Expression Analysis of CCL27 and CCL28 mRNA in Lesional and Non-Lesional Skin of Dogs with Atopic Dermatitis

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Abstract. Chemokines are important regulators of the selective recruitment of inflammatory cells into sites of allergic inflammation. Since canine atopic dermatitis (AD) shares many clinical features with human AD, patterns of chemokine production in dogs may also be similar with those in humans. The aim of this study was to examine mRNA expression of CCL27 and CCL28 in lesional skin of dogs with AD to demonstrate similarity of chemokine production with human counterparts. RNA was extracted from skin biopsy specimens of 12 dogs with AD. The mRNA expression of CC chemokines (CCL4, CCL19, CCL20, CCL21, CCL24, CCL27 and CCL28) was analyzed by quantitative real-time PCR and was compared between lesional and non-lesional skin. Seven types of chemokines examined were constitutively expressed in both lesional and non-lesional skin. It was found that mRNA expression levels of CCL27 and CCL28 among the chemokines were significantly different between lesional and non-lesional skin (P<0.05). Expression level of CCL27 mRNA in lesional skin was significantly lower than that in non-lesional skin. On the other hand, CCL28 mRNA expression in lesional skin was found to be higher than that in non-lesional skin. These results suggest that CCL28 but not CCL27 may play important roles in immunopathogenesis of canine AD, indicating that experimental canine study may provide additional information that can be extrapolated to human AD.

Key words: atopic dermatitis, canine, chemokines, real-time RT-PCR.

Chemokine receptors are expressed on various types of leukocytes and regulate specific or selective cell trafficking [35]. Specific expression profiles of chemokine receptors have been demonstrated in two different subsets of helper T-cells, Th1 and Th2 cells [1]. Regarding specific chemokine receptors, it has been shown that Th1 cells selectively express CXCR3 [23] and CCR5 [11] whereas Th2 cells express CCR3 [24] and CCR4 [5]. Thus, the difference of expression patterns of the chemokine receptors between Th1 and Th2 cells may cause selective infiltration or migration of the cells into inflammatory sites, depending on the chemokines produced in allergic lesions. In human AD, it was found that CCR4 expression on CD4⁺ cells was up-regulated [21] and correlated with disease severity [32]. In lesional skin of human AD, CCR4⁺ cells were found to be markedly infiltrated [18], and thus may be involved in the production of a functional ligand for CCR4, thymus and activation regulated chemokine (CCL17/TARC), in the lesion. The plasma CCL17 level was increased in human AD and correlates with disease severity [9]. In lesional skin of human AD, keratinocytes were the major cell source of CCL17 production [29]. In HaCaT cells, CCL17 production was up-regulated by stimulation with inflammatory cytokines including IL-1β, IFN-γ and TNF-α [31]. These results suggest that CCR4/CCL17 interaction plays an essential role in the immunopathogenesis of AD in humans.

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It has been shown that cutaneous lymphocyte-associated antigen (CLA) was preferentially expressed in skin homing T cells [2]. Since CLA is a ligand for E-selectin, which is prominently expressed on inflamed vascular endothelium [25], interaction of CLA and E-selectin most likely mediates the selective infiltration of skin homing T cells. Recent studies demonstrated that CLA⁺ cells exclusively expressed on CCR10 [3] and CCR10⁺ cells were predominantly infiltrated in humans with AD [3]. Cytokine profile of CCR10⁺ cells has not been fully characterized; however, these are less likely categorized as Th2 cells because only 27% of CCR10⁺ cells co-expressed CCR4 in atopic lesion [30]. To date, cutaneous T cell attracting chemokine (CCL27/CTACK) [4] and mucosa-associated epithelial chemokine (CCL28/MEC) [33] have been identified for the ligands of CCR10. Previous studies indicated that both CCL27 [3] and CCL28 [8] were strongly expressed in atopic lesions in humans. The plasma CCL27 [10] and CCL28 [8] levels were increased in human AD and correlated with disease severity. These results indicated that interaction of CCR10-CCL27 or CCL28 may be important to regulate chemotaxis of skin-homing T cells.

Canine AD shares a number of clinical features with human AD [16]. House dust mite allergen, which is the most common environmental allergen in humans, has been identified as the most important allergen in canine AD [17]. It has been speculated that a predisposing genetic factor may exist in dogs with AD, since there is a high incidence of the disease in certain breeds and families [28]. Histopathological analysis revealed that CD4⁺ cells were markedly infil-
trated in lesional skin of canine AD [26]. A recent study indicated that IL-4 mRNA was preferentially expressed in lesional skin of dogs with AD [20]. In lesional skin of canine AD, expression of CCL17 [12] and CCR4 mRNA [14] was detected in conjunction with the expression of inflammatory cytokines including IL-1β, IFN-γ and TNF-α [12]. Furthermore, it was also found that the number of CCR4+ cells was increased in peripheral CD4+ cells from dogs with AD [13]. A recent study also demonstrated that keratinocytes were major CCL17-producing cells in lesional skin of dogs with AD [15]. These studies indicate that the immunopathogenesis of canine AD may be similar to that of human AD, suggesting that canine AD can be used as an animal model for spontaneous allergy rather than a mice model.

In this study, the possible association between allergic reaction and CCL27 and CCL28 was investigated to further clarify similarities in immunopathogenesis between canine and human AD.

MATERIALS AND METHODS

Dog cases with AD: This study was approved by the Institutional Animal Care and Use Committee at Gifu University. Twelve dogs referred to the Veterinary Medical Teaching Hospital of Gifu University were diagnosed with AD according to Willemse’s criteria for clinical diagnosis of canine AD [34]. All of the dogs with AD had compatible historical and clinical findings of AD including seasonal or recurrent and chronic pruritis. Dogs with other skin diseases causing pruritus such as infestation of parasites and infection of bacteria or fungi were excluded based on routine dermatologic examinations and a therapeutic trial of antibiotics. Food hypersensitivity was excluded based on the negative result by food elimination test, which was performed with commercial prescription diets for 8 weeks. In the present study there were no dogs showing improvement of clinical signs by the food elimination. All dogs showed positive reaction to Dermatophagoides farinae and D. pteronyssinus based on intradermal skin testing, which are the most common environmental allergens for AD in Japan [17].

Skin biopsies and isolation of total RNA: Skin biopsies were carried out using a 6-mm disposable punch (DERMAPUNCH®, Nipro Medical Industries Ltd., Tokyo, Japan) under local analgesia with 2% lidocaine hydrochloride (Xylocaine®, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan). If necessary, dogs were sedated by intramuscular injection of medetomidine (Domitor®, Orion Corporation, Espoo, Finland) at a dose of 0.04 mg/kg and midazolam (Dormicur®, Roche, Basel, Switzerland) at a dose of 0.3 mg/kg. A pair of skin biopsy samples was obtained from sites of both lesional and non-lesional skin in each dog with AD. Gross findings of the lesional skin were generally consistent among the dogs and included lichenification and hair loss showing characteristic chronic lesion rather than acute lesion. All skin samples were immediately submerged in RNA later (Qiagen Inc., Valencia, CA, U.S.A.) and stored at −20°C until the extraction of total RNA. Following homogenization of skin samples, total RNA was extracted using a commercially available kit (RNeasy Plus Mini Kit, Qiagen Inc., Valencia, CA, U.S.A.) and stored at −80°C until expression analysis.

Quantitative Real-Time PCR: Transcription of chemokine mRNA was quantified by one-step real-time PCR (OneStep RT-PCR Kit, Qiagen Inc., Valencia, CA, U.S.A.) according to manufacturer’s instructions. Template amplification and detection were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). Sequences of the primer and probe pairs (QuantiTect Probe RT-PCR Kit, Qiagen Inc., Valencia, CA, U.S.A.) for canine CCL4, 19, 20, 21, 24, 27, 28 and GAPDH are listed in Table 1. A comparative CT method was used for quantification of chemokine mRNA expression as previously reported [12]. For each sample, the CT values for the target amplicon (chemokines) and the calibrator (GAPDH) were determined to report the relative transcription of the amplicon cDNA against calibrator cDNA, respectively. The CT value of the calibrator was subtracted from the CT value of the target chemokine (delta CT) to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the reverse transcription. All samples were examined in duplicate and the mean value of delta CT was calculated. The amounts of chemokine mRNA were calculated by 2^(-delta CT), resulting in the evaluation of the samples as n-fold difference relative to that of GAPDH mRNA.

Statistical analysis: Paired t-test was used for statistical analysis of chemokine expression between lesional and non-lesional skin. A value of P<0.05 was considered to be statistically significant. Statistical analysis was performed by using the JMP IN 5.1.2 program (SAS institute inc., Cary.

Table 1. Sequences of primers and probes for quantitative real-time sequence detection system

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Probe (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL4/Mip1β</td>
<td>TCCCTTCTCCCTCGTAGTCA</td>
<td>GCTGCTGTCACAAAGTAGAAC</td>
<td>TCCCTTCTACTGCTGTCTG</td>
</tr>
<tr>
<td>CCL19/Mip3β</td>
<td>TGGTGCCAAAGTGCTGCTGA</td>
<td>CAGTCGACGAGGCTCCCTGA</td>
<td>TGGTGCTTACACCTGTCCC</td>
</tr>
<tr>
<td>CCL20/SLC</td>
<td>ATGTCAGTGGCAAGAAGAT</td>
<td>GGTAGTGAAAGAAGACAGCAA</td>
<td>GAAGGCTAGAAGGAAAG</td>
</tr>
<tr>
<td>CCL24/EC02X1N2</td>
<td>GCTGATCGGAAATGGAGGAGGG</td>
<td>GGTAGTGAAAGAAGACAGCAA</td>
<td>GGGTCCTGAAACTGATG</td>
</tr>
<tr>
<td>CCL27/TACTA</td>
<td>AGGAGTCTGGCAGCAGGATTG</td>
<td>TCTCTTGTGGCAAGGCTGTTG</td>
<td>GAGGAGTCTGGCAGCAGG</td>
</tr>
<tr>
<td>CCL28/MEC</td>
<td>CTGCAGAAGGTCGAGCAGAAT</td>
<td>TCTCTTGTGGCAAGGCTGTTG</td>
<td>GAGGAGTCTGGCAGCAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTGCAGAAGGTCGAGCAGAAT</td>
<td>TCTCTTGTGGCAAGGCTGTTG</td>
<td>GAGGAGTCTGGCAGCAGG</td>
</tr>
</tbody>
</table>
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Table 2. Means and standard errors of 2-ΔCT of chemokine mRNA expressed in lesional and non-lesional skin samples of dogs with AD

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Lesional skin (Mean ± SEM)</th>
<th>Non-lesional skin (Mean ± SEM)</th>
<th>P value (lesional vs non-lesional)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL4/Mip1β</td>
<td>1.6 ± 0.5 x 10^-3</td>
<td>0.9 ± 0.2 x 10^-3</td>
<td>0.1961</td>
</tr>
<tr>
<td>CCL19/Mip3β</td>
<td>2.9 ± 0.8 x 10^-3</td>
<td>2.6 ± 0.7 x 10^-3</td>
<td>0.5572</td>
</tr>
<tr>
<td>CCL20/LLARC</td>
<td>8.1 ± 2.6 x 10^-2</td>
<td>14.8 ± 7.0 x 10^-3</td>
<td>0.4034</td>
</tr>
<tr>
<td>CCL21/SLC</td>
<td>4.3 ± 2.2 x 10^-2</td>
<td>2.2 ± 0.7 x 10^-2</td>
<td>0.3807</td>
</tr>
<tr>
<td>CCL24/eotaxin2</td>
<td>6.0 ± 3.2 x 10^-2</td>
<td>7.8 ± 2.9 x 10^-3</td>
<td>0.7053</td>
</tr>
<tr>
<td>CCL27/CTACK</td>
<td>6.4 ± 2.3 x 10^-2</td>
<td>22.9 ± 5.0 x 10^-2</td>
<td>0.0266</td>
</tr>
<tr>
<td>CCL28/MEC</td>
<td>1.8 ± 0.5 x 10^-2</td>
<td>0.4 ± 0.1 x 10^-3</td>
<td>0.0242</td>
</tr>
</tbody>
</table>

NC, U.S.A."

RESULTS

Dogs with AD: Twelve dogs were diagnosed with AD based on the clinical diagnostic criteria proposed by Willemse [34]. The dogs with AD consisted of 4 males (1 neutered) and 8 females whose age ranged from 2 to 11 years old (6.1 ± 4.0 years old). All of the dogs with AD had various degrees of pruritic skin lesions with signs such as erythema, hair loss, papules, and lichenification on the ventral abdomen. Lesional skin samples were originated from ventral abdomen, whereas non-lesional skin samples were obtained from lateral thorax, which gross findings did not show any abnormality.

Quantitative Real-Time PCR: Quantitative Real-Time PCR was performed to evaluate mRNA expression of CC chemokines, using total RNA samples from both lesional and non-lesional skin samples. Expression of mRNA for the chemokines examined was observed in all samples. Values of 2-ΔCT of the chemokines are summarized in Table 2. Statistical analysis revealed that no difference of the expression levels of CCL4, 19, 20, 21 and 24 between lesional and non-lesional skin samples. Of the 12 cases, eight cases indicated lower expression of CCL27 in lesional compared to non-lesional skin (Fig. 1A), which was statistically significant (P=0.027). On the other hand, expression levels of CCL28 in lesional skin were statistically higher than those in non-lesional skin from most cases except for one case (P=0.024) (Fig. 1B).

DISCUSSION

It was reported that skin-infiltrating lymphocytes express CCR10 in patients with psoriasis, atopic or contact allergic dermatitis [3]. Effector function of CCR10+ cells has not been determined based on cytokine production because CCR10+ cells showed mixed IL-2/IL-4 expression pattern and minor proportion expressed CCR4 [30]. Since its ligands, CCL27 and CCL28, were preferentially expressed in inflamed skin [3, 8] and their serum levels were increased in humans with AD [8, 10], CCR10-CCL27 or CCR10-CCL28 may play an important role on T-cell skin homing. Further investigation demonstrated that CCR10+ cells did not express CD27 and CCR7, markers of naive cells [27]. Thus, CCR10+ cells could be associated with effector but not trafficking phenotypes. Initially, CCR10 would be necessary for cutaneous homing of T cells; however, CCR10+ T cells...
cells were determined to have no advantage over CCR4+ T cells in T cell homing [27]. A recent study also suggested that CCR10 may play more important roles for the migration of keratinocyte precursor cells from bone marrow [6]. In the present study, we confirmed lower expression of CCL27 mRNA compared with non-lesional skin. This result is consistent with a human study reporting lower expression of CCL27 in chronic skin lesion compared with normal skin [19]. Taken together with previous results in humans, CCL27 may be less associated with chemotraction for T-cells infiltrating chronic lesional skin.

CCL28 is the latest CC chemokine that was identified by searching the Human Genome Science and GenBank dbEST databases [33]. Expression of CCL28 has been confirmed in mucosal epithelial surfaces such as skin and colon [33]. Like CCL27, CCL28 was initially determined as a ligand for CCR10, and a later study demonstrated that CCL28 could also induce chemotaxis of eosinophils expressing CCR3 [22]. CCL28 was strongly expressed in epidermal keratinocytes of humans with AD [8], suggesting an involvement of CCL28 in the pathogenesis of human AD. However, no study was conducted to compare levels of mRNA expression between lesional and non-lesional skin. The present study is the first to analyze expression levels in skin of the dogs with AD, indicating higher expression of CCL28 mRNA in lesional skin compared to non-lesional skin. Although little is known about which CCR1 or CCR3 can preferentially bind to CCL28, a recent study indicated that CCL28 regulated eosinophil recruitment via CCR3 in allergic inflammation [7]. In this study, histopathological examination was carried out in all cases, showing infiltration of eosinophils in lesional skin (data not shown); nevertheless, no difference was found on mRNA expression level of CCL24/eotaxin2, which is one of the chemokine recruiting eosinophils. It may be possible that CCL28 may play an essential role for infiltration of eosinophils, not T-cells, in atopic skin lesions. To prove this hypothesis, expression analysis of not only other chemokines for eosinophils such as CCL11/eotaxin, CCL26/eotaxin3 and CCL5/RANTES, but also CCR3 and CCR10, should be investigated in future prospective studies.

In conclusion, expression analysis of ligands for CCR10 suggests that CCL28 but not CCL27 may play important roles in immunopathogenesis of AD, indicating that experimental canine study may provide additional information that can be extrapolated to human AD.

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