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Effects of FK506 (tacrolimus hydrate) on chronic oxazolone-induced dermatitis in rats

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Abstract

Chronic allergic contact dermatitis was induced in rat ear by repeated application of oxazolone. This dermatitis was accompanied by sustained ear swelling and marked epidermal hyperplasia. In the induced ear, there was marked inflammatory cell infiltration into the dermis site and the interferon-γ amount increased in both protein and mRNA, while the interleukin-4 amount changed minimally. Topical administration of FK506 (tacrolimus hydrate) dramatically suppressed ear swelling and epidermal hyperplasia as well as the increase in interferon-γ expression. Betamethasone valerate also showed suppressive effects, but 1,25-dihydroxyvitamin D_3 (calcitriol) had no effect. These results suggest that interferon-γ plays an important role in dermatitis and this model could be a useful pharmacological model for chronic dermatitis featuring epidermal hyperplasia in which interferon-γ plays a crucial role, such as psoriasis. FK506 demonstrating suppressive effects as potent as those of betamethasone valerate shows potential as a topically usable drug for such skin disorders.

Keywords: Psoriasis; FK506 (tacrolimus hydrate); Animal model; Allergic contact dermatitis

1. Introduction

Psoriasis is a common, chronic and inflammatory skin disorder, however, the pathophysiological mechanism has not yet been well characterized. Good animal models, indispensable for developing new drugs, have not been developed either. Recently, severe combined immunodeficient (SCID) mice transplanted with psoriatic human skin have been proposed as a useful model, not only for investigating the pathophysiology of psoriasis, but for the assessment of potential antipsoriasis therapies (Boehncke et al., 1999; Dam et al., 1999; Zeigler et al., 2001). However, this model requires specific tissue, i.e. psoriatic skin, which is not easily obtained, making it difficult to conduct the mass screening necessary for pharmacological assessment. Thus, an appropriate animal model for pharmacologically assessing drug candidates is needed.

One of the most prominent features of psoriasis is epidermal hyperplasia, which can be a useful pharmacological index for the disease. A single application of 12-O-tetradecanoilphorbol 13-acetate (TPA) has been reported to induce skin inflammation accompanied by epidermal hyperplasia due to enhanced keratinocyte proliferation, which is assessed by the measurement of ornithine decarboxylase activity (Sato et al., 1996). This model has been used to estimate antipsoriasis drug action, however, it is a simple acute inflammation model and has been shown to be induced independently of T cell involvement in its onset (Reynolds et al., 1998), making use of the model limited. T cells are now mainly classified into two types, interleukin-2, interferon-γ producing type 1 (Th1 and Tc1) cells and interleukin-4, interleukin-5 producing type 2 (Th2 and Tc2) cells. Psoriasis patients have been shown to have type 1 bias in lesion skin and peripheral blood (Austin et al., 1999), and are thought to develop type 1 cytokine networks, resulting in keratinocyte hyperplasia and angiogenesis (Nickoloff, 1991). Among type 1 T cell-producing factors, interferon-γ has been indicated to be crucial to the pathogenesis of psoriasis (Bonish et al., 2000; Ovigne et al., 2001). Therefore, a chronic inflammation model, easily produced without requiring specific tissue and depending on interferon-γ producing type 1 T cells,

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would much better meet the requirements for a model of psoriasis.

We now attempted to develop a chronic skin inflammation model that depends on interferon-γ and induces considerable epidermal hyperplasia in rat ear on repeated application of oxazolone. We then evaluated the effects of FK506 (tacrolimus hydrate), a potent immunosuppressant currently used for preventing allograft rejection in various solid organ transplantations by systemic administration, as well as for treatment of atopic dermatitis by topical administration (Nakagawa et al., 1994; Ruzicka et al., 1997; Boquniewicz et al., 1998). The effect of FK506 was then compared to that of drugs now used clinically in the treatment of psoriasis.

2. Materials and methods

2.1. Animals

All experiments were approved by the Fujisawa Pharmaceutical Animal Experiment Committee and were carried out according to guidelines for animal experiments at Fujisawa Pharmaceutical Sprague–Dawley rats (female, 6 weeks old) were purchased from Nippon SLC (Hamamatsu, Japan). They were housed under specific-pathogen-free conditions in our animal facility, fed a standard laboratory diet, given water ad libitum and used for experiments after preliminary housing for 1 to 2 weeks.

2.2. Drugs and reagents

FK506 was prepared at Fujisawa Pharmaceutical (Osaka, Japan). Betamethasone valerate and calcitriol were obtained from Sigma (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. All drugs were dissolved in ethanol to the appropriate concentrations. Oxazolone (Sigma) was dissolved in ethanol to a concentration of 2% for initial sensitization or in vehicle [acetone:olive oil (4:1)] to 1.6% for subsequent application to the ear. The enzyme-linked immunosorbent assay (ELISA) kits for quantitative analysis of cytokines were purchased from PharMingen (San Diego, CA, USA).

2.3. Experimental protocol

Sprague–Dawley rats were sensitized by application of 300 μl of 2% oxazolone to the abdomen and then a total of 60 μl of 1.6% oxazolone was applied to both sides of the ear every 3 days starting from 7 days after sensitization. Ear thickness was measured using a dial thickness gauge (Ozaki Seisakusho, Tokyo, Japan) 72 h after each application of oxazolone. FK506, betamethasone valerate and calcitriol were applied in a total volume of 60 μl to both sides of the ear 30 min before and 3 h after each application of oxazolone.

2.4. Histopathological study and measurement of epidermal thickness in the ear

Rat ears were excised 72 h after the last application of oxazolone and fixed in 10%-buffered formalin solution, embedded in paraffin by standard methods, cut into 3-μm sections and stained with hematoxylin–eosin, then assessed under light microscopy. After the microscopic fields were photographed, epidermal thickness measured as the distance from the bottom of the stratum corneum to the basement membrane in the interfollicular epidermis (Reynolds et al., 1998) was determined from the mean of four random fields for which 5 measurements were averaged. For immunohistochemical studies, the ear was embedded in optimal cutting temperature (OCT) compound (Miles, Elkhard, IN, USA), snap frozen in dry ice-acetone, and cut into 5-μm slices. Cryosections were sequentially incubated with mouse monoclonal antibody against rat CD4 (Serotec, Raleigh, NC, USA) or mouse monoclonal antibody against rat CD8β (PharMingen), followed by goat anti-mouse IgG conjugated with peroxidase (Jackson, West Grove, PA, USA). The reaction products were visualized with 3-amino-9-ethylcarbazole. For staining of eosinophils, 3-μm paraffin wax sections were treated with 0.1% Congo red solution (Merck, Darmstadts, Germany).

2.5. Measurement of interferon-γ and interleukin-4 in the ear

Samples of ear tissue extract for ELISA were prepared as described by Kitagaki et al. (1997). Briefly, ears were

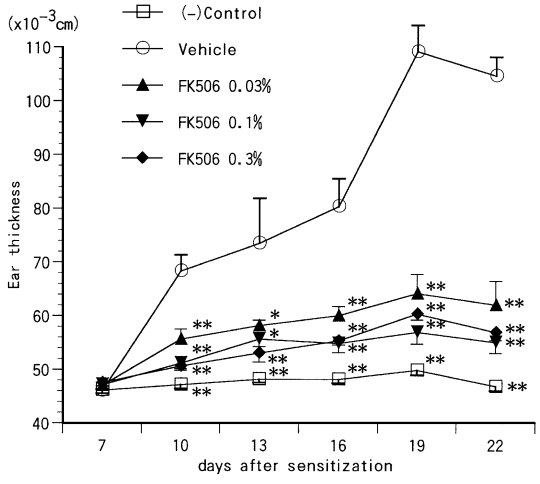

Fig. 1. Effects of FK506 on the change in ear thickness of rat ear induced by repeated application of oxazolone. Sprague–Dawley rats were sensitized by one application of 2% oxazolone to the abdomen followed by 1.6% oxazolone application to both sides of the ear every 3 days starting after a 7-day sensitizing period. As a negative control, no application followed sensitization. Vehicle (ethanol) or FK506 was applied to the ear 30 min before and 3 h after each application of oxazolone. Ear thickness was measured 72 h after each application of oxazolone. Values represent means ± S.E.M. for eight rats. *$P<0.05$, **$P<0.01$: significantly different from the vehicle group (Dunnett's multiple comparison test).

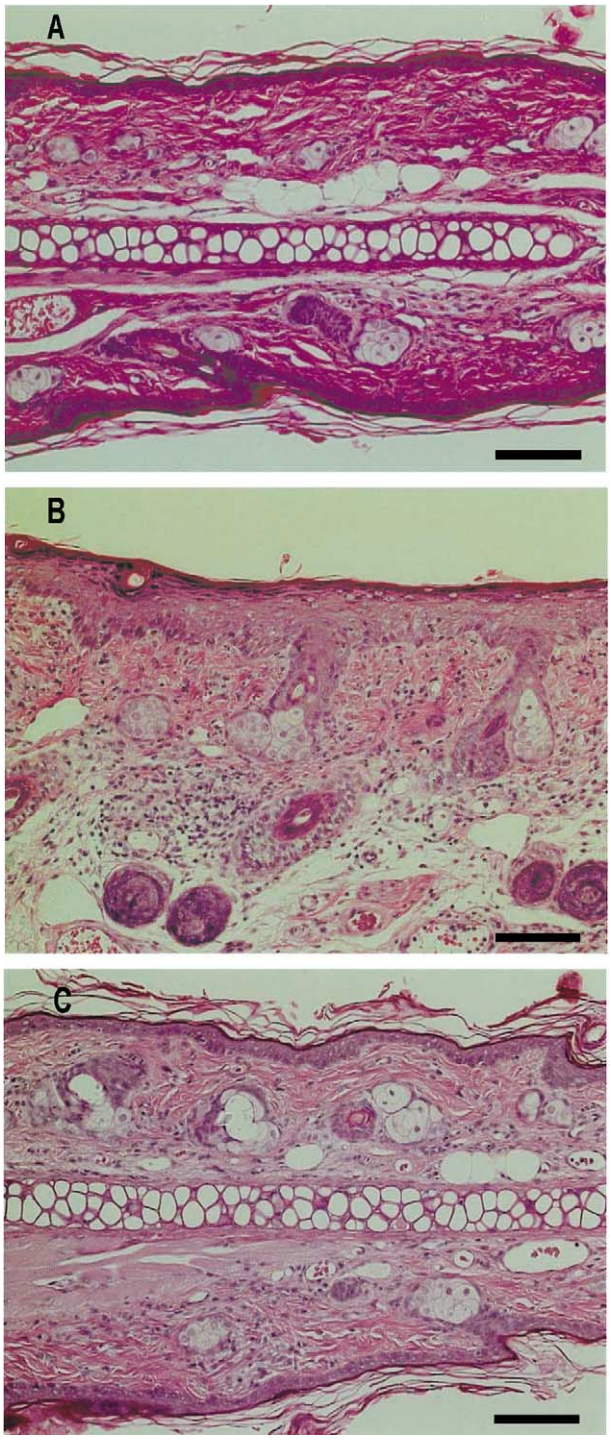

Fig. 2. Histopathological pictures of rat ear after repeated application of oxazolone with or without FK506. Rat ears were excised 72 h after the last application of oxazolone and stained with hematoxylin–eosin. As a negative control, rats were only sensitized with 2% oxazolone to the abdomen followed by no application to the ear (A). 1.6% Oxazolone was applied to both sides of the ear every 3 days starting after a 7-day sensitizing period and vehicle (ethanol) (B) or 0.1% FK506 (C) was applied to the ear 30 min before and 3 h after each application of oxazolone. Scale bars, 100 μm.

excised 6 h after the last application of oxazolone and homogenized with 1 ml of 0.1% Tween-20 in phosphate-buffered saline (PBS). Samples were then frozen at −30 °C for 30 min, thawed in a 37 °C water bath for 15 min with the freezing and thawing procedure repeated once, then were sonicated for 15 s and centrifuged for 5 min at 13,000 × *g*. The supernatants were collected and kept at −30 °C until measurement of cytokines.

2.6. Analysis of interferon-γ and interleukin-4 mRNA expression in the ear by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from ears 6 h after the last application of oxazolone by using TRIzol reagent (GIBCO BRL, Caitherburg, MD, USA), according to the manufacturer's instructions. First-strand cDNA was generated using random primers (GIBCO BRL) from 5 μg of total RNA. Each cDNA sample was amplified in a total volume of 25 μl reaction mixture containing cDNA template, dNTP (Amersham Pharmacia Biotech, Amersham, UK), DNA polymerase (KOD Dash; TOYOBO, Osaka, Japan) and primers. Primer sequences were designed as described by Fujimura et al. (1998): interferon-γ sense, 5′-AGA-GCC-TCC-TCT-TGG-ATA-TCT-GG-3′ and antisense, 5′-GCT-TCC-TTA-GGC-TAG-ATT-CTG-GTG-3′ (product size, 309 bp); interleukin-4 sense, 5′-TCT-CAC-GTC-ACT-GAC-TGT-A-3′ and antisense, 5′-CTT-TCA-GTG-TTG-TGA-GCG-T-3′ (product size, 406 bp); and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) sense, 5′-GCA-TGG-CCT-TCC-GTG-TTC-CTA-C-3′ and antisense, 5′-ACT-CCT-TGG-AGG-CCA-TGT-AGG-C-3′ (product size, 318 bp). Ampli-

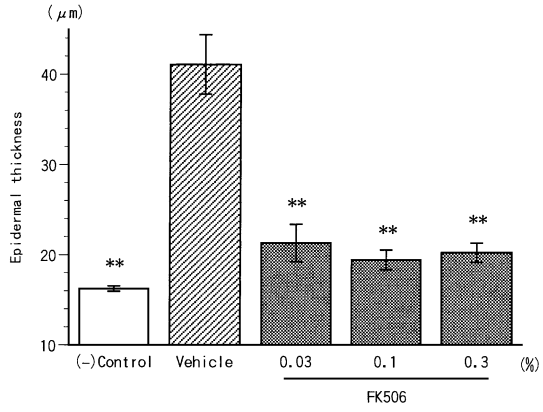

Fig. 3. Effects of FK506 on epidermal thickness induced in rat ear by repeated application of oxazolone. Rats were sensitized by one application of 2% oxazolone to the abdomen followed by 1.6% oxazolone application to both sides of the ear every 3 days starting after a 7-day sensitizing period. As a negative control, no application followed sensitization. Vehicle (ethanol) or FK506 was applied to the ear 30 min before and 3 h after each application of oxazolone. Ears were excised 72 h after the last application of oxazolone and epidermal thickness was measured after staining with hematoxylin–eosin. Values represent means ± S.E.M. for eight rats. $^{**}P < 0.01$: significantly different from the vehicle group (Dunnett's multiple comparison test).

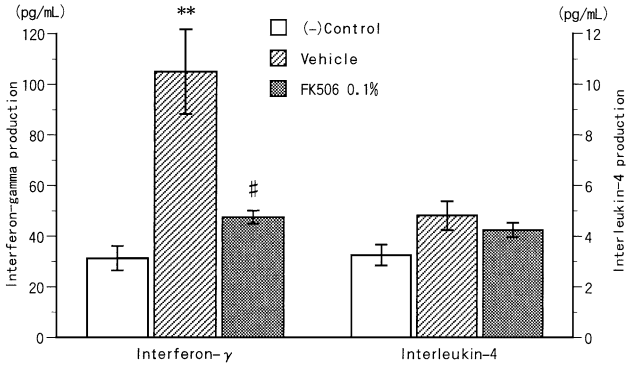

Fig. 4. Effects of FK506 on interferon-γ and interleukin-4 production in induced rat ear by repeated application of oxazolone. Rats were sensitized by one application of 2% oxazolone to the abdomen followed by 1.6% oxazolone application to both sides of the ear every 3 days starting after a 7-day sensitizing period. As a negative control, no application followed sensitization. Vehicle (ethanol) or 0.1% FK506 was applied to the ear 30 min before and 3 h after each application of oxazolone. Ears were excised 6 h after the last application of oxazolone, and interferon-γ and interleukin-4 levels in homogenized ear tissue were measured by ELISA. Values represent means ± S.E.M. for five rats. $^{**}P<0.01$: significantly different from the negative control group (Student's *t*- or Aspin–Welch test). $\#P<0.05$: significantly different from the vehicle group (Student's *t*- or Aspin–Welch test).

fication was performed for 36 cycles for interferon-γ and interleukin-4, 22 cycles for G3PDH (glyceraldehyde-3-phosphate-dehydrogenase) in a thermal cycler; 95 °C for 15 s, 65 °C for 30 s and 72 °C for 60 s. Polymerase chain reaction (PCR) product was applied to 2.2% agarose gel, resolved by electrophoresis, and visualized by ethidium bromide staining. The intensity of each band was obtained and the ratio to the intensity of G3PDH as an internal control for RT-PCR was calculated.

2.7. Statistical analysis

The data were expressed as the means ± S.E.M. Statistical analyses were made using Dunnett's multiple comparison test or Student's *t*- or Aspin–Welch test for two-sample comparison. A *P* value less than 0.05 was considered to indicate a significant difference.

3. Results

We applied oxazolone to the ear of Sprague–Dawley rats every 3 days, starting from 7 days after sensitization with oxazolone, onto the shaved abdomen, based on the result of a preliminary experiment showing that a single application of oxazolone to sensitized rats gave a peak value for ear swelling after 72 h (data not shown). The ear with oxazolone applied repeatedly exhibited erythema (reddening of the skin), edema and/or induration, and sometimes abrasion. When ear thickness was measured as an index of skin inflammation, it increased as application was repeated and reached its maximum 19 days after sensitization (Fig. 1). For histopathological analysis, we excised the ear at 22 days and stained it with hematoxylin–eosin (Fig. 2). Note that the ear with oxazolone applied (B) swelled so dramatically that the entire section could not be shown, as it was for the other ear sections (A and C) although the magnification was the same. Also the ear showed prominent epidermal hyperplasia and marked infiltration of inflammatory cells, consisting of monocytes, granulocytes and macrophages, mainly into the dermis and some into epidermis, whereas only a thin epidermal layer and sparse cells were observed in negative control ear. Additional immunohistochemical assessment revealed that CD4- and CD8-positive cells were detectable in the dermis site of the oxazolone-applied ear but not in the negative control; no eosinophils were detected in either case. Measurement of epidermal thickness to assess epidermal hyperplasia showed that oxazolone application resulted in a significant, two- to three-fold increase of epidermal thickness compared to the negative control (Fig. 3).

To investigate the change in cytokine expression accompanied by the onset of dermatitis, interferon-γ and interleukin-4, which are representative type 1 and type 2 cytokines, respectively, were measured using ELISA and RT-PCR analyses. The production of interferon-γ protein increased greatly in the ear with oxazolone applied, whereas the increase in interleukin-4 was only slight and not significant (Fig. 4). Likewise, RT-PCR analyses showed a clear expression of interferon-γ mRNA in the ear with oxazolone but not in the negative control (Fig. 5). We could not detect

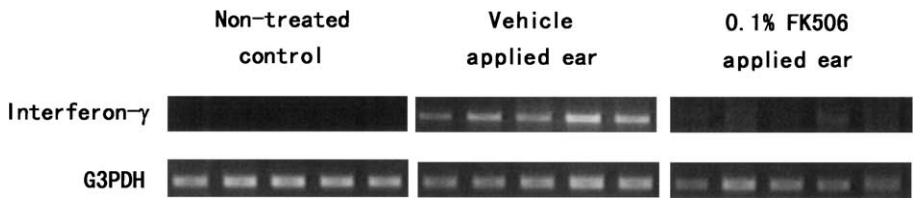

Fig. 5. Effects of FK506 on the expression of interferon-γ mRNA in rat ear induced by repeated application of oxazolone. Rats were sensitized by one application of 2% oxazolone to the abdomen followed by 1.6% oxazolone application to both sides of the ear every 3 days starting after a 7-day sensitizing period. As a negative control, no application followed sensitization. Vehicle (ethanol) or 0.1% FK506 was applied to the ear 30 min before and 3 h after each application of oxazolone. mRNA was extracted from rat ear 6 h after the last application of oxazolone and the expression of interferon-γ mRNA was analyzed by RT-PCR. When the intensity of each band was obtained and the ratio to the intensity of G3PDH as an internal control for RT-PCR was calculated, (–) Control; 0.00 ± 0.00, Vehicle applied ear; 0.44 ± 0.04, 0.1% FK506 applied ear; 0.04 ± 0.02 (means ± S.E.M. for five rats), the difference between the vehicle group and the 0.1% FK506 group was significant at $P<0.01$ (Student's *t*- or Aspin–Welch test).

interleukin-4 mRNA in either the oxazolone or the negative control ear (data not shown).

Next, we evaluated the effects of FK506 on this model by topical administration after solution in ethanol. FK506 at all doses (0.03%, 0.1%, 0.3%) potently suppressed ear swelling at each time-point as well as the epidermal hyperplasia at 22 days, with a suppressive rate of 80% or more (Figs. 1 and 3). These results were accompanied by the observation that not only inflammatory cell infiltration but also elevation of interferon-γ expression was significantly suppressed in both protein and mRNA by the administration of FK506 (Figs. 2, 4 and 5).

Finally, we compared the effects of FK506 to those of calcitriol and corticosteroid, which are now clinically used for psoriasis and other skin disorders. As shown in Fig. 6, betamethasone valerate, a potent corticosteroid, significantly suppressed the increase in ear thickness as well as in epidermal thickness, with almost equal efficacy to FK506. In contrast, calcitriol had no effect in this model.

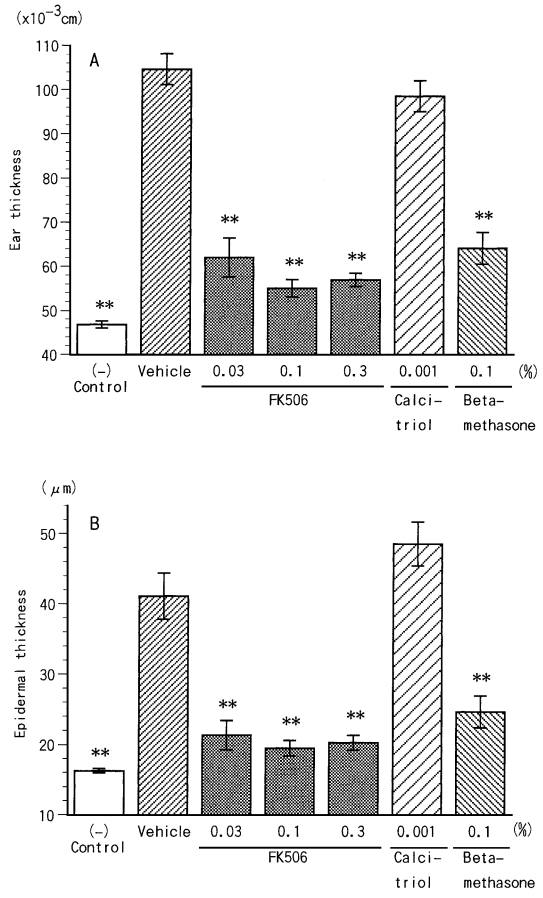

Fig. 6. Effects of FK506, betamethasone valerate and calcitriol on ear thickness (A) and epidermal thickness (B) induced in rat ear by repeated application of oxazolone. Rats were sensitized by one application of 2% oxazolone to the abdomen followed by 1.6% oxazolone application to both sides of the ear every 3 days starting after a 7-day sensitizing period. As a negative control, no application followed sensitization. Vehicle (ethanol), FK506, betamethasone valerate or calcitriol was applied to the ear 30 min before and 3 h after each application of oxazolone. Ear thickness was measured 72 h after the last application of oxazolone. Then, ears were excised and epidermal thickness was measured after staining with hematoxylin–eosin. Values represent means ± S.E.M. for eight rats. $^{**}P<0.01$: significantly different from the vehicle group (Dunnett's multiple comparison test).

4. Discussion

We now induced chronic-contact dermatitis in the ear of Sprague–Dawley rats by repeatedly applying oxazolone. This dermatitis was accompanied by sustained ear swelling, prominent epidermal hyperplasia and marked infiltration of inflammatory cells consisting of monocytes, granulocytes and macrophages but no eosinophils. Additionally, there was an increased interferon-γ amount in both protein and mRNA in the induced ear, while the interleukin-4 amount changed only minimally. Interferon-γ has been shown to activate various types of cells, resulting in inflammatory events (Issekutz et al., 1988; Barker et al., 1989), and to induce thickened epidermis due to the increase in keratinocyte proliferation (Carroll et al., 1997). Therefore, it is reasonable to conclude that ear edema and the prominent epidermal hyperplasia in our model were induced by interferon-γ.

Topical treatment with FK506 dramatically suppressed not only the increase in ear thickness but epidermal thickness. In addition, the enhanced expression of interferon-γ in skin with oxazolone applied was also suppressed. In vitro FK506 has potent suppressive effects on the production of various cytokines from human PBMC including interferon-γ at a dose of 0.1 ng/ml or more (Sakuma et al., 2001), while the direct effect on keratinocytes is not so potent, that is FK506 does not suppress the proliferation of keratinocytes from humans (Duncan, 1994; Kaplan et al., 1995) and rats (our unpublished observation) at a dose of as much as 1 μg/ml. Accordingly, we hypothesized that the suppressive effect of FK506 on epidermal hyperplasia may be mediated by the suppression of interferon-γ produced by T cells. However, we do not eliminate the possibility of involvement of factors other than interferon-γ as FK506 modifies various events important for inducing skin inflammation such as antigen presentation (Panhans-Gross et al., 2001), keratinocyte activation (Michel et al., 1996), and the expression of adhesion molecules (Homey et al., 1998).

Betamethasone valerate, a potent corticosteroid used clinically in the treatment of psoriasis and other skin disorders, also suppressed the increase in ear thickness and epidermal thickness to an extent similar to that with FK506. Corticosteroids are well known to have potent anti-inflammatory effects, however, topical use can cause intense skin atrophy (Schäfer-Korting et al., 1996; Oikarinen et al., 1998), one of the serious side-effects limiting their use for chronic skin diseases. Repeated application of corticosteroids on dorsal skin of rats also causes dramatic skin atrophy (Hisatomi et al., 1997), which suggests that betamethasone valerate may have suppressed ear thickness and/or epidermal

hyperplasia in our model through atrophogeneic effects in addition to anti-inflammatory effects.

In contrast, calcitriol whose derivatives are also used clinically against psoriasis, had no effect in our model, although it has been reported to have anti-proliferative effects on keratinocytes (Matsumoto et al., 1990; Sato et al., 1996), and has been demonstrated to have immunosuppressive effects as well (Bagot et al., 1994; Barna et al., 1997; Larsson et al., 1998). We do not know the reason for this, however, it has been shown that topical application of calcitriol and its derivatives has no effect on epidermal hyperplasia in mice, or sometimes even enhances it if applied repeatedly (Gniadecki et al., 1995). We therefore speculate that the in vitro effect of calcitriol is hard to reproduce in animal models.

In conclusion, this model can be a useful pharmacological model for chronic dermatitis featuring epidermal hyperplasia in which interferon-γ plays a crucial role, like psoriasis (Bonish et al., 2000; Ovigne et al., 2001). It has the advantages of being easy to use, and not to require specific tissue, animals or techniques that are necessary for human psoriatic skin-SCID mouse transplant and other models (Carroll et al., 1995; Blessing et al., 1996; Cook et al., 1997; Schön et al., 2000). Based on results with this model, FK506 displays great potential for becoming a novel, topically usable drug for skin disorders.

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