tf (Wang et al., 1997). In this report, we characterize the stability and biological activity of In-Tf. Our results demonstrate that s.c. or orally delivered In-Tf can produce a slow but prolonged hypoglycemic effect in STZ-induced diabetic rats.

Experimental Procedures

Materials

Recombinant human insulin and human apo-transferrin were purchased from Sigma (St. Louis, MO). *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was obtained from Pierce Chemical Company (Rockford, IL). Sephacryl S-200 was purchased from Pharmacia (Uppsala, Sweden). Broad range protein marker was purchased from BioRad Laboratories Inc. (Richmond, CA). All other chemicals were purchased from Sigma. The Sprague-Dawley female rats (about 10 weeks old and 220–240 g) used in our experiments were obtained from Harlan (San Diego, CA).

Preparation of In-Tf

In-Tf was prepared by a similar procedure as previously described (Shah and Shen, 1996) with a few minor modifications. Recombinant human insulin was covalently linked to iron-loaded human Tf via a disulfide linkage with a bifunctional cross-linking agent, SPDP. Two milliliters of diferric-Tf solution (20 mg/ml, pH 7.0) was reacted with SPDP (700 μ g in *N,N*-dimethylformamide) at 4°C for 30 min and the reaction mixture was dialyzed overnight against PBS (pH 8.0). The final ratio of 3-(2-pyridyldithio)propionate:Tf was determined to be 2:1. After sulfhydryl-containing Tf (40 mg) was generated from Tf-3-(2-pyridyldithio)propionate upon dithiothreitol treatment, it was reacted with SPDP-modified insulin (5.8 mg) at 4°C for 2.5 h. The reaction was stopped by adding 2 mg of *N*-ethylmaleimide (NEM) to the reaction mixture followed by dialysis in PBS (pH 8.0) for 18 h. The conjugate was purified by gel filtration on Sephadex G-50 in PBS (pH 8.0).

Characterization of In-Tf

HPLC Analysis. Analysis of In-Tf was performed by using a computer-controlled gradient high-performance liquid chromatographic (HPLC) system (Rainin Instruments, Woburn, MA) equipped with a variable-wavelength ultraviolet/visible detector. The gradient system used in this study consisted of a mobile phase A (water solution with 0.1% trifluoroacetic acid and 10% acetonitrile), and a mobile phase B (acetonitrile solution with 0.09% trifluoroacetic acid, 2% water, and 5% tetrahydrofuran). The gradient system was programmed by increasing the portion of mobile phase B from 20 to 42% within 30 min. The sample was injected into a VYDAC protein

C4 column. The HPLC system was run at a flow rate of 1 ml/min. The ultraviolet detector was set at 214 nm.

Gel Filtration Chromatography Study. In-Tf (3 mg/ml) or a mixture of Tf (3 mg/ml) and human insulin (1 mg/ml) was separated using a Sephacryl S-200 column (2 × 23 cm) equilibrated and eluted with PBS (pH 7.4). Protein peaks in collected fractions (1 ml each) were detected by measuring absorbance at 280 nm.

SDS-PAGE Analysis. SDS-PAGE was performed according to the method of Laemmli (1970). Bands were detected by the Coomassie blue stain, and the molecular weight was estimated by comparison with protein standards. The gel was scanned using a charge-coupled device camera-based scanning densitometer and a BioImage software package (Ann Arbor, MI) to estimate the quantity of each band.

In Vitro Liver Metabolism of In-Tf

A fresh liver, taken from a normal Sprague-Dawley female rat, was cut into slices approximately 2 mm in width. In-Tf (equivalent to 250 μ g/ml insulin) was incubated with the liver slices (1 g of wet tissue/ml of incubation medium) at 37°C in a water bath shaker. The medium consisted of Dulbecco's modified Eagle's medium/F-12 with HEPES buffer (pH 7.5) and 1 mg/ml BSA. For experiments with proteinase inhibitor cocktail (PIs; consisting of 2 μ g/ml pepstatin A, 20 μ g/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, 2 μ g/ml leupeptin, 20 μ g/ml *N*-tosyl-L-lysine chloromethyl ketone, 20 μ g/ml soybean trypsin inhibitor, 20 μ g/ml *N*-tosyl-L-arginine methyl ester, 20 μ g/ml *N*-benzyl-L-arginine methyl ester, and 348 μ g/ml phenylmethylsulfonyl fluoride) or NEM, the liver slices were preincubated with PIs or NEM at 37°C for 30 min. An aliquot of 50 μ l was taken from the incubation medium at 0, 5, 10, 20, 30, 60, 90, and 120 min, and subjected to human insulin-specific radioimmunoassay (RIA) (Linco Research, Inc., St. Louis, MO).

Diabetic Animal Model

Female Sprague-Dawley rats were housed in stainless steel metabolic cages and fed with rodent chow. After an initial 5-day acclimation period, the rats were fasted for 24 h before inducing diabetes mellitus. STZ solution (60 mg/ml) was freshly prepared in acetate buffer (pH 4.5) and used within 1 h. After the baseline blood glucose level was determined, rats were injected i.p. with STZ at 60 mg/kg. Five days after STZ treatment, the rats with a fasted plasma glucose level >300 mg/dl were selected as diabetic rats for further investigations.

Studies of Hypoglycemic Effect

Subcutaneous Injection of In-Tf. Diabetic rats were fasted for 12 h before the treatment. Insulin (0.35 U/kg), In-Tf (equivalent to 0.35 U/kg insulin), or placebo (saline) in PBS solution was injected s.c. to the diabetic rats. Blood samples were collected from the tails of the treated rats at predetermined time points. The blood glucose level was measured using a ONE TOUCH blood glucose monitoring system (Lifescan, Inc., Milpitas, CA), and the hypoglycemic effect was expressed as the percentage change of the blood glucose level from the initial value.

Oral Administration of In-Tf. The diabetic rats were fasted for 12 h and then were orally administered with insulin, In-Tf, or placebo (PBS) in NaHCO₃ solution (30 mg/ml) by using a gavage needle. The doses of In-Tf ranged from 6.7 to 80 U/kg insulin. The treated rats were kept in metabolic cages, with free access to water only. Blood samples were collected from the tails of treated rats at predetermined time points. The blood glucose level was measured as described above. The hypoglycemic effects were expressed as the percentage change of the blood glucose level from the initial value.

Gel Filtration Chromatography of Rat Serum

Blood samples were obtained from rats at 30 min, 2 h, or 4 h after the oral administration of ¹²⁵I-insulin (80 U/kg) or ¹²⁵I-In-Tf (equiv-

alent to 80 U/kg insulin). The serum (2 ml) from each blood sample was applied to a Sephacryl S-200 column (2 × 24 cm) equilibrated and eluted with PBS (pH 7.4). The radioactivity in each fraction (1 ml) was detected by using a gamma counter, and the distribution of serum protein in collected fractions was estimated by the absorbance at 280 nm. The Sephacryl S-200 column was calibrated by applying a mixture of ^{125}I -In-Tf, ^{125}I -insulin, and ^{125}I -Tf in normal rat serum to identify the radioactive peaks in the samples.

Statistical Analysis

Results were evaluated using the Student's *t* test. Values were considered statistically significant if $P < .05$. All data are expressed as mean $\pm$ S.E.

Results

Characterization of In-Tf. HPLC chromatogram of purified In-Tf (Fig. 1) showed a single peak with a retention time of 17.0 min. Under the same conditions, the retention times of insulin and Tf were 10.1 and 15.5 min, respectively. These results indicated that there was no free insulin or Tf in the conjugate. Results from Sephacryl S-200 gel filtration also indicated that there were no detectable free insulin and Tf in the purified In-Tf (Fig. 2).

SDS-PAGE showed that the minimal molecular mass of In-Tf was around 85 kDa (Tf is 80 kDa and insulin is 5.8 kDa) (Fig. 3A) and that A and B chains of insulin and Tf in In-Tf conjugate could be dissociated when the disulfide linkage was reduced with mercaptoethanol (Fig. 3B). The insulin A chain (~2.5 kDa) and B chain (~3.5 kDa) localized in the front of the gel as a single band. The gel scanning results of the reduced In-Tf indicated that the molar ratio of insulin and Tf in the conjugate was 1:1 (Fig. 3B).

Liver Metabolism of In-Tf. To further investigate whether In-Tf could release free insulin in the tissue, the metabolism of In-Tf in rat liver slice was studied and released insulin levels were quantitated using a human insulin-specific RIA kit. As shown in Fig. 4, free insulin was detected 5 min after incubation. When the liver slices were

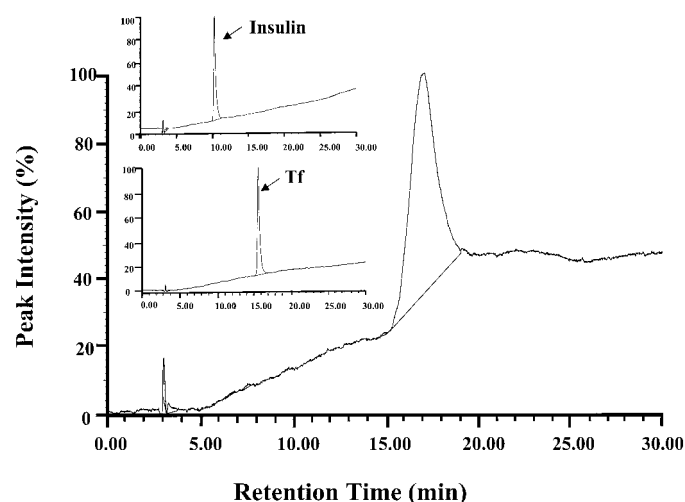


Fig. 1. HPLC chromatogram of In-Tf. The insets are the HPLC chromatograms of recombinant human insulin and iron-loaded human Tf. In-Tf, Tf, or insulin (1 μg each) was analyzed by HPLC and the retention times of In-Tf, Tf, and insulin were 17.03, 15.52, and 10.13 min, respectively. When 15 μg of In-Tf was applied to the same HPLC, there were no detectable free insulin or Tf, which indicated that the purity of In-Tf was at least 93%.

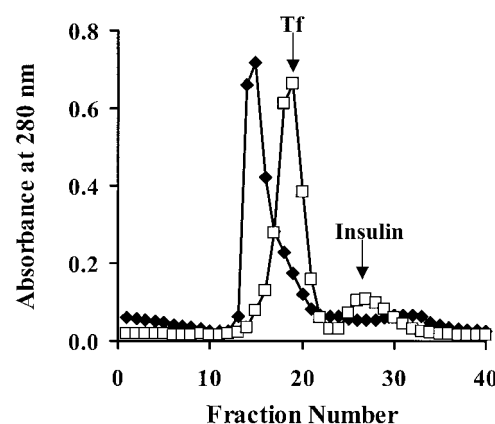


Fig. 2. Sephacryl S-200 gel filtration chromatogram of In-Tf. Three milligrams of In-Tf (◆) or a mixture of 3 mg of Tf and 1 mg of human insulin (□) were applied to a Sephacryl S-200 column (2 × 23 cm) with PBS (pH 7.4) as eluent. The fractions (1 ml each) were collected and proteins were detected by measuring absorbance at 280 nm.

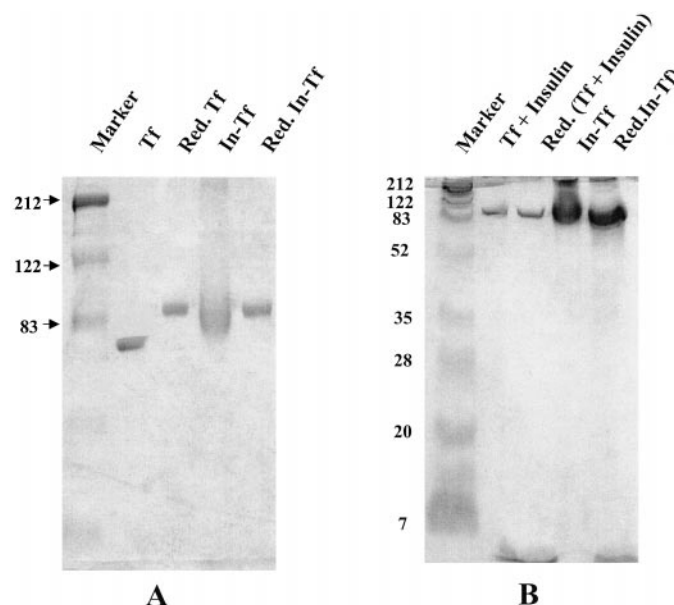


Fig. 3. SDS-PAGE analysis of In-Tf. Purified In-Tf (2 μg), Tf (1 μg), or human insulin (2 μg) were boiled in 15 μl of 1% SDS (with or without 0.2 M mercaptoethanol) for 5 min and applied to 8% polyacrylamide gels (A) or 12.5% polyacrylamide gels (B) with a 4% stacking gel. After electrophoresis, protein bands were detected by Coomassie blue stain. Insulin and Tf released from the In-Tf after mercaptoethanol reduction were quantitated by densitometer scan and the ratio of insulin and Tf was calculated. Red. in the figure represents that the sample was reduced by mercaptoethanol before loading.

pretreated with PIs, the free insulin level released from In-Tf increased progressively until 10 min after incubation and was higher than that of samples without PIs treatment throughout all time points (Fig. 4). On the other hand, the production of free insulin was inhibited by pretreating the liver slices with a sulfhydryl-reactive agent NEM (1.5 mg/ml) (Fig. 4).

Hypoglycemic Effects of s.c. Injected In-Tf. The biological activity of In-Tf conjugate was investigated in diabetic rats. The 12-h-fasted rats with a plasma glucose level around 300 mg/dl were used (Table 1). As shown in Fig. 5, s.c. injection of human insulin at 0.35 U/kg had a maximum hypoglycemic effect (−50% change of baseline) at 3 h post

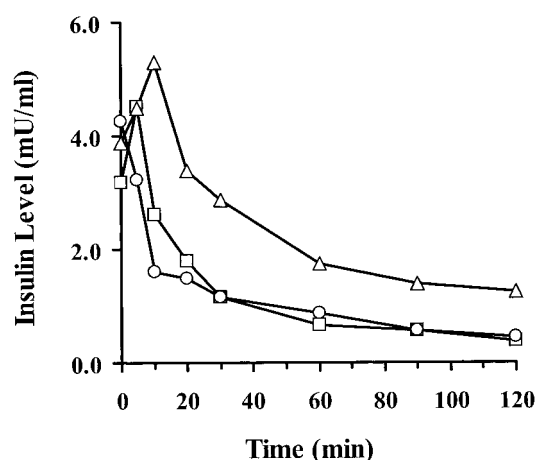


Fig. 4. Release profiles of insulin from In-Tf incubated with rat liver slices with or without PIs or NEM pretreatment. A fresh liver was obtained from a female Sprague-Dawley rat, and cut into slices 2 mm in width. Liver slices (1 g wet tissue/ml incubation medium) with PIs (Δ) or NEM ($\circ$) for 30-min pretreatment were compared with those without pretreatment ($\square$). An aliquot of 50 μ l was taken from the incubation medium at 0, 5, 10, 20, 30, 60, 90, and 120 min, and subjected to human insulin-specific RIA. Each point represents an average of duplicate samples.

TABLE 1

Blood glucose level of diabetic rats with s.c injection of PBS, insulin, or In-Tf

Rats were fasted for 12 h before drug treatment and fed with excess food immediately after the experiment was terminated. The average glucose level of normal rats after a 12-h fast is 50 ± 7 mg/dl ($n = 14$). The dose of insulin or In-Tf was 0.35 U insulin/kg. PBS (pH = 7.4) was used as the placebo. Results are expressed as the mean $\pm$ S.E. ($n = 3$ or 4).

Treatment	Glucose Level		
	0 h before Injection	11 h after Injection	10 h after Experiment Termination with Feeding
	mg/dl		
PBS	292 ± 30	267 ± 20	392 ± 14
Insulin	373 ± 4	312 ± 24	412 ± 25
In-Tf	378 ± 21	$104 \pm 16^{**}$	422 ± 18

$^{**} P < .01$, compared with group injected with insulin or PBS.

administration and the blood glucose level was recovered to baseline after 7 h. However, s.c. injection of In-Tf at the same dose had a more intensive and prolonged effect on reducing blood glucose level in diabetic rats. The blood glucose level decreased by -70% of control at 9 h and was maintained at this level (104 ± 16 mg/dl, Table 1) until 11 h when the experiment was terminated. The plasma glucose level recovered to 422 ± 18 mg/dl (Table 1) at 10 h after the rats were fed with food, suggesting that In-Tf did not induce a severe hypoglycemia.

Hypoglycemic Effects of Orally Administered In-Tf.

No significant decrease in blood glucose levels was observed in STZ-induced diabetic rats after oral administration of either PBS (placebo) or 80 U/kg human insulin formulated in 30 mg/ml NaHCO_3 solution (Fig. 6). In contrast, oral administration of In-Tf formulated with 30 mg/ml NaHCO_3 solution caused a slow but significant decrease in blood glucose level (Fig. 6). This hypoglycemic effect of orally administered In-Tf was dose dependent. In-Tf at a dose equivalent to 80 U insulin/kg showed a 70% reduction of the glucose level at 11 h from the initial level of 333 ± 13 to 87 ± 28 mg/dl. NaHCO_3 in the formulation was used to neutralize the gastric acid and

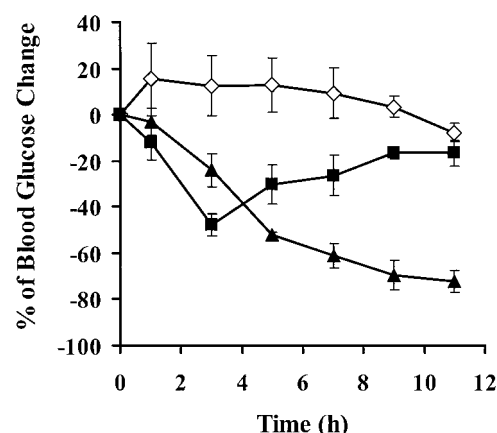


Fig. 5. Hypoglycemic effect of In-Tf injected s.c. to STZ-induced diabetic rats. Blood samples were collected from the tails of the treated rats at predetermined time points. The blood glucose level was measured using a ONE TOUCH blood glucose monitoring system and the hypoglycemic effect was expressed as the percentage change of the blood glucose level from the initial value shown in Table 1. The dose of insulin ($\blacksquare$) or In-Tf ($\blacktriangle$) was 0.35 U/kg. PBS was used as the placebo ($\diamond$). The initial blood glucose levels were 292 ± 30 ($n = 3$), 373 ± 4 ($n = 3$), and 378 ± 21 ($n = 4$) for the diabetic rats treated with PBS, insulin and In-Tf, respectively. Results are expressed as the mean $\pm$ S.E. ($n = 3-4$, $^{**} P < .01$).

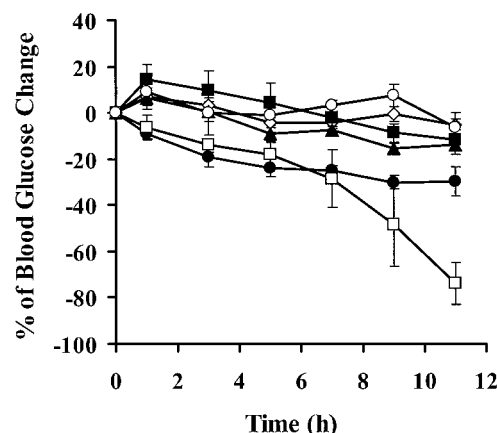


Fig. 6. Changes of blood glucose levels in STZ-induced diabetic rats after oral administration of In-Tf. Blood samples were collected from the tails of the treated rats at predetermined time points. The blood glucose level was measured using a ONE TOUCH blood glucose monitoring system and the hypoglycemic effect was expressed as the percentage change of the blood glucose level from the initial value (about 350 mg/dl). Hypoglycemic effects of oral administration of In-Tf at 6.7 ($\blacksquare$), 13.5 ($\blacktriangle$), 27 ($\bullet$), and 80 ($\circ$) U insulin/kg were compared with insulin (80 U/kg) ($\circ$) and PBS control (placebo) ($\diamond$). The initial blood glucose levels of the diabetic rats treated with PBS, insulin, 6.7, 13.5, 27, and 80 U insulin/kg In-Tf were 316 ± 16 ($n = 3$), 330 ± 21 ($n = 3$), 311 ± 21 ($n = 3$), 332 ± 6 ($n = 3$), 364 ± 29 ($n = 4$), and 333 ± 13 ($n = 4$), respectively. Results are expressed as the mean $\pm$ S.E. [$n = 3$ or 4, $^{**} P < .01$; t test for the comparison of oral administration of insulin (80 U/kg) and In-Tf (80 U/kg insulin)].

protect insulin as well as In-Tf from degradation in the stomach.

Detection of In-Tf in Plasma. Figure 7 shows the elution profiles of the radioactivity in a Sephacryl S-200 column loaded with serum obtained from ^{125}I -In-Tf- or ^{125}I -insulin-administered rats. In-Tf was detected in the serum of the rats at 4 h after oral administration of ^{125}I -In-Tf (80 U insulin/kg) (Fig. 7A). This peak (fraction 17–20) represented 10.2% of total radioactivity in the serum and most of the rest radioactivity (71.7%, fraction 52–61) was in the small molecular

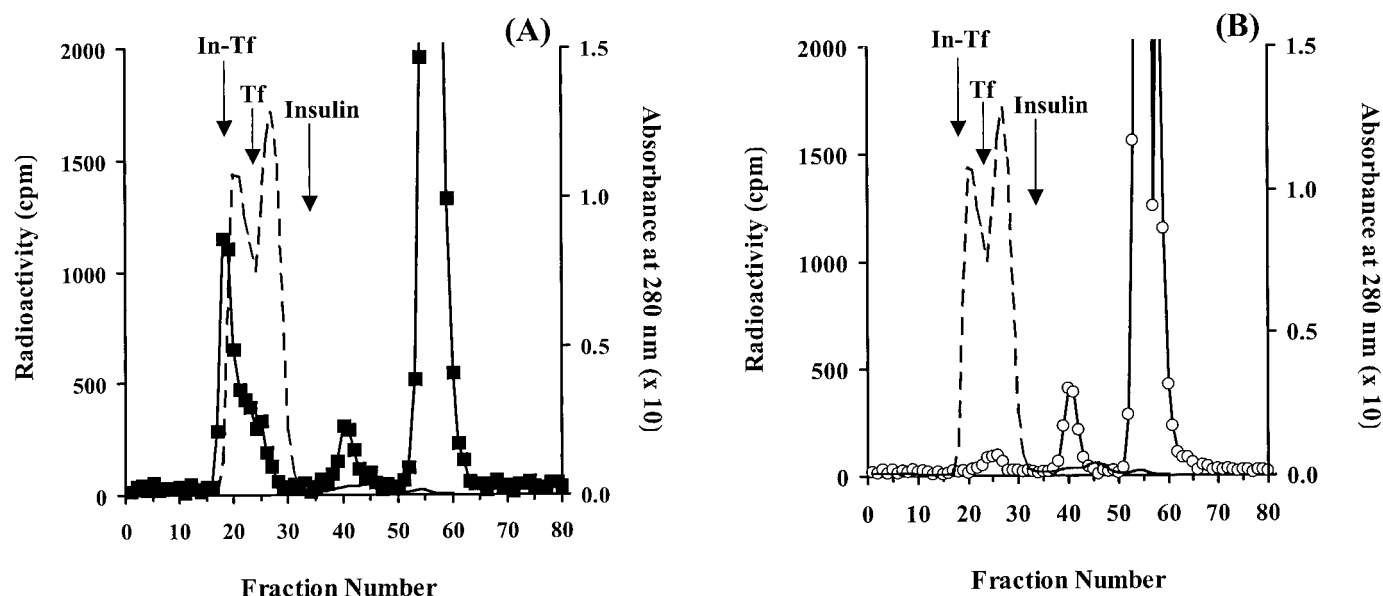


Fig. 7. Gel filtration chromatogram of rat serum. The serum samples were collected from the rats at 4 h after p.o. administration of ^{125}I -In-Tf (A) or ^{125}I -insulin (B) and a 2-ml sample was applied to a Sephacryl S-200 column (2×24 cm) with PBS (pH 7.4) as an eluent. Each fraction (1 ml) was subjected to radioactivity measurement (measured by gamma counter) and UV spectrum assay (measuring absorbance at 280 nm). A, elution profile of radioactivity (■) and protein absorbance (—) of pooled serum taken from the rats at 4 h after p.o. administration of ^{125}I -In-Tf. The radioactivity peak in the In-Tf region (fraction 17–20) was 10.2% of the total and the majority of radioactivity (71.7%) was located in the small molecular position (fraction 52–61). B, elution profile of radioactivity (○) and protein absorbance (—) of serum taken from the rats at 4 h after p.o. administration of ^{125}I -insulin. No free insulin was detected and 90.8% radioactivity was in the small molecular area (fraction 52–61). The standard In-Tf (fraction 17–20), Tf (fraction 23–26), or insulin (fraction 33–36) was identified by analyzing the serum mixed with ^{125}I -In-Tf, ^{125}I -Tf, and ^{125}I -insulin, and indicated by arrows (↓) ($n = 3$ of each experimental group).

area. There was no detectable In-Tf or free insulin in the rat serum sample of 30 min or 2 h after oral administration of In-Tf in these gel filtration studies (data not shown). Our preliminary results of human insulin-specific RIA showed that, 4 h after oral administration, the free human insulin level in plasma was higher in the In-Tf-treated rats (134 ± 39 $\mu\text{U/ml}$, $n = 4$) than in the insulin-treated (69 ± 21 $\mu\text{U/ml}$, $n = 3$) or PBS-treated (46 ± 20 $\mu\text{U/ml}$, $n = 3$) controls. However, this low level of free insulin was not detectable in the radioactive-labeling studies by gel filtration because it was below the detection limit.

Discussion

Recombinant human insulin was conjugated to Tf with a disulfide bond. The advantage of disulfide linkage is that it can be cleaved after the conjugate is absorbed into the bloodstream, thereby giving rise to free insulin to elicit its therapeutic action (Thorpe et al., 1988). The product from the conjugation reaction was heterogeneous, as indicated by a broad band in SDS-PAGE (Fig. 3, A and B). It is likely that cross-linking of Tf may occur during the conjugation reaction, even though the average molar ratio of insulin to Tf was estimated to be one in the final conjugate, In-Tf.

The data presented in Fig. 4 provide direct evidence that the disulfide bond in the In-Tf could be reduced and free insulin could be released in the liver. To get a deeper insight into the release pattern of insulin from In-Tf, we further investigated the liver metabolism of In-Tf with or without PIs or NEM treatment. Initially during incubation, without any treatment, free insulin could be detected by RIA, but it was degraded rapidly (Fig. 4). The degradation of released insulin could be partially inhibited by PIs. With liver pretreated by

NEM, a sulfhydryl-reactive agent and an inhibitor of the insulin-degrading enzyme (Bai et al., 1995, 1996), no free insulin was detected in the RIA (Fig. 4), suggesting the involvement of disulfide reduction reaction in the insulin release.

Results obtained from s.c. administration of In-Tf in diabetic rats indicated that In-Tf is more effective than native insulin in reducing blood glucose levels (Fig. 5). Furthermore, the profile of the hypoglycemic activity of In-Tf was strikingly different from that of insulin. A delayed onset, but an extensively prolonged effect was observed in In-Tf-treated diabetic rats (Fig. 5). After s.c. injection of insulin, at a dose of 0.35 U/kg, a nadir of $\sim 50\%$ of control glucose levels was achieved in 3 h, and this hypoglycemic effect was completely abolished at 9 h. However, a gradual decrease of blood glucose level was observed in In-Tf-treated diabetic rats, and a 70% decrease of the control blood glucose level was observed 11 h after the injection of In-Tf (Fig. 5). The experiment was terminated by feeding the experimental rats because the rats have been fasted for a total 23 h at that time. It is very likely that the hypoglycemic activity of In-Tf could last much longer than 11 h according to the activity trend. However, we observed that the blood glucose level of treated rats regained to the initial level 10 h after the termination of the experiment (Table 1). This observation indicates that the hypoglycemic effect of injected In-Tf last longer than 11 h but shorter than 24 h. The difference of response between In-Tf and insulin could be attributed to several factors. First, it is possible that the hypoglycemic activity of In-Tf is dependent on the release of free insulin from the Tf-conjugate. In this case, the delayed onset and prolonged activity suggests that In-Tf may have a longer plasma half-life and that insulin is slowly released from the conjugate. This assumption is consistent with the

fact that the plasma half-life of Tf in mice (40 h) (Li and Kaplan, 1997) is significantly longer than that of insulin (10 min) (Jones et al., 1984). The reduction of the disulfide linkage in protein conjugates such as immunotoxins (Winkler et al., 1990) has been demonstrated in the plasma. The second possibility is that In-Tf may bind to Tfr in interstitial tissues. Such a binding would produce a depot effect and, consequently, a sustained release of insulin or In-Tf from the tissues into the blood may occur. A depot effect may also be generated due to the large molecular size of In-Tf, resulting in an increased absorption time and a decreased clearance rate in the bloodstream. It is noteworthy that a protraction of insulin action has been reported when insulin was conjugated to fatty acids, and the binding of the fatty acid-insulin conjugates to plasma proteins was suggested to be responsible for the prolonged hypoglycemic activity (Markussen et al., 1996; Myers et al., 1997). The third possibility is that different distribution profiles may exist among target tissues between native insulin and conjugated insulin; similar phenomena have been observed in certain polyethylene glycol-insulin conjugates (Neubauer et al., 1983). After injection, a substantial part of insulin is degraded in the liver and kidneys (Ferranni et al., 1983), whereas only a smaller part is taken up by muscles where most of the glucose use occurs (DeFronzo, 1988). However, In-Tf may have given rise to a relative specificity for certain tissues and consequently produced a prolonged and efficacious insulin level in interstitial fluids.

We have previously reported that In-Tf was transported across Caco-2 cells via Tfr-mediated transcytosis (Shah and Shen, 1996). On the other hand, insulin receptor-mediated transport of free insulin was not detected in Caco-2 cells (Shah and Shen, 1996). The Caco-2 cell line is a well-known cell culture model of intestinal epithelium for screening oral drug absorption (Audus et al., 1990; Quaroni and Hochman, 1996; Gan and Thakker, 1997). The finding that In-Tf, but not insulin, can be transported across Caco-2 cells via receptor-mediated transcytosis suggests that an increase of GI absorption can be achieved when insulin is conjugated to Tf. To confirm the GI absorption of In-Tf, the conjugate was administered orally to STZ-induced diabetic rats. As shown in Fig. 6, a dose-dependent response to In-Tf on the hypoglycemic activity in diabetic rats was observed, whereas insulin at the highest dose (80 U/kg) did not show any effect. These results indicate that In-Tf may overcome the enzymatic and the transport barrier of the GI tract to achieve the action of insulin. Interestingly, the profile of hypoglycemic effect of orally administered In-Tf at higher doses was similar to that of s.c. administered In-Tf. Both administration routes for In-Tf demonstrated a delayed onset with prolonged activity in reducing blood glucose levels. The fact that an apparent nadir of the plasma glucose level in rats with oral administration of In-Tf was observed at 11 h or longer is particularly intriguing. It is unlikely that In-Tf can be retained in the GI tract for an extensively long period of time without degradation or excretion. Therefore, we speculate that In-Tf must be absorbed by the intestinal epithelium as an intact conjugate. A direct absorption of the intact In-Tf in the GI tract is further supported by the finding that the radioactive In-Tf, but not Tf or insulin, was detected from the rat serum at 4 h after orally administered with ^{125}I -In-Tf (Fig. 7). The fact that no radioactive In-Tf was detected from the rat serum at

30 min or 2 h after orally given with ^{125}I -In-Tf was consistent with the delayed onset effect of In-Tf. However, we cannot rule out the possibility that In-Tf may be accumulated inside the body at a specific site such as the liver or other glucose-using organs where it is slowly released either as the intact conjugate or as free insulin. Although in vitro metabolism study showed that In-Tf could release free insulin in the liver, insulin could also be released from In-Tf in the bloodstream or at other specific sites with disulfide reduction activity because free insulin was detected by RIA in plasma of rats at 4 h after oral administration In-Tf.

Taken together, our results demonstrate that conjugation to Tf can markedly improve the hypoglycemic effect of insulin in STZ-induced diabetic rats. This conjugate, In-Tf, is slow in onset of action when s.c. injected into diabetic rats and therefore can avoid hyperinsulinemia. In-Tf is capable of maintaining low blood glucose levels at least 4 times longer than insulin. In-Tf can be absorbed by intestinal epithelium and can exhibit a hypoglycemic effect when orally administered to diabetic rats. It should be emphasized here that even though a hypoglycemic effect was maintained in rats with oral or s.c. In-Tf treatment, the lowest blood glucose level, i.e., around 100 mg/dl, was close to the normal value (around 50 mg/dl), and the rats were not at a risk of severe hypoglycemia. The finding that a prolonged effect of In-Tf on maintaining the blood glucose level at normal ranges is important for considering optimal therapy for the diabetic patients (Galloway and Chance, 1994). Therefore, with appropriate formulations, such as the addition of transcytosis enhancers (Shah and Shen, 1996), Tf can potentially be developed as a unique carrier for the oral delivery of insulin, as well as other peptide drugs.

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