製剤を筆者らの調査した範囲で表4に示した。狭義のDDS製剤といえないものも含まれているし，また外国で実用化されている重要なものが抜けているかもしれないが，主なものはおおむね記載されていると思う。前述したように日本で開発されたものの中に画期的なものが多いが，最近実用化され，臨床で極めて有用なものはダウノルビシンのリポソーム製剤，アムホテリシンのリポソーム製剤，PEG化インターフェロンなど，外国で開発されたものである。最近，日本の医薬品開発が種々の理由で遅れていることがおそらく関係しているのであろうが，最近の日本のものは徐放剤，経口崩壊錠など特に画期的なものでないもののみで，この表4に入れるのも多少疑問があるといえるかもしれない。しかし，現在臨床の最終段階にきている日本産のDDS製剤もあり，今後の発展をぜひ期待している。

## おわりに

以上，DDS製剤についてこれまでの大きな流れ，日本の研究のあゆみ，日本の貢献，そして主要なDDS製剤について述べた。


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# Transdermal delivery of $CaCO_3$-nanoparticles containing insulin

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Abstract

Objective: This study evaluates the pharmacokinetic and pharmacodynamic effects of a transdermally delivered insulin using a novel $CaCO_3$-nanoparticles in normal and diabetic mice.

Methods: $CaCO_3$-nanoparticle encapsulating insulin (nanoinsulin) was transdermally applied to the back skin of normal ddY and diabetic dB/dB and kkAy mice after fasting for 1 h. Serum insulin levels of ddY mice were analyzed by EIA and blood glucose of normal and diabetic mice was monitored using Diasensor.

Results: Maximum serum insulin was 67.1 ± 25.9 μIU/ml at 4 hr with 200 μg of transdermal nanoinsulin in ddY mice, whereas that after subcutaneous (sc) injection of 3 μg monomer insulin was 462 ± 20.9 μIU/m at 20 min. Transdermal nanoinsulin decreased glucose levels in a dose dependent manner. A maximum decrease in blood glucose of 48.3 ± 3.9 % (ddY), 32.5 ± 9.8 % (dB/dB), 26.2 ± 7.6 % (kkAy) after 6 hr was observed with 200 μg of transdermal nanoinsulin, whereas that of 64.1 ±

1.0 % (ddY), 57.9 ± 3.4 % (dB/dB), and 24.1 ± 6.7 % (kkAy) was observed after 1 hr with 3 μg of sc monomer insulin. Insulin bioavailability until 6 hr with transdermal nanoinsulin in ddY mice was 0.9% based on serum insulin level and 2.0% on pharmacodynamic blood glucose-lowering effects.

Conclusions: This $CaCO_3$-nanoparticle system successfully delivered insulin transdermally, as evidenced by a significant sustained decrease in blood glucose in normal and diabetic rats. These results support the feasibility of developing transdermal nanoinsulin for human applications.

## Introduction

Therapeutic insulin for diabetes is typically administered via subcutaneous injection and a patient might require injections over their life-time. A viable non-injectable insulin delivery route would thus dramatically improve both compliance and the quality of life in patients with diabetes. Various alternative routes including rectal, intrapulmonary, intrauterine, oral, ocular, nasal, buccal, and dermal, have thus been considered for the administration of insulin (1,2). The noninvasive transdermal route in particular is an attractive candidate for the steady and sustained delivery of insulin into the blood in a pain free manner, and the main advantages of the transdermal routes are the low proteolytic activity of the skin and the possibility of continuously delivering drugs with short-half-lives and prolonged effects by continuous absorption. But, one of the major disadvantages in transdermal drug delivery is the low penetration rate through the skin. The diffusion barrier for most substances is localized in the upper layer of the skin, the stratum corneum. Various

attempts have been made to facilitate the transdermal permeation of insulin through the rate-limiting barrier of the lipophilic stratum corneum and to overcome the diffusion resistance across the skin tissues.

Skin poration with electrical, acoustic, thermal or photomechanical means can yield excellent delivery results, but generally suffers from the problem of temporary barrier destruction and potentially of dermal immunization. These physical methods might be more suitable for short–term rather than sustained delivery of insulin. Chemical absorption enhancers such as bile salts and sodium salicylate have also been used to facilitate insulin transport (3). Additionally, to increase the stability of a peptide, the use of enzyme inhibitors must be considered. But methods involving chemical disruption of the skin might also cause chronic pathological changes.

Special ultra-adaptable lipid aggregates, so-called Transfersomes characterized by their extreme surface hydrophilicity, membrane flexibility and shape deformability, and biphasic lipid system

incorporated in a patch has been developed for transdermal delivery of insulin (4, 5, 6).

Recently, nanoparticles with a proper size and chemically appropriate surface properties has been reported to penetrate the skin (7, 8). If nanoparticulate carriers can cross the stratum corneum, they can act as micro-reservoirs of a drug and provide sustained drug delivery. We have recently developed a simple method for incorporating drugs into solid calcium carbonate nanoparticles, and this $CaCo_3$ – nanopreparation showed a sustained release of incorporated drugs (9, 10).

Here we describe a novel transdermal $CaCO_3$- nanoparticle suitable for insulin delivery across the skin.

## Materials and Methods

### 1. Materials

Recombinant human insulin and Tween 80 were purchased from ICN Pharmaceutical, Inc. (Costa Mesa, CA, USA). Other reagents and solvents were purchased from Wako Pure

Chemicals Industries Ltd. (Osaka, Japan) and used without further purification.

2. Preparation of nanoparticles (nanoinsulin)

Ten microliters of 30 mM HCl aqueous solution dissolved 500 μg zinc chloride was added to 1 ml of 10 mM HCl aqueous solution dissolved 1 mg insulin to form an insulin-zinc complex. After the addition of 36 μl of ethanol/water (7/3, v/v) dissolved 500 μg sodium myristate and 100 μl of 2 % Tween 80 acetone solution, the suspension of an insulin-zinc complex was stirred with a magnetic stirrer for 15 min at RT. The resulting suspension dissolved an insulin-zinc complex was added drop-wise to 5 ml of distilled water to obtain nanoparticles formed from insulin, zinc, Tween 80, and sodium myristate. After 30 min of incubation at RT under stirring, 15 μl of 5 M calcium chloride aqueous solution was added and the suspension was continuously stirred for 30 min at RT. Finally, the nanoparticles formed from $CaCO_3$, insulin, zinc, Tween 80 and sodium myristate, were prepared by

the addition of 15 μl of 1 M sodium carbonate aqueous solution and further incubation for 30 min at RT under stirring. The resulting nanoparticles suspension was centrifuged for 5 min at 20,000 g to separate the nanoparticles from unencapsulated insulin and the precipitated nanoparticles were freeze-dried without any additive.

3. Characterization of nanoinsulin

The particle size and distribution were determined by a dynamic light scattering method (FPAR-1000, Otsuka Electronics, Ltd., Osaka, Japan). The recovery efficiency of insulin was calculated by measuring the amount of unencapsulated insulin. The suspension of nanoparticles was centrifuged for 5 min at 20,000 g and insulin in the supernatant was determined by HPLC using a reversed-phase column Symmentry 300TM C4 column (Waters Co., Massachusetts, USA) with UV detection at 280 nm, solvent water-acetonitrile with 0.1 % TFA, linear gradient 5-100 % acetonitrile. The main peak showed in the chromatogram of

monomer insulin was used for quantification of insulin.

4. *In vivo* animal experiment

The Animal Ethics and Research Committee of Jikei University School of Medicine approved the protocols for the animal experiments. Eight week-old normal male ddY mice were obtained from Nihon SLC (Shizuoka, Japan) and 10 week-old diabetic male dB/dB and 12 week-old diabetic male kkAY mice were obtained from Nihon Clea (Shizuoka, Japan). All mice were kept under normal laboratory conditions for 1 week. The day before the experiments, the hair of the upper back of each mouse was carefully removed with an electric clipper and a razor without breaking the skin. Then, the sample was applied to this 1.5-cm diameter square site.

5. Pharmacokinetics of serum insulin

Nanoinsulin (200 μg as of insulin) or monomer insulin (200 μg as

of insulin) dispersed in 100 mg of white Vaseline was applied transdermally in ddY mice (n=20, each) and whole blood was obtained from the abdominal artery just prior to and 2, 4, 6 and 8 hr after the application (n=4, each). As controls, monomer insulin was subcutaneously (sc) (3 μg) (n= 30) applied and whole blood was obtained just before and 0.3, 0.6, 1, 1.5 and 3 hr after the administration (n= 5, each).

The level of serum insulin was analyzed using ELISA reader (AIA-600 ; Toso, Tokyo Japan) and expressed as IU/ml. The detection limit was 0.25 μIU/ml in our hand. And the baseline insulin concentration in mice without treatment was up to 7.8 μIU /mL. The area under the serum concentration-time curve of insulin (AUC-I) was calculated by trapezoid calculation and expressed as μIU・hr/ml. And the relative bioavailability (BA-I) of transdermal insulin delivery was calculated in comparison with sc administration and was expressed as

$$BA\text{-}I = Dose_{sc}/Dose_{transedemal} \times AUC\text{-}I_{transdermal}/AUC\text{-}I_{sc} \times 100.$$

The relative BA-I calculated using each AUC for 0-6 hr after

dosing was expressed as $BA\text{-}I_{0\text{-}6\ h}$.

6. Pharmacodynamics of blood glucose response

Nanoinsulin (0, 50, 100, 200 μg) or monomer free insulin dispersed in 100 mg of white Vaseline was applied transdermally to normal ddY mice (n=3, each) and blood was obtained from the tail vein just prior to and 2, 4, and 6 hr after the application. As a control, monomer insulin (3 μg) (n=3) was administered sc and blood was obtained just prior to and 0.5, 3 hr after the administration. Furthermore, 200 μg of nanoinsulin was transdermally applied to normal (n=7) and diabetic dB/dB (n=5) and kkAy (n=7) mice, whereas 3 μg (in ddY mice) and 5 μg (in diabetic mice) of monomer insulin was injected sc as controls. All glucose values were normalized at t=0 (insulin treatment was initiated) as a basis and nadir level was calculated. AUC was calculated by trapezoid calculation and expressed as μIU・hr/ml. They can be followed only up to 6 hr, after that glucose levels start to decrease even in untreated mice. And the relative BA of

transdermal insulin delivery was calculated in comparison with sc administration, and the relative BA calculated using each AUC for 0-6 hr after dosing was expressed as $BA_{0\text{-}6\,h}$.

7. Statistical analysis

Results are expressed as mean ± SEM values. Statistical significance was evaluated using Student *t*-test. A *P* value <0.05 was considered significant.

## Results

1. Physical properties of nanoinsulin

The nanoparticles formed from calcium carbonate and containing insulin were successfully prepared in this report. Although insulin-zinc complex (Insulin Lente) is known as a crystalline suspension with slow release characteristics, we employed zinc to make soluble monomer insulin hydrophobic to form solid particles. It was found that the nanoparticles had a diameter ranging from 300 to 500 nm by the analysis of the dynamic light

scattering. The recovery efficiency of insulin (ratio of insulin encapsulated in the nanoparticles to whole insulin in feed) was approximately 50 %. As released insulin from particle had shown a single monomer peak in HPLC, insulin was kept quite stable and not be aggregated nor denatured in this nanoparticle preparation (data not shown) .

2. Serum concentration of insulin

The concentration profile of serum insulin is shown in Fig. 1. The maximum concentration with transdermal nanoinsulin (200 μg) was 67.1 ± 25.9 μIU/ml at 4 hr, whereas that with sc monomer insulin (3 μg) was 462.6 ± 20.9 μIU/ml at 0.3 hr. AUC-I with nanoinsulin and sc monomer insulin was 264.8 and 431.3 μIU・hr/ml, respectively. And the relative BA-I with transdermal nanoinsulin was 0.9% based on insulin level.

3. *In vivo* hypoglycemic effects

The time course profile of the blood glucose response in ddY mice

is shown in Fig. 2. The average glucose concentration of 133.9 mg/dl at t=0 was defined as 100 % in Figure 2. A dose-dependent decrease of blood glucose was observed with transdermal nanoinsulin, whereas transdermal monomer insulin did not have any effect. With 200 μg of transdermal nanoinsulin, the maximum drop of blood glucose was 48.3 ± 3.9 % at 4 hr, whereas it was 64.1 ± 1.0 % at 0.3 hr with sc monomer insulin. The blood glucose could be followed only up to 6 hr, since fasting itself caused a drop of glucose after 6 hr in control mice. Neither transdermally-applied blank Ca-nanoparticles nor saline had a hypoglycemic effect.

The pharmacodynamic response of blood glucose in normal and diabetic mice is summarized in Table 1. The AUC with transdermal insulin (200 μg) and sc monomer insulin (3 μg) was 179 and 133 μIU·hr/ml, respectively in normal ddY mice. And the AUC with transdermal nanoinsulin (200 μg) and sc monomer insulin (5 μg) was 115 and 156 μIU·hr/ml, in diabetic dB/dB mice, and 100 and 74 μIU·hr/ml, in diabetic kkAY mice. Consequently,

the relative BA in each mouse model was 2.0% (ddY), 1.8% (dB/dB), and 3.4% (kkAy).

## Discussion

Since nanoparticles using $CaCO_3$ improved the transdermal delivery of insulin, these results suggest that nanoinsulin could penetrate through the stratum corneum and the epidermis, then reach the dermis to the blood circulation. This observation might be due to their ultrafine particle size and their surface characteristics, while PLGA-microparticles (5 μm) could penetrate through the stratum corneum and reach the epidermis, but was not able to reach the dermis (11).

It has also been suggested that calcium carbonate is a safe and viable drug carrier for the intranasal delivery of various drugs including insulin (12). In case of intranasal insulin deliver, a rapid and short-acting pharmacological effect is shown against postprandial hyperglycemia. Meanwhile, the serum glucose levels reduced slowly after transdermal administration of

nanoinsulin in comparison with subcutaneous injection, reflecting the difference in insulin absorption rates. Nanoparticles might remain in the dermis and slowly release free insulin *in situ*, since rhodamine-labeld-$CaCO_3$-nanoparticles, dermal penetration was detectable within 1 hr (data not shown). The systemic BA of insulin correlated well with the pharmacodynamic responses as determined by glucose-level measurement, although further study is required to clarify the higher BA on phamacodynamic response (BA) than that on pharmacokinetic study (BA-I). Comparative studies with the subcutaneous administration of insulin demonstrate that the insulin that was delivered transdermally by $CaCO_3$-nanoparticles achieved a therapeutically effective concentration that efficiently controlled blood glucose levels in diabetic mice. Although insulin BA with the transdermal Biphasic-insulin patches was 20-30% (6), that with nanoinsulin was only 2%. But this nanoinsulin was quite stable and easy to be prepared compared with liposome. Furthermore, the aggregation or denaturation of

insulin did not occur. The results to date indicate problems related to poor absorption, high proteolytic degradation, and/or variable delivery times. Consequently, BA is low, and response times are difficult to predict accurately.

Although the penetration mechanism of the nanoparticles through the skin is not fully known and requires further research, increased skin permeation may be achieved using an absorption enhancer such as benzyl benzoate which was employed in poly n-butylcyanoacrylate (PNBCA) nanocapsules, or oleic acid/D-limonne (7, 13, 14). Flexible vesicles with sodium cholate also promoted the transfer of insulin through the skin *in vivo* when applied onto the mice skin non-occlusively (15). Novel drug carriers, $CaCO_3$-nanopartiucles, provide a means for the non-invasive transdermal delivery of therapeutic agents, and nanoinsulin is thought to be a safe and highly available system enabling more effective insulin absorption.

Acknowledgements

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