NOTE *Internal Medicine*

Production of GM-CSF Mediated by Cysteine Protease of Der f in Canine Keratinocytes

Tsuyoshi KIMURA[1)], Machiko SEKIDO[2)], Naoki CHIMURA[1)], Sanae SHIBATA[2)], Naho KONDO[2)], Harumi KAMISHINA[2)], Hiroaki KAMISHINA[2)] and Sadatoshi MAEDA[2)]*

[1)]*The United Graduate School of Veterinary Sciences, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan*
[2)]*Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan*

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ABSTRACT. House dust mite (HDM) allergens are the most common allergens for induction of IgE-mediated hypersensitivity. Recently, epicutaneous sensitization with HDM allergens has been emphasized in the development of atopic dermatitis (AD) by producing various soluble factors in keratinocytes. Among the soluble factors, GM-CSF is a key molecule that activates Langerhans cells, antigen-presenting cells in the epidermis. In the present study, we investigated the effects of *Dermatophagoides farinae* (Der f) on GM-CSF production in a canine keratinocyte cell line, CPEK. CPEKs were found to produce GM-CSF upon stimulation by Der f. The GM-CSF production was suppressed by addition of a cysteine protease inhibitor. The present results suggest that cysteine protease-derived Der f may be an initiator of allergic inflammation by inducing the production of GM-CSF in keratinocytes.
KEY WORDS: atopic dermatitis, canine, Der f, GM-CSF, keratinocytes.

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Canine atopic dermatitis (AD) is a common inflammatory skin disorder in dogs. It is a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features [8]. Most cases of canine AD are associated with type 1 hypersensitivity to house dust mites (HDMs), including *Dermatophagoides farinae* and *D. pteronyssinus* [10, 11, 19].

Mucosal epithelium in respiratory and alimentary tracts has been considered the major route of HDM exposure. In a canine model of AD, however, HDM allergens were demonstrated to penetrate the epidermis, inducing pruritic dermatitis with increase of mite-specific IgE [22]. This result suggests that epicutaneous exposure of allergens also plays an important role in the initiation of allergic inflammation.

Keratinocytes are the major cellular components in epidermis. Keratinocytes have been shown to produce various cytokines associated with allergic inflammation [6]. Granulocyte macrophage colony-stimulating factor (GM-CSF) is one of the cytokines produced by keratinocytes. GM-CSF is known as a pivotal cytokine to enhance antigen presentation in Langerhans cells [9]. GM-CSF also promotes the survival of eosinophils and monocytes/macrophages, and enhances the proliferation of keratinocytes, contributing to the perpetuation of inflammation and epidermal hyperplasia in the chronic lesions with AD [4]. There were a greater number of GM-CSF mRNA-expressing cells detected by in situ hybridization in AD lesions [4]. It was also shown that keratinocytes from uninvolved skin of AD patients exhibited markedly increased spontaneous and PMA-stimulated release of GM-CSF [21]. Since GM-CSF was also demonstrated to inhibit IL-12 production in mouse Langerhans cells [25], it may be involved in the induction of the Th2-dominated immune environment in AD. Therefore, down-regulation of GM-CSF production should be focused on more to develop a novel therapy for AD.

Dermatophagoides farina (Der f) and *D. pteronyssinus* are the major environmental allergens in both human and canine AD. In particular, Der f has been shown as the most sensitized HDM in canine AD [10, 19]. Der f 1, a major allergen of Der f, belongs to the papain-like cysteine protease family [5]. Its proteolytic activity has been suggested to be linked to the allergenicity of HDMs [19]. In human keratinocytes, proteolytic activity of HDM allergen has been reported to up-regulate the release of GM-CSF [16, 20]. Recently, in canine keratinocyte cell line CPEK, transcription levels of GM-CSF mRNA were significantly enhanced by stimulation with Der f 1 [18]. These results suggest that HDM allergens may be an initiator for allergic inflammation by stimulating GM-CSF production in canine keratinocytes. However, no study has reported the production of GM-CSF protein in canine keratinocytes activated by HDM allergens. In this study, therefore, we investigated whether the proteolytic activity of Der f would up-regulate the release of GM-CSF in canine keratinocytes.

A cell line derived from the epidermis of an adult beagle, CPEK (CELLnTEC Advanced Cell Systems, Bern, Switzerland), was cultured in a 24-well culture plate (Nunclon Multidish, Nunc Inc., Roskilde, Denmark) in CnT-09 (CELLnTEC Advanced Cell Systems) until approximately 100% confluence at 37°C under 5% CO_2. In this study, 5th- to 10th-passage CPEK was used. When cultured

*CORRESPONDENCE TO: MAEDA, S., Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, 1–1 Yanagido, Gifu 501–1193, Japan.
e-mail: sadat@gifu-u.ac.jp

CPEK had reached 100% confluence in CnT-09, the cells were washed two times with Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, St. Louis, MO, U.S.A.) and cultured in serum-free medium (Opti-MEM I reduced-serum medium, Invitrogen Corp., Carlsbad, CA, U.S.A.) 24 hr before stimulation with Der f 1. Solution of Der f 1 (0.1, 1 and 10μg/m*l*) was prepared by dissolving lyophilized Der f (concentration of Der f 1: 378 μg/m*l*, Greer Laboratories, Lenoir, NC, U.S.A.) with DPBS. For inhibition assays, the solution of Der f 1 was pre-incubated with a cysteine protease inhibitor [trans-Epoxysuccinyl-L-Leucylamido-(4-Guanidino) Butane (E-64, Sigma-Aldrich)] at 37°C for 15 min. The cells were cultured for 24 or 48 hr in various culture conditions with DPBS (control), the Der f 1 solution or the Der f 1 solution pretreated with the cysteine protease inhibitor. After the culture, cell viability was estimated by a Trypan Blue dye exclusion method. The culture supernatants were subjected to ELISA for measurement of GM-CSF protein using a commercial kit (DuoSet Canine GM-CSF, R&D Systems, Minneapolis, MN, U.S.A.). The data were statistically analyzed by Tukey-Kramer's HSD Test for the different culture periods or Dunnett's test for different culture conditions. All analyses were performed using a statistical software package (JMP version 5.1.2; SAS Institute, Cary, NC, U.S.A.). A P value <0.01 was regarded as statistically significant.

Addition of Der f 1 (10 μg/m*l*) and the protease inhibitor did not affect the cell morphology and viability in the cultured cells (3.5×10^5/well), which was demonstrated by the proportions of living cells at more than 75% (data not shown). The stimulation with Der f 1 induced the production of GM-CSF, which was increased proportionally with both concentration of Der f 1 and culture period (Fig. 1). The production of GM-CSF was completely inhibited by the addition of the cysteine protease inhibitor at a concentration of 10^{-7} M (Fig. 2).

The present study demonstrated that Der f 1 induced GM-CSF production in CPEKs, which was completely inhibited by the cysteine protease inhibitor.

Since Der f 1, one of the major allergens of Der f, has been identified as a cysteine protease, its specific inhibitor (E-64) was used to investigate whether proteolytic activity of Der f 1 would be essential for production of GM-CSF. However, it is also known that the cysteine protease inhibitors decrease the proteolytic activity of not only exogenous but also endogenous cysteine proteases such as cathepsins [17]. Thus, these endogeneous cyteine proteases might be associated with the production of GM-CSF in the present study. In humans, 11 members of cathepsin have been identified [26]. Among the members, E64 suppresses the activity of cathepsin B, H and L by an irreversible binding to their active thiol [3, 17]. These cathepsins can be activated by various inflammatory stimuli such as TNF-α [7], however, the mechanisms are not fully understood. So far, no study has been conducted to demonstrate that the cathepsins would be activated by exogenous cysteine proteases including Der f 1. Taken together with earlier studies, it is plausible that E64 mainly inhibited proteolytic activity of Der f 1, suggesting that the production of GM-CSF was most likely mediated by

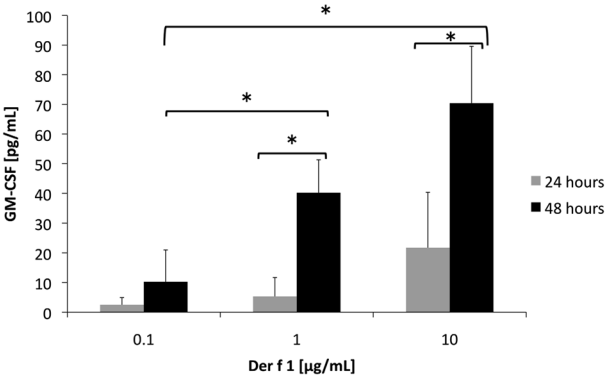

Fig. 1. Production of GM-CSF from CPEK stimulated with Der f 1. The data represent the mean + SD of three independent experiments. The significance of differences (*: P<0.01) was determined with Tukey-Kramer's HSD Test.

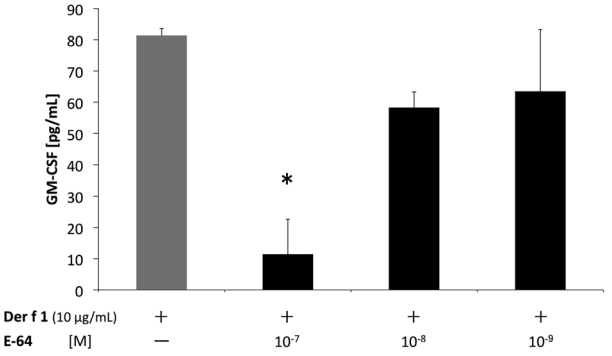

Fig. 2. Inhibitory effect of a cysteine protease inhibitor on production of GM-CSF. The data represent the mean + SD of three independent experiments. The significance of differences (*: P<0.01) from the control was determined by Dunnett's test.

the exogenous cysteine protease derived from Der f 1 itself.

As the mechanism of CPEK activation mediated by cysteine protease, protease activated receptor (PAR) would be involved. PAR is a G protein-coupled seven transmembrane receptor, which is activated by specific protease. In mammals, four isotypes have been identified for PARs (PAR 1–4) [27]. Among them, it has been reported that human keratinocytes express PAR-2 on their cell surface [12]. Once extracellular N terminus peptide chain of PAR-2 is cleaved by cysteine protease, the cleaved N terminus binds to extracellular second loop of PAR-2 molecule, initiating a signal transduction [23].

In human keratinocytes, nuclear factor-kB (NF-kB) plays an essential role for GM-CSF production [14]. Since NF-kB can be activated via PAR-2 [14, 15], it is plausible that a signaling pathway via NF-kB may also be involved in GM-CSF production in keratinocytes activated by Der f 1. Furthermore, in human lymphocytes, transcriptional factors such as nuclear transition of nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1) were shown to

be necessary for GM-CSF production [13]. Recent studies demonstrated that a signaling pathway via NFAT has been found in human keratinocytes [1, 2]. These results prompted us to investigate further the association of NF-kB, NFAT or AP-1 with the production of GM-CSF in keratinocytes activated by Der f 1.

In this study, we employed monolayer culture with CPEK which showed phenotypic similarity to the basal keratinocytes [24]. However, results from one cell line do not always reflect a fact because keratinocytes exhibit their authentic functions by forming the highly organized structure as an epidermal sheet. Thus, it will be necessary to perform a prospective study with a primary culture or an epidermal tissue culture to evaluate effects of differentiation of keratinocytes or cross-talking to other cellular components on production of GM-CSF.

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