Penetration and Absorption of Tacrolimus (FK506) Ointment in the Skin After a Single Dermal Application to the Rat

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Summary: The penetration and absorption of tacrolimus (FK506) in the skin of rat after a single topical application of ^{14}C -labeled FK506 (^{14}C -FK506) ointment under occlusive dressing was studied by semi-microautoradiography of the skin and by counting radioactivity in the sliced sections of the skin. Stability of FK506 in the skin was investigated *in vivo* and *in vitro*.

1. The distribution of the radioactivity of ^{14}C -FK506 in rat skin was observed by semi-microautoradiography after a single dermal application of the ointment. In the intact skin, radioactivity of the ointment was mainly distributed in the stratum corneum of the epidermis during application for 24 hours. In the damaged skin, radioactivity was observed in the epidermis and dermis after application for 2 hours, and was evenly distributed in the dermis and subcutaneous connective tissues after application for 8 hours. Radioactivity was detected in the hair follicles of both the intact and damaged skin, but that was negligible in the sebaceous gland of the intact skin or was less than in the follicles of the damaged skin.

2. After a single dermal application of ^{14}C -FK506 ointment to rats, the penetration of FK506 was investigated by counting radioactivity in the sliced section of skin. In the intact skin, maximal radioactivity was observed in the section of the epidermis throughout the application period for 24 hours. In the damaged skin, maximal radioactivity was detected in the dermis after application for 2 hours, and radioactivity in the sections was decreased thereafter and only a small amount of radioactivity was observed after application for 24 hours.

3. After application of ^{14}C -FK506 ointment for 24 hours, levels of radioactivity were compared with those of FK506 in the skin, and the unchanged FK506 accounted for about 90% of radioactivity detected.

4. The oxidative metabolism of FK506 was studied in the skin and liver microsomes. Formation of the *in vitro* major metabolite, the 13-O-demethylated metabolite, in the skin microsomes was several-fold less intensive than in the liver microsomes.

Key words: Tacrolimus, FK506, Ointment, Penetration, Absorption, Rat skin

Introduction

Tacrolimus (FK506) is a 23-membered macrolide antibiotic, produced by *Streptomyces tsukubaensis*. FK506 exhibited a very potent immunosuppressive action and has been marketed as an immunosuppressant for the prevention and control of rejection in organ transplantation.¹⁾ In addition to being an immunosuppressor in solid organ transplantation, FK506 has shown immunosuppressive effect in a variety of autoimmune diseases. Its activity in these disorders make FK506 a prime candidate for atopic dermatitis. Clinical trials of FK506 ointment are in progress for the treatment of atopic dermatitis.²⁾

The rodents were used for the toxicological and pharmacological studies during the development of FK506.

We evaluated the systemic properties about absorption, distribution and excretion after topical application of FK506 ointment to rat.^{3,4)} A lot of information of the basic systemic properties after intravenous and oral administration to rat had been accumulated for the development of Prograf[®],^{5,6)} and rats are suitable animals for the further evaluation of FK506 ointment. For the development of ointment formulation of a drug, information about penetration of the drug into the skin is essential. In this study, we reported the penetration and absorption of FK506 in the skin after a single dermal application of ^{14}C -FK506 ointment to rat.

Materials and Methods

Materials

FK506, ^{14}C -labeled FK506 (^{14}C -FK506), ^{14}C -FK506

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ointment, the 13-O-demethylated metabolite (M-I), the 31-O-demethylated metabolite (M-II), the 15-O-demethylated metabolite (M-III), and the 12-hydroxylated metabolite (M-IV) were obtained as described previously.⁷⁾ Specific activity of ¹⁴C-FK506 was 574 kBq/mg or more and its radiochemical purity was more than 97% by thin layer chromatography. β -Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-P DH) were purchased from Sigma Chemical Co. Testosterone was obtained from Nakarai Tesque and used after purification by reverse phase high performance liquid chromatography (HPLC). Standards of testosterone metabolites were purchased from Steraloid. 7-Ethoxycoumarin and hydroxycoumarin were obtained from Nakarai Tesque.

Animals and Treatment

Male Sprague-Dawley strain rats (7–8 weeks old) were purchased from Clea Japan Inc. Animals were used without fasting.

On the day before dermal application, rats were anesthetized with diethyl ether and the dorsal hair was removed by clipping with electric clippers and by shaving with an electronic shaver. The damaged skin was prepared by tape-stripping ten times with adhesive tape.

Semi-Microautoradiography

Rats were anesthetized with diethyl ether and 0.5% ¹⁴C-FK506 ointment (574 kBq/mg FK506) was applied on the dorsal skin (2 × 4 cm/250 g rat) with a stainless steel spatula at a dose level of 0.5 mg FK506/100 mg ointment/kg rat. The applied site was covered with a vinyl sheet and the animal body was wrapped with an elastic bandage to prevent removal of the sheet. The maximal period of application was 24 hours. Animals were killed by exsanguination under anesthesia with diethyl ether at 2, 8 or 24 hours after the start of application. The remaining ointment on the application site was removed by sweeping five times with individual cottons wet with slightly hot water, and the swept skin was dissected. The dissected skin was embedded in embedding material (O.C.T. Compound, Tissue-Tek), frozen in liquid propane cooled with liquid nitrogen, and cut in 5 μ m sections with a cryo-stat (CM3000, Leica). The sections were attached to a microscopic slide glass which was previously coated with emulsifier (NR-M2, Konica), and exposed for 63 days at around -80°C in a black box. After the exposure, the specimens were fixed with 10% formalin, washed with water, and developed with Konicadol (Konica) followed by stopping, fixing and washing the development. The samples were dried, stained with hematoxylin and eosin stain, spread with Entelanew (E. Merck), and observed with a microscope.

Penetration Study

Rats were anesthetized with diethyl ether and 0.1% ¹⁴C-FK506 ointment (643 kBq/mg FK506) was applied on the dorsal skin with a stainless steel spatula. The damaged skin was prepared on half of the clipped skin as described above and the ointment was applied to an equal area of the damaged and intact skin (1.5 × 3 cm, each) of the same animal at a dose level of 0.045 mg FK506/45 mg ointment/each site. After application, the site applied was covered with a vinyl sheet and the animal body was wrapped with an elastic bandage to prevent removal of the sheet. Animals were killed after application for 2, 8 or 24 hours by exsanguination under anesthesia with diethyl ether. The remaining ointment at the application site was removed by sweeping five times with individual cottons wet with slightly hot water, the swept skin was dissected, and the damaged and intact skin was separated by cutting. Each skin was prepared at about 2 mm thickness and frozen. The frozen skin was punched and the columnar skin (base, 1 cm²; and height, 2 mm) was prepared. The columnar skin was embedded in embedding material O.C.T. Compound, frozen as described above, and cut in 20 μ m sections from the surface of the skin. Each sliced section was mixed with 10 mL of Econofluor after dissolving with 0.2 mL of Soluene 350. Radioactivity in the treated sections was counted as described previously.⁷⁾

The rest of the punched sample was embedded, frozen, and cut in 10 μ m sections, attached to the slide glass, dried, and stained with hematoxylin and eosin stain. Distribution of the epidermis, dermis, and other skin constituents was observed with a microscope.

Analysis of Radioactivity and FK506 in Rat Skin

Rats were anesthetized with diethyl ether and 0.5% ¹⁴C-FK506 ointment (574 kBq/mg FK506) was applied to the dorsal skin (2 × 4 cm/250 g rat) with a stainless steel spatula at a dose level of 1.6 mg FK506/320 mg ointment/kg rat. After application, the site applied was covered with a vinyl sheet and the animal body was wrapped with an elastic bandage to prevent removal of the sheet. Animals were killed after 24 hours application and 10% (w/v) homogenates of the dissected skin were prepared in 0.1 N HCl/ethanol (1/1, v/v). FK506 in the homogenates was assayed as described previously,⁷⁾ after extraction to a mixture of n-hexane/isoamyl alcohol (40/1, v/v). For measurement of radioactivity, the homogenates (100 μ L) were dissolved in 1 mL of Soluene 350 and mixed with 10 mL of Hionicfluor, and radioactivity was counted as described previously.⁷⁾

Metabolism of FK506, Testosterone and 7-Ethoxycoumarin *In vitro*

Microsomes of the skin and liver of rats were prepared as follows. After removing the dorsal hair by clipping, rats were killed by decapitation. The dorsal skin was dissected and homogenized with 3.5 volume (v/w)

Fig. 1 Semi-microautoradiograms of the intact skin after a single dorsal application of ^{14}C -FK506 ointment to rat. Distribution of radioactivity was investigated after application to ^{14}C -FK506 ointment for (A) 2, (B) 8, and (C) 24 hours. Dermal sites of the skin are designated as follows: c, corium; e, epidermis; fc, fat cells; hf, hair follicle; ml, muscular layer; sg, sebaceous gland; and st, subcutaneous tissue.

of 1.15% KCl solution (containing 1 mM EDTA and 0.1 mM dithiothreitol) with Polytron homogenizer under ice cold condition. Skin microsomes were prepared by differential centrifugation and pellets washed at 105000 g were homogenized in 100 mM (pH 7.4) K-phosphate buffer (containing 20% glycerol, 1 mM EDTA and 1 mM dithiothreitol). Hepatic microsomes were prepared as described previously.⁸⁾ Reaction media contained 100 mM (pH 7.4) K-phosphate buffer, NADPH generating system (0.5 mM NADP, 5 mM G-6-P, 1 U/mL G-6-P DH and 5 mM MgCl₂), microsomal suspension, and substrate. Concentrations of ¹⁴C-FK506, and testosterone and 7-ethoxycoumarin were 10 μ M, and 500 μ M, respectively. Metabolite formation of ¹⁴C-FK506, testosterone and 7-ethoxycoumarin was determined as described previously.^{7,9)} Microsomal protein was measured by the method of Lowry *et al.*¹⁰⁾

Results

Semi-Microautoradiography

The penetration of radioactivity in 0.5% ¹⁴C-FK506

ointment was studied in the intact and damaged skin after a single dermal application to rats under occlusive dressing. After application on the intact skin for 2 hours (Fig. 1), silver particles which denote radioactivity were observed most abundantly in the epidermis, and especially in the stratum corneum. The particles were located in the upper layer of the dermis similar to or slightly lower to the radioactive level of the epidermis but were located at the back ground level in the lower layer. No particles were observed in the subcutaneous tissue, muscle layer, sebaceous gland and hair follicles. After application for 8 hours, the particles in the epidermis except in the stratum corneum were fewer than those in the epidermis after application for 2 hours, more particles were observed in the dermis and subcutaneous tissue after application for 8 hours than for 2 hours. The particles were detected in the hair follicles but not in the muscle layer, sebaceous gland and fat cells. After application for 24 hours, the particles were observed abundantly in the stratum corneum but not in the other sites.

In the damaged skin to which ¹⁴C-FK506 ointment

Fig. 2 Semi-microautoradiograms of the damaged skin after a single dorsal application of ¹⁴C-FK506 ointment to rat. Distribution of radioactivity was investigated after application to ¹⁴C-FK506 ointment for (A) 2, (B) 8, and (C) 24 hours. The sites of the skin are designated as in the legend of Fig. 1.

was applied for 2 hours (**Fig. 2**), silver particles were observed in the epidermis and dermis, and the levels and gradient of the particles were higher than those in the intact skin with the same application period. The particles in the subcutaneous tissue and muscle layer were slightly higher than those of background level. These were detected in the hair follicles of the dermis but not in the sebaceous gland. After application for 8 hours, the particles were generally higher in the damaged skin than those of the intact skin. The particles were evenly distributed in the dermis and subcutaneous connective tissue, and the particles in the hair follicles were more abundant than those in the sebaceous gland and fat cells. After application for 24 hours, the particles detected were generally few in the damaged skin but were slightly more abundant in the regenerated stratum corneum.

Penetration Study

After a single dermal application of 0.1% ^{14}C -FK506 ointment under occlusive condition, radioactivity in the sliced sections of the intact and damaged skin was deter-

Fig. 3 Distribution of radioactivity in the sections of intact skin after a single dorsal application of ^{14}C -FK506 ointment to rat

Distribution of radioactivity in skin slices was estimated after application to ^{14}C -FK506 ointment for (○) 2, (□) 8, and (△) 24 hours. Each point represents mean + S.E.

mined. In the intact skin (**Fig. 3**), the distribution of radioactivity in the samples collected after application for 2 and 8 hours was almost the same and peaked at 60 μm depth from the skin surface with a concentration of about 50 $\mu\text{g eq./cm}^3$ skin. After application for 24 hours, the distribution of radioactivity was similar to that applied for 2 and 8 hours. In the respective section at the same depth, the level of radioactivity in the sections after application for 24 hours was about half or one third of the level of the sections applied for 2 and 8 hours. The distribution of radioactivity in the sections at a range of 0 to 80 μm was almost flat and in the sections deeper than 80 μm gradually decreased. In the damaged skin (**Fig. 4**), maximal radioactivity was detected in the section at around 140 μm depth with a concentration of 20 $\mu\text{g eq./cm}^3$ skin after application for 2 and 8 hours. The distribution of radioactivity was flatter than that of the intact skin at respective depth, and also the range of flat level was wider. Radioactivity in the sections after 2 hours application was higher than that after application for 8 hours, and only a small amount of radioactivity was observed in the sections after application for 24 hours.

Concentrations of Radioactivity and FK506 in Rat Skin

After a single dermal application of 0.5% ^{14}C -FK506

Fig. 4 Distribution of radioactivity in the sections of damaged skin after a single dorsal application of ^{14}C -FK506 ointment to rat

Distribution of radioactivity in skin slices was estimated after application to ^{14}C -FK506 formulation for (○) 2, (□) 8, and (△) 24 hours. Each point represents mean + S.E.

Table I Concentration of radioactivity and FK506 in rat skin at 24 hours after single dermal application of 0.5% ^{14}C -FK506 ointment

Rat number	Radioactivity	FK506	Ratio
	(ng eq./g)	(ng/g)	
1	5453.5	5166.6	0.9474
2	3288.0	2800.0	0.8516
3	3915.2	3519.6	0.8990
Mean	4218.9	3828.7	0.8993
S.D.	1114.2	1213.2	0.0277
C.V. (%) ^{a)}	26.4	31.7	3.1

^{a)} : C.V. (%) = Mean/S.D. $\times$ 100

ointment to rat intact skin under occlusive dressing, levels of radioactivity and FK506 in the skin were determined (**Table I**). After application of the ointment for 24 hours, mean concentrations of radioactivity and FK506 were about 4200 ng eq./g and 3800 ng/g, respectively, and the unchanged FK506 accounted for about 90% of radioactivity in the skin. Although there were wide individual differences in concentrations of radioactivity and FK506, the ratio of FK506 to radioactivity was quite constant.

Table II Oxidative metabolism of FK506, testosterone and 7-ethoxycoumarin by skin and liver microsomes of rats

Substrate	Reaction	Activity	
		(pmol/min/mg protein)	
		Skin	Liver
FK506	M-I formation	0.071	194
Testosterone	2 α -Hydroxylation	n.d.	1700
	2 β -Hydroxylation	n.d.	250
	6 β -Hydroxylation	1.3	2330
	7 α -Hydroxylation	n.d.	150
	16 α -Hydroxylation	n.d.	2020
	16 β -Hydroxylation	n.d.	30
7-Ethoxycoumarin	O-Deethylation	0.31	1030

Values represent mean of duplicate experiments.

n.d. : not detected.

Oxidative Metabolism of FK506 by Microsomes

Oxidative metabolism of FK506, testosterone and 7-ethoxycoumarin was studied in skin and liver microsomes of rats (**Table II**). Formation rate of M-I, the *in vitro* major metabolite of FK506, was 0.071 and 194 pmol/min/mg protein in the skin and liver microsomes, respectively. Although 2 α -, 2 β -, 6 β -, 7 α -, 16 α - and 16 β -hydroxylating activities of testosterone were detected in the hepatic microsomes, only 6 β -hydroxylation activity was detected in the skin microsomes. Oxidative metabolism of FK506, testosterone and 7-ethoxycoumarin was much less in the skin microsomes than that by the hepatic microsomes.

Discussion

The distribution and penetration of radioactivity in the skin was studied after a single dermal application of ^{14}C -FK506 ointment to rats. About 90% of radioactivity was accounted for by the unchanged FK506 in the skin collected after application of the ointment for 24 hours. Oxidative metabolism of FK506 by skin microsomes was very low when compared to that by liver microsomes. These results indicate that the distribution and penetration of radioactivity observed in this study shows that the unchanged FK506 in rat skin is stable for at least 24 hours.

The transdermal absorption route of drugs is classified into two pathways; one is the transepidermal route through the stratum corneum and the other is the trans-appendageal route through the pilosebaceous unit or eccrine gland.¹¹⁾ The stratum corneum functions as a barrier of drug absorption in the transepidermal route, and the absorption is extensively enhanced under the condition without the stratum corneum, such as the damaged skin prepared by removing the stratum corneum with adhesive tape. After application of ^{14}C -FK506 ointment to

the intact skin, FK506 was mainly distributed in the stratum corneum of the epidermis during application of the ointment for 24 hours. In the damaged skin, FK506 was observed in the epidermis and dermis after application for 2 hours, and was evenly distributed in the dermis and subcutaneous connective tissues after application for 8 hours. Radioactivity was detected in the hair follicles of the intact and damaged skin, but that in the sebaceous gland was negligible in the intact skin or less than in the follicles of the damaged skin. These results support our previous conclusions that the stratum corneum of rat skin acts as a barrier against the absorption of FK506 in the ointment, and that FK506 in the ointment is absorbed through the transepidermal route of the rat.^{3,4)}

After a single dermal application of ^{14}C -FK506 ointment to rats, distribution of radioactivity in the sliced sections differed between the intact and damaged skin. The distribution was steeper in the intact skin with a peak distribution at the epidermis, and radioactivity was more evenly distributed in the damaged skin after application for 2 and 8 hours. FK506 was mainly distributed in the stratum corneum from the results of semi-microautoradiography of the intact skin. These results indicate that the high distribution of FK506 in the stratum corneum affects on the radioactivity in the outer few sliced sections of the intact skin. As reported previously,³⁾ excretion of radioactivity in the urine and feces was respectively 0.4 and 4.2% of dose after application to the intact skin and 2.4 and 53.6% to the damaged skin. Radioactivity remaining in the ointment after application for 24 hours was 69.6 and 38.3%, respectively, on the intact and damaged skin, and radioactivity detected at the application site was 22.6 and 4.7% in the intact and damaged skin. Although the level of radioactivity in the sliced sections of the damaged skin was lower than that of the intact skin, the large bioavailability was observed in the damaged skin. The results of the present and previous study indicate that radioactivity is absorbed more rapidly through the damaged skin than the intact skin, and absorbed radioactivity is excreted mainly in the feces as observed in the result after intravenous injection of ^{14}C -FK506.⁶⁾

Microsomes from rat skin catalyzed O-deethylation of 7-ethoxycoumarin and hydroxylation of testosterone but their specific activities were much lower than those of liver microsomes as previously reported.¹²⁾ In this study, only 6 β -hydroxylation of testosterone was detected by skin microsomes but no hydroxylation at other positions. The results suggest that cytochrome P450 3A (CYP3A) is present in the rat skin but its activity and/or content is very low. CYP3A was reported to catalyze oxidative metabolism of FK506 in rat and human.¹³⁾ Contribution of microsomes or CYP3A in rat skin to the metabolism of FK506 during the penetration of FK506 through the skin should be very little or negligible, considering the very low specific activity of FK506

metabolism by rat skin microsomes. This suggestion is supported by the fact that unchanged FK506 occupied a major portion of radioactivity in the skin after a single dermal application of ^{14}C -FK506 formulation.

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