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Effects of 0.1% Tacrolimus on Canine Skin Mast Cells and Eosinophils

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Abstract : Five dogs were used to determine whether 0.1% tacrolimus ointment application for one day would inhibit IgE-mediated late-phase reactions (LPRs). It was consisted of three periods: one period without therapeutic administration (control) and two periods of treatment with either the tacrolimus ointment or vehicle. Induction of IgE-mediated LPRs was induced by intradermal injections of 0.05 ml (0.14 mg/ml) of solution of goat anti-canine IgE polyclonal antibodies. Each section for mast cells (MCs) and eosinophils (EPs) was stained with acidified toluidine blue, and Luna's stain, respectively. Assessment of anti-inflammatory effect of tacrolimus ointment composed of cell counts of MC and EP from lesions of induced LPR. In normal canine biopsies, the number of dermal MCs and EPs were 12.3 ± 1.4 cells/mm^2 and 3.1 ± 1.3 cells/mm^2, respectively. MC counts dramatically decreased at time dependent manner after anti-IgE administration. However, the number of MCs on 6 hours after challenge was significantly less decreased in the groups treated with the tacrolimus, as compared with control and vehicle group. The number of EPs on 24 hours after challenge was significantly lower in the group treated with the tacrolimus than in the control and vehicle groups. In conclusion, this study revealed that 0.1% tacrolimus ointment in dogs may exert a potent anti-inflammatory effect on inhibition of MC degranulation and also secondary prevention of EP infiltration during LPR.

Key words **:** tacrolimus, mast cell, eosinophil, late-phase reaction, atopic dermatitis, dog.

Introduction

Atopic dermatitis (AD) is defined as a hereditary, IgE-mediated hypersensitivity to environmental allergens characterized by intense pruritus, induration, a distinctive clinical morphology, and distribution of skin lesions, high IgE levels, mast cell (MC) degranulation and eosinophilia (28,29). The pathogenesis of AD may be the result of an allergen-specific process mediated by Langerhans cells. In acute phase of AD lesions, Langerhans cells release of IL-1 results in activation specific T-helper lymphocyte subset including CD4+ Th2 lymphocytes. Activated CD4+ Th2 cells are associated with the production of IL-4, IL-5, and IL-13. These cytokines are propensity to influencing IgE synthesis and MC degranulation, promoting eosionophil (EP) differentiation and survival, inducing vascular endothelial adhesion molecules, thus contribute to advance chronic lesion (32). In late chronic lesion, there is an increase of Th1 cytokines such as IL-2, IFN-γ, and IL-12 (3,32). In dogs, the pathogenetic basis of AD is similar with humans (25).

The intradermal administration of anti-canine IgE in both normal and atopic dogs can give rise to the immediate-phase and late-phase reaction (LPR) (26). Immediate-phase reaction (IPR) triggered by MC degranulation is associated with the acute phase of AD lesion. During this phase, MC degranulation can induce not only pro-inflammatory mediators such as histamine, and platelet activating factor but also various cytokines such as IL-3, IL-4, IL-5, IL-6, GM-CSF, TNF-α, and IFN-γ. This sequela can be accompanied with LPR characterized by infiltration of EPs, basophils, and mononuclear cells including memory T-cells. A LPR can develops between 6 and 24 hours after anti-IgE injection, characterized by increased skin thickening, diffuse edema, pruritus, and erythema (11, 26,41). These characters are similar with AD. Therefore, LPRs are excellent model to determine drug efficacy for AD.

Therapy for AD is composed of the avoidance of allergen, the establishment of skin barriers, the use of anti-inflammatory drugs, allergen-specific immunotherapy or antimicrobial agents (27,36). Despite hyposensitization is the only therapy that can prevent the development of further allergy, it is usually not seen clinical improvement until the first 3-12 months of continued on therapy (8). Antihistamine such as terfenadine and diphenhydramine inhibited immediate-phase reaction but not LPR. Topical corticosteroids are the most potent treatment for AD. However, more severe patients and chronic use may be associated with significant side effects including atrophy, scaling, comedones, alopecia, and pyoderma correlates with steroid potency (33,37).

Tacorlimus or, FK-506 (FR900506), an antibiotic of the macrolide lactone isolated from fungus *Streptomyces tsukubaensis*, is an immunosuppressive agent with a spectrum of activity similar to that of cyclosporine A (14,17,23). The use

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of tacrolimus or cyclosporin A in atopic patients is, however, restricted due to some serious adverse effects including nephrotoxicity, neurotoxity, diabetogenicity, and arterial hypertension (18,22). Therefore, many researchers tried to topical use of these drugs. They revealed that tacrolimus could be effective due to lower molecular weight and lipophilicity, and higher potency compared with cyclosporin A (22). Advantages of tacrolimus topical agent are associated with minimal systemic absorption, and an excellent safety profile (29). It was approved for use in humans with AD in Japan in November 1999 and tacrolimus (FK-506) ointment, Protopic, commercially available in Korea.

MCs are crucial role in immediate phase and acute phase of AD lesions; EPs play a pivotal role in late-phase and chronic phase of AD lesions. Therefore, the present study was designed to determine whether a 0.1% tacrolimus ointment could prevent degranulation of MCs and infiltration of EPs in LPR in normal canine skin. This is the first study investigating the anti-inflammatory effects on one day administration of tacrolimus in the LPR-induced by anti-IgE antibodies.

Materials and Methods

Subjects and study design

Five dogs (3-5 year old) were used in this study. Two of them were Maltese, two were Shih tzu, and the rest was Pekingese. Two of them were female and three were male. This study was performed as a randomized, crossover, and placebo-controlled for the purpose of rule out the active ingredient. It was divided into three periods (Table 1): one period without therapeutic administration (control), and two similar periods of treatment with either the active or vehicle of one day, separated by a wash-out period of 12 days (30). Dogs with presence of other pruritic skin diseases and the presence of secondary ear and skin infections were excluded.

Application of the ointment

Active(0.1% Protopic® Fugisawa, Japan) or placebo ointment were given on days 7 to 8, and days 20 to 21 during 24 hours (Fig 1). Five dogs were treated first with the active product and then with the placebo. The right side of the thorax was used for the first application and the left side of the thorax for the second application. The active product or vehicle was applied twice a day on day 7, and 20 and once a day the next following day on 8, and 21 before collection specimens.

Table 1. Mast cell and eosinophil counts in normal dogs. The number of mast cells and eosinophils was counted in both 10 consecutive superficial dermis and deep dermis. Counts are given per mm^2

	Superficial Dermis	Deep Dermis	Dermis Total
Mast Cell Count per mm^2	18.9 ± 2.1*	5.7 ± 1.2	12.3 ± 1.4
Eosinophils Count per mm^2	3.1 ± 1.6	3.1 ± 2.0	3.1 ± 1.3

Mean ± SE
*Significantly higher than counts from deep dermis

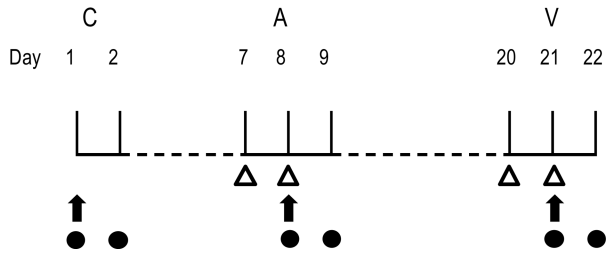

Fig 1. Schematic representation of the protocol of the study. Open triangles: application of FK-506 ointment or vehicle. Closed arrows: injection of anti-IgE. Closed circles: collection of skin biopsy specimens 6, 12, and 24 hours post administration antiserum. A: Active ingredient (FK-506 ointment), V: Vehicle, C: Control

Generation of IgE-mediated LPR

For each procedure, an area of approximately 10 cm × 5 cm on the lateral thorax was clipped using electric clippers and a No. 40 clipper blade. Anesthesia was induced with medetomidine (750 mcg/kg) intravenously (12,39). Induction of IgE-mediated LPRs was induced by intradermal injections of 0.05 ml of solution of goat anti-canine IgE polyclonal antibodies (Goat anti-Canine IgE, USBiological, USA) containing 0.14 mg/ml (26). Three injection sites, more than 2 cm apart in two horizontal rows, were made and marked with a permanent marker pen (30). To avoid self-trauma at the site of injection, we let them wear elizabath collar and shoes made by coban and cotton, and were kept on the dogs until the biopsies had been obtained.

Biopsy procedure and handling

Each biopsy specimen (8 mm) was collected from normal appearing skin on the lateral thorax before injection, Samples were collected by the same method at 6, and 24 hours post-injection from skin sites. After sampling, each specimen was immediately placed in Bouins solution for routine embedding in paraffin. The staining methods for each time from three groups were used acidified toluidine blue, and Luna's stain. The control sections of the healthy dogs were initially assessed to ensure that there was no histological evidence of skin disease by examination of the Luna's stained sections.

Cell enumeration

MCs and EPs were counted in the dermis of each section using a Nikon microscope at 10 × 40, and 10 × 100 magnification, respectively. Ten consecutive fields were examined both in the superficial dermis and in the deeper dermis which positioned at least two high power field (HPF) depth below the dermal-epidermal junction, where possible. The cell counts were expressed as the number of cells per mm^2.

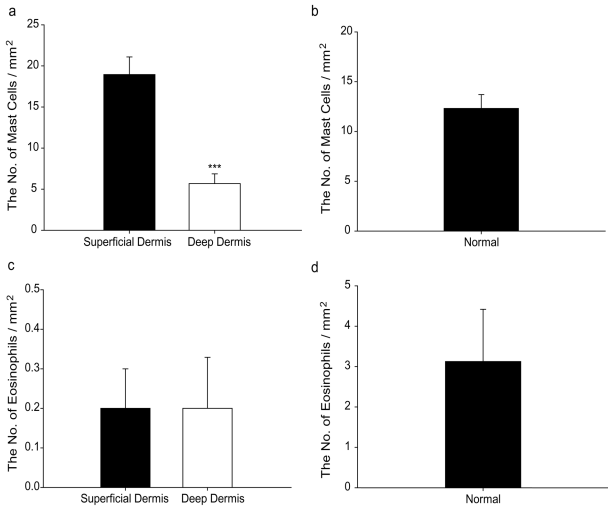

Fig 2. The number of mast cells and eosinophils in normal canine skin. Differences were judged as significant if the p value was ≤ 0.05(*), p ≤ 0.01(**), p ≤ 0.001(***) as determined by the Mann-Whitney U/Wilcoxon.

Statistical methods

The counts of MCs and EPs in three groups were compared by student *t*-test or Mann-Whitney *U*/Wilcoxon test using computer software (SAS Institute Inc., Cary, NC, USA.). Differences were judged as significant if the *p* value was ≤ 0.05(*), *p* ≤ 0.01(**), *p* ≤ 0.001(***) as determined by *t*-test or Mann-Whitney *U*/Wilcoxon.

Results

MC and EP counts in normal and LPR canine skin

All histological analysis was performed using the counts from formalin fixed tissues. Toluidine blue staining permitted the visualization of MCs characterized with oval shape, and metachromatic granuled cell with large round nuclei (Fig 3a-d and f). EPs were revealed with Luna's stain. All specimens included intra- or paracellular eosinophilic granules (Fig 4a-f).

Table 2. Mast cell counts in normal canine skin at 6 and 24 hours after intradermal injection of goat anti-canine IgE in control, vehicle and tacrolimus

Mast cell Count / mm²		Superficial Dermis	Deep Dermis	Dermis Total
Normal		18.9 ± 2.1*	5.7 ± 1.2	12.3 ± 1.4
6 hr later	Control	4.8 ± 1.2	3.1 ± 1.1**	3.9 ± 0.8**
	Vehicle	4.6 ± 1.1	2.7 ± 0.8*	3.7 ± 0.7*
	Tacrolimus	5.5 ± 1.5	4.6 ± 1.0	5.1 ± 0.9
24 hr later	Control	2.4 ± 0.9	3.2 ± 0.7	2.8 ± 0.6
	Vehicle	2.3 ± 0.6	3.2 ± 0.9	2.7 ± 0.6
	Tacrolimus	3.1 ± 1.0	3.7 ± 1.0	3.4 ± 0.7

Mean ± SE
Significantly lower than counts from tacrolimus (*$p < 0.05$; **$p < 0.01$)

The MC and EP counts obtained from thorax. The number of normal MCs and EPs is 12.3 ± 1.4, and 3.1 ± 1.3, respectively (Fig 2b, d, and Table 1). In consistent with previous study, our data showed that MC counts in the superficial dermis were significantly higher than the deep dermis ($p < 0.000001$). The number MCs in superficial and deep dermis was 18.9 ± 2.1 and 5.7 ± 1.2, respectively (Fig 2, and Table 1). However, EP counts in the superficial and deep dermis have no significant difference (Fig 2, and Table 1).

MC counts during cutaneous LPR in canine skin

The MC counts in normal specimens were 12.3 ± 1.4 cells/mm² (Fig 3b, and Table 2). After anti-IgE administration, MC counts dramatically decreased in a time dependent man-

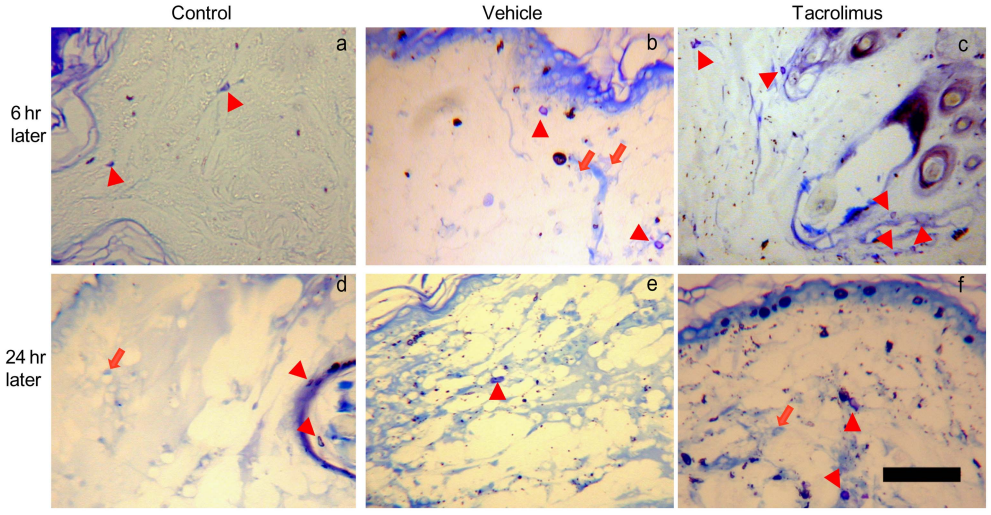

Fig 3. Mast cell counts in canine dog skin at 6 and 24 hours after intradermal injection of goat anti-canine IgE in control, vehicle and tacrolimus. Red arrow head: mast cell; Red arrow: Degranulated mast cell. Toulidine blue stain, bar = 80 μm.

Table 3. Eosinophi counts in normal canine skin at 6 and 24 hours after intradermal injection of goat anti-canine IgE in control, vehicle and tacrolimus

Eosinophil Count / mm^2		Superficial Dermis	Deep Dermis	Dermis Total
Normal		3.1 ± 1.6	3.1 ± 2.0	3.1 ± 1.3
6 hr later	Control	135.9 ± 30	129.7 ± 20.4	132.8 ± 18.0
	Vehicle	128.1 ± 19.6	104.7 ± 22.6	116.4 ± 14.9
	Tacrolimus	121.1 ± 24.7	119.5 ± 22.7	120.3 ± 16.6
24 hr later	Control	144.5 ± 27.8**	125.8 ± 19.3***	135.2 ± 16.9***
	Vehicle	150.8 ± 19.4***	118.0 ± 19.3***	134.4 ± 13.7***
	Tacrolimus	70.3 ± 12.9	37.5 ± 8.8	53.9 ± 8.0

Mean ± SE
Significantly higher than counts from tacrolimus (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

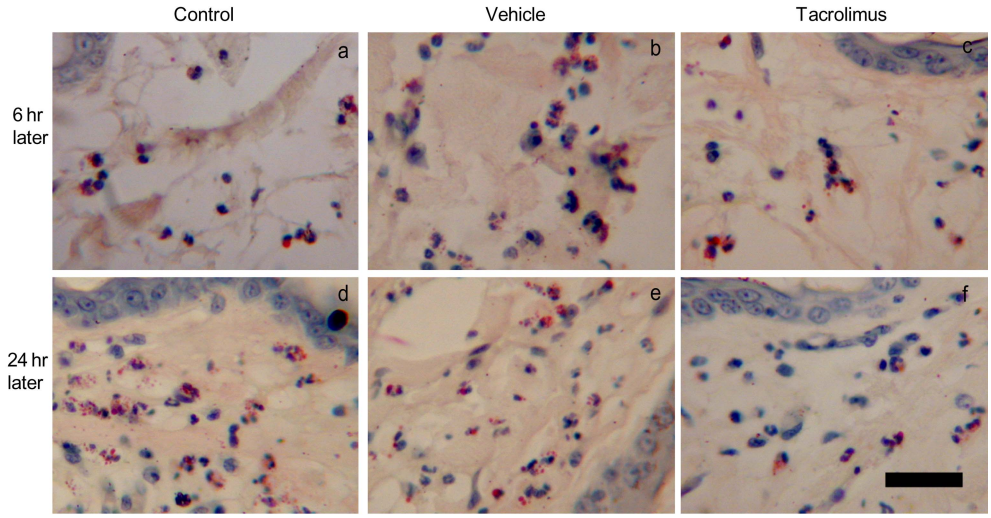

Fig 4. Eosinophil counts in normal canine skin at 6 and 24 hours after intradermal injection of goat anti-canine IgE in control, vehicle and tacrolimus.Luna's stain, bar = 80 μm.

ner (Table 2). However, the number of MCs on 6 hours after challenge was significantly less decreased in the groups treated with the tacrolimus, as compared with control ($p < 0.01$) and vehicle ($p < 0.05$) group (Fig 3a-c; red arrowhead, f and Table 2). On 24 hours after chanllege, the number of MCs had no significant difference (Fig 3d-g; red arrowhead, f, and Table 2).

A fainter cytoplasmic color or scattered matachromatic granules were regarded as MC degranulation following activation by anti-IgE antibodies (Fig 3d-f; red arrow) and this cells excluded MC counts due to their obscure morphology.

EP counts during cutaneous LPR in canine skin

The EP counts in normal specimens were 3.1 ± 1.3 cells/mm^2 (Fig 4d, and Table 3). Post anti-IgE challenge in control group, EP counts increased about 42 fold than non-challenged specimens. The number of EPs on 24 hours after anti-IgE challenge was significantly lower in the group treated with the tacrolimus, as compared with control ($p < 0.001$) and vehicle ($p < 0.001$) group (Fig 4a-g, and Table 3). This data revealed that the EP infiltration in the group administered with the tacrolimus ointment was significantly decreased. The pattern of EP infiltration was interstitial pattern (Fig 4c-d) and perivascular pattern (Data not shown). During anti-IgE mediated LPR, we couldn't found epidermal EP micro-aggregates but frequently found in neurtrophil infiltration (Fig 4a; red arrow). Eosinophilic granules were observed in 10 × 100 magnification (Fig 4e-f).

Discussion

This study showed that 0.1% tacrolimus ointment resulted in anti-inflammatory effect on the LPR during anti-IgE-mediated hypersensitivity. Several uncontrolled studies and randomized, placebo-controlled trial in adult and pediatric patients with moderate-to-severe AD have reported that treatment with topical tacrolimus results in markedly diminished pruritus and skin inflammation (1,4). The efficacy of tacrolimus for treatment of canine AD appears to be similar in magnitude to treatment in human AD except uncontrolled pruritus (2,20,21).

The tacrolimus (FK-506) / FKBP complex inhibits calcineurin and cascade block the dephosphorylation of the nuclear factor of activated T-cell protein and the expression of Th1 (IL-2, IL-3, GM-CSF, IFN-γ, and TNF-α) as well as Th2 cytokines (IL-4, IL-5, and IL-13) without impairing their response to exogenous lymphokine (5,23,32,38). Meanwhile, the Th2 cytokines predominate in the acute phase of AD, whereas in chronic AD lesions are characterics of Th1 and Th0 cytokine predominance (3). Consequently, the suppressive capacity of tacrolimus on the Th1 and Th2 T-cells could result in more effective control of AD.

During LPR, cells with paracellular granules and a fainter cytoplasmic color were frequently observed and were regarded as MC degranulation following activation by anti-IgE antibodies (26). MC degranulation was consistent our observation time for 24 hours after anit-IgE challenge. These degranulated cell number were excluded in MC counts due to its obsecure morphological features. In normal dogs, the density of MCs found in this study was 12.3 ± 1.4 cells/mm^2. Canine MC counts showed various number depending on their biopsy region. Auxilia *et al*. revealed that the density of MCs in thorax is 37.5 cells/mm^2 (1). Kube P *et al*, MC density of approximately 50-250 mast cells/mm^2 was observed in sections obtained from the forehead, but the sampling region is different our study (16). Our study showed that the density of MCs in the thorax was 12.3 cells/mm^2. The reason for these higher counts compared to our data, it is likely to be related to the selection of anesthesia (this study used to medetomine to minimize its degranulation), subtle differences in staining methodes. Furthermore, the age of in this study was 3 to 5-year-old, but 7 out of 10 dogs for the study of Auxilla *et al*. were older than 10 years (1). The fact that peritoneal MCs increase in number with increasing age of the rat raise the possibility of positive relationship between MC density and age (40). More elder age may be implicated longer exposture time for allergen.

Meanwile, we also observed that MC degranulation seems to be lasted not only IPR, but also LPR. After IgE-mediated stimuli, MC counts from three groups were decreased which are compatible with previous study (41). However, the number of MCs in the tacrolimus group was significantly less decreased at 6 hours after anit-IgE challenge that of control and vehicle groups. In addition, this pattern was more apparent in deep dermis. This data showed that MC degranulation induced by IgE-mediated stimuli can be blocked by 0.1% tacrolimus ointment.

Although tacrolimus ointment have no decreased effect on wheal diameter which is related with immediate MC degranulation (4), the tacrolimus effect on significantly decreased MC degranulation on 6 hours after challenge could lead to dramatically reduced EP infiltration on 24 hours after challenge. These patterns strongly indicate the different degranulation or infiltration time kinetics between MC and EP. We hypothesized that many MC degranulation can lead to many EP infiltration during LPR and eventually lead to the exacerbate magnitude of chronic AD lesion. In fact, percutaneous absorption of tacrolimus ointment in canine had higher blood levels at 6 hour post application and this patterns strongly appeared repeated application (21). Although there were no references about blood levels on 24 hour after tacrolimus application in dogs, the mean disposition half-life ($t_{1/2}$) estimated from day 1 blood-concentration data in human was about 8.5-9.4 hour (31). Our data showed that MC degranulation was significantly decreased at 6 hour post application, but not at 24 hour. We had no try to the extent of MC degranulation at 8 or 12 hour post application. However, if the pharmacokinetics of 0.1% tacrolimus ointment is similar with human, the efficient concentration of tacrolimus one day application in dogs could be depleted every 8 hour after (10). Therefore, tacrolimus ointment had no effect on MC degranulation at 24 hour post application whereas, significantly decreased MC degranulation at 6 hour post application could lead to EP infiltration at 24 hour post application. Therefore, three times a day application of 0.1% tacrolimus ointment can lead to reduced MC degranulation and EP infiltration.

Although our study didn't investigate the tacrolimus effect on T-cell infiltration, our histopahtological data strongly suggested that the effect of 0.1% tacrolimus ointment play a substantially role in reduced MC degranulation and EP infiltration as well as this effect has a different time kinetics. Based on our histological data, use of tacrolimus ointment three times a day application may result in cost increase but more effective to the chronic AD lesions. In addition, the use of topical tacrolimus has been associated with minimal risk of skin infections in the human and dogs and that has been considered a major advantage over the use of glucocorticoids (6,21).

EPs are regarded as one of the important effector cells at the site of allergic lesion, however, there have been few reports about the direct effect of tacrolimus on EPs. Previous reports demonstrated that chronic AD lesions are involved in extensive dermal deposition of EP (9). The number of activated EPs was correlated with increased number of CD45RO+, the memory T-cell phenotype (7). CD45RO+ may orchestrated the chief cell during the events leading to the LPR by the production of cytokine such as IL-3, IL-4, IL-5, and GM-CSF which eventually lead to EP growth, maturation, and differentiation (7,9,13). In our study, EPs appeared to be critical infiltrating cell to the development of the LPR. Some researchers suggest that EPs come in early and that the deposition of cationic proteins persists for up to 56 hours or 8 days following anti-IgE challenge (19,33). In normal dog, the influx of EPs was gradually increased during 24 hours. Our histopathologic data revealed that EPs are also present in the skin of normal dogs and that the number was significantly increased at 6, and 24 hours after anti-IgE injection. The infiltration of EPs from tacrolimus applied group was significantly suppressed. Tacrolimus blocked the rate of IL-5 gene transcription which plays a critical role in EP growth, maturation and differentiation (24). Tacrolimus prevent infiltration of inflammatory cells such as EPs by inhibiting chemokine release from EPs such as eotaxin and major cationic protein-1

(15). The EP cationic proteins, particularly major basic protein may play a direct role in enhancing basophil and MC activation. Therefore, it can be indicated the interaction between MCs and EPs exacerbate the magnitude of disease activity. Further studies are needed to confirm the correlation between repeated exposure like allergen or microbial antigen and the severities degrees of the lesion or the magnitude influx of EP infiltration.

The principle finding in this study was that the application of the 0.1% tacrolimus ointment for one day inhibited infiltration of MCs and EPs provoked by post injection of anti-IgE into canine skin. Collectively, 0.1% tacrolimus ointment has direct or indirect and rapid anti-inflammatory effects on MCs, and EPs by inhibition on T cell activation, the release of pro-inflammatory mediators and cytokines. These results suggested that tacrolimus can be an effective remedy for AD in point of blocking MC degranulation and reducing EP infiltration, proliferation, and activity.

Acknowledgement

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개에서 0.1% FK-506 연고의 피부 비만세포와 호산구에 미치는 영향

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요 약 : Goat-anti-canine IgE polyclonal antibody로 유발한 개 피부염에 대한 0.1% FK-506 연고의 항염효과를 평가하였다. 임상적으로 건강한 성견 5두를 사용하여 실험군은 대조군, 위약적용군, FK-506 적용군으로 구분하였다. 항염효과는 팽진의 크기, 피부생검 검사를 통한 비만세포와 호산구수를 측정하여 평가하였으며 비만세포는 Toluidine blue, 호산구는 Luna 염색법을 사용하였다. 염증유발 15분 후의 팽진의 크기는 FK-506 적용군에서 유의성 있게 감소하였다 ($p < 0.01$). 6, 12, 24 시간 후 염증유발부위에 대한 피부생검을 각각 실시하였다. 정상피부의 비만세포는 12.32 ± 3.27 cells/mm^2 이었으며 12시간 후에 FK-506 적용군은 5.79 ± 0.86 cells/mm^{2}으로 대조군의 1.89 ± 0.49 mm^2, 위약군의 1.74 ± 0.50 에 비해 유의하게 증가하였다($p < 0.01$). 정상피부의 호산구는 2.73 ± 2.24 cells/mm^2 이었다. 대조군, 위약 적용군, FK-506 적용군의 호산구 수는 12 및 24 시간 후 FK-506 적용군에서 대조군과 위약 적용군에 비해 유의하게 증가하였다($p < 0.01$). 따라서 FK-506 연고는 급성염증과 아토피증상과 유사한 후기염증에서 항염효과 있는 것으로 사료된다.

주요어 : FK-506, 비만세포, 호산구, 개