Studies on pathophysiological roles of chemokine in canine allergic diseases

（イヌのアレルギー性疾患の病態におけるケモカインの役割に関する研究）

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General Introduction

Atopic dermatitis (AD) in humans has been thought to be a Th2-type disease rather than a Th1-type disease because IgE synthesis is mainly up-regulated by Th2 cytokines such as IL-4, IL-5 and IL-13 produced by Th2 cells (Grewe et al., 1998; Kimura et al., 1999). Atopic skin lesion in humans can be characterized with the infiltration of various types of inflammatory cells such as lymphocytes, monocytes, eosinophils and mast cells (Leung and Soter, 2001). Of the lymphocytes infiltrating in the lesional skin, Th2 cells were shown to be predominant in the acute phase (Hamid et al., 1996; Thepen et al., 1996). Not only in lesional skin but also in peripheral blood, Th2 cells were increased in human AD (Nakazawa et al., 1997). These results suggest that Th2 cells play essential roles in the immunopathogenesis of human AD.

Canine AD is one of the important diseases in small animal practice. Dogs with AD have severe pruritus and develop skin lesions showing erythema, hair loss, hyperpigmentation and lichenification at ventral abdomen, thigh or axilla. Positive results in the intradermal testing and *in vitro* allergen-specific IgE assay have shown that canine AD occurs as a type-I hypersensitivity mediated by allergen-specific IgE (Lian and Halliwell, 1998; Masuda et al., 2000). An immunohistochemical study revealed that CD4+ T-cells were predominantly found in skin lesion of canine AD (Sinke et al., 1997). Furthermore, it was reported that expression of IL-4 mRNA was enhanced in the lesional skin of canine AD (Nuttall et al., 2002a; Olivry et al., 1999). These results suggest that the pathogenesis of AD may be associated with a Th2-type mechanism, indicating similarities in the pathogenesis of AD between dogs and humans.

Recent studies suggested that the migration of Th2 cells could be regulated by CC chemokines (Bleul and Boehm, 2000). Thymus and activation-regulated chemokine (TARC) is known to be one of the CC chemokines, and its interaction with CC chemokine receptor 4 (CCR4) plays an important role in the initial migration of Th2 cells to the site of allergic inflammation (Imai et al., 1999; Imai et al., 1996b), since CCR4 is selectively expressed on Th2 cells. In human AD, it was found that CCR4 expression on CD4+ cells was up-regulated (Okazaki et al., 2002) and correlated with the disease severity (Wakugawa et al., 2001). In the lesional skin of human AD, CCR4+ cells were found to be predominantly infiltrated (Nakatani et al., 2001), which could be attracted by TARC in the lesion. Immunoreactive TARC was detected in atopic skin lesions not only in humans (Kakinuma et al., 2001) but also in NC/Nga mice, which are an animal model for studying human AD (Vestergaard et al., 1999). It was also reported that the serum TARC level in patients with AD was higher than that in patients with psoriasis and was correlated with the disease severity (Kakinuma et al., 2001). Epidermal keratinocytes have been shown to be a major source of TARC production up-regulated by inflammatory cytokines such as IL-1β, IFN-γ and TNF-α *in vitro* (Vestergaard et al., 2000a; Vestergaard et al., 2001). These cytokines were shown to be secreted in chronic skin lesions in human AD (Leung and Soter, 2001) and induce regional production of TARC in the skin (Kakinuma et al., 2001; Vestergaard et al., 2001; Vestergaard et al., 1999), resulting the development of allergic inflammation. These studies suggest that TARC and CCR4 may be a key chemokine and its receptor,

respectively, for initiating allergic inflammation in AD, and could be used as reliable markers to evaluate disease severity.

The present study, which consists of 4 chapters, was conducted in order to examine an involvement of the chemokine in the immunopathogenesis of canine AD. In Chapter 1, molecular cloning of TARC was performed and its mRNA expression was examined in the lesional skin of dogs with AD. In Chapter 2, molecular cloning of CCR4 was carried out and its mRNA expression was examined in the lesional skin of dogs with AD. In Chapter 3, proportion of CCR4+ cells in peripheral blood CD4+ cells was investigated in dogs with AD and experimentally sensitized with Japanese ceder pollen. In Chapter 4, a monoclonal antibody directed to canine TARC was developed to identify a major cell source of TARC production in the atopic skin lesions.

Chapter 1

Molecular cloning of thymus and activation-regulated chemokine (TARC) and its mRNA expression in dogs with atopic dermatitis

Abstract

Thymus and activation-regulated chemokine (TARC) is known as a functional ligand for CC chemokine receptor 4 (CCR4), which is selectively expressed on Th2 lymphocytes and induces selective migration of the cells to allergic lesions. In this study, I cloned canine TARC cDNA from canine thymus by reverse transcription-polymerase chain reaction (RT-PCR) with rapid amplification of cDNA ends (RACE) method. The canine TARC clone contained a full-length open reading frame encoding 99 amino acids and included four cysteine residues characteristic to CC chemokine family. The canine TARC cDNA showed 77.5%, 67.4%, and 68.5% amino acid sequence similarity with human, mouse and rat homologues, respectively. Expression of TARC mRNA was detected not only in thymus but also in spleen, lymph node, lung and heart of the various normal dog tissues examined. Furthermore, TARC mRNA was found to be selectively expressed in lesional skin of the dogs with AD, but not in non-lesional skin of the dogs with AD or the normal skin of the healthy dogs. The expression levels of IL-1β, IFN-γ and TNF-α in the lesional skin were also significantly higher than those in the non-lesional skin of the dogs with AD. However, IL-4 mRNA was not detected in any of the skin samples in this study. The present results suggest that TARC and inflammatory cytokines such as IL-1β, IFN-γ and TNF-α may play roles in the pathogenesis of canine AD as well as that of human AD.

1. Introduction

Atopic dermatitis (AD) in humans is a chronic inflammatory skin disease that is associated with type-I hypersensitivity mediated by IgE (Leung and Soter, 2001). Atopic dermatitis has been thought to be a Th2-type disease rather than a Th1-type disease because IgE synthesis is mainly up-regulated by Th2 cytokines such as IL-4, IL-5 and IL-13 produced by Th2 cells (Grewe et al., 1998; Kimura et al., 1999). Atopic skin lesion can be characterized according to the infiltration of various types of inflammatory cells such as lymphocytes, monocytes, eosinophils and mast cells (Leung and Soter, 2001). Of the lymphocytes infiltrating in lesional skin, Th2 cells were shown to be predominant in the acute phase (Hamid et al., 1996; Thepen et al., 1996). Recent studies suggested that the migration of Th2 cells could be regulated by CC chemokines and their receptors (Bleul and Boehm, 2000). Thymus and activation-regulated chemokine (TARC) is known to be one of the CC chemokines, and its interaction with CC chemokine receptor 4 (CCR4) plays an important role in the initial migration of Th2 cells in allergic inflammation (Imai et al., 1999; Imai et al., 1996b), since CCR4 is selectively expressed on Th2 cells. Immunoreactive TARC was detected in atopic skin lesions not only in humans (Kakinuma et al., 2001) but also in NC/Nga mice, which are an animal model for studying human AD (Vestergaard et al., 1999). It was also reported that the serum TARC level in patients with AD was higher than that in patients with psoriasis and was correlated with disease severity (Kakinuma et al., 2001). Epidermal keratinocytes have been shown to be a major cell source of

TARC production up-regulated by inflammatory cytokines such as IL-1β, IFN-γ and TNF-α *in vitro* (Vestergaard *et al.*, 1999; Vestergaard *et al.*, 2001). These cytokines have been shown to be among the predominant cytokines secreted in chronic skin lesions in human AD (Leung, 2000) and to induce regional production of TARC in the skin (Vestergaard et al., 2000a; Vestergaard et al., 2001; Vestergaard et al., 1999) and contribute to the worsening of allergic inflammation. These results suggest that TARC may be a pivotal chemokine for initiating allergic inflammation in AD, and could be used as a reliable marker to evaluate the disease severity.

Canine AD is one of the important diseases in small animal practice. Positive results of intradermal testing and *in vitro* allergen-specific IgE assay have shown that canine AD occurs as a type-I hypersensitivity mediated by allergen-specific IgE (Lian and Halliwell, 1998; Masuda et al., 2000). An immunohistochemical study revealed that CD4+ T-cells were predominantly found in skin lesion of canine AD (Sinke et al., 1997). Furthermore, it was reported that IL-4 mRNA was frequently expressed in lesional skin of canine AD (Nuttall et al., 2002a; Olivry et al., 1999). These results suggest that the pathogenesis of AD may be associated with a Th2-type mechanism, indicating similarities in the pathogenesis of AD in dogs and humans. However, further studies are still necessary to clarify the immunopathological mechanisms of canine AD.

In Chapter 1, I performed molecular cloning of canine TARC and examined the mRNA expression of TARC, IL-1β, IL-4, IFN-γ and TNF-α in the skin of dogs with AD and

compared the expression levels with those in non-lesional and normal skin of both atopic and healthy dogs in order to determine whether there is an association between TARC and these cytokines in the pathogenesis of canine AD.

2. Materials and Methods

2.1 Molecular cloning of canine TARC and its expression in various normal canine tissues and skin samples from dogs with AD and healthy dogs

2 1.1. Preparation of cDNAs

Total RNA was extracted from thymus, heart, lung, liver, spleen, kidney, adrenal gland, colon, small intestine, lymph node and skin of a healthy dog kept for experimental purposes, and skin samples (lesional, non-lesional and normal skin) from dogs with AD and healthy dogs by use of the acid guanidine-phenol-chloroform method (RNAzol: Biotecx, Houston, TX, USA). A cDNA sample was synthesized from 0.5 μg of total RNA with a commercially available kit (RNA PCR Kit: PERKIN ELMER, Branchburg, NJ, USA).

2.1.2. PCR primers

Oligonucleotide primers to amplify a central region of canine TARC cDNA were designed based on the sequences conserved between human (Imai et al., 1996b) and mouse (Lieberam and Forster, 1999) TARC cDNAs: forward primer, 5′-CACTTCAGATGCTGCTCCTGG-3′ (nucleotide (nt) 36-56 in mouse TARC cDNA [4]) and reverse primer, 5′-TGTTGGGGTCCGAACAGATG-3′ (nt 284-265 in human

TARC cDNA (Imai et al., 1996b)). To amplify the 5′ region of canine TARC cDNA, another primer pair was prepared: forward primer, 5′-TCCCCTCCTGGGCTCCTGGCACC-3′ (nt 31-52 in human TARC cDNA (Imai et al., 1996b)) and reverse primer, 5′-GGCAGCACTCCCGGCCCACG-3′ (nt 103-84 in canine TARC cDNA obtained in this study). To amplify the 3′ region of canine TARC cDNA, the rapid amplification of cDNA ends (RACE) method was employed with the gene specific primer, 5′-TAGAGTACTTCAAAGGAGCCAT-3′ (nt 104-124 in canine TARC cDNA obtained in this study) and the universal amplification primer, 5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′ (3′ RACE System for Rapid Amplification of cDNA Ends: Life Technologies, Rockville, TX, USA). To amplify the full length of canine TARC cDNA, a primer pair was prepared: forward primer, 5′-ATGATTCCCTTGAAGATGCT-3′ (nt 1-20 in canine TARC cDNA obtained in this study) and reverse primer, 5′-TCAGGACTCTTGGGGCCCTC-3′ (nt 300-281 in canine TARC cDNA). As an internal control, canine GAPDH cDNA was amplified, using a primer pair: forward primer, 5′-CTCATGACCACAGTCCATGC-3′ (nt 514-533 in canine GAPDH cDNA, GenBank/EMBL/DDBJ accession number AB038240) and reverse primer, 5′-TGAGCTTGACAAAGTGGTCA-3′ (nt 925-906 in canine GAPDH cDNA).

2.1.3 Polymerase chain reaction (PCR) amplification

The reaction was performed in a volume of 50 μl with an RNA PCR Kit (PERKIN

ELMER, Branchburg, NJ, USA) containing a pair of primers (1.5 μl each). PCR amplifications were performed by 35 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min) and polymerization (72°C, 7 min). The PCR products were electrophoresed on a 1.5% agarose gel and extracted from the gel. These DNA fragments were cloned into a plasmid vector using the pGEM-T Easy Vector system (Promega, Madison, WI, USA). *Escherichia coli* INVαF′ (Invitrogen, San Diego, CA, USA) cells were transformed with the ligation mixture and plated onto 2×TY agar plates containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (36 μg/ml) and isopropyl β-D-galactoside (40 μg/ml). Plasmid DNAs were prepared with a QIAGEN plasmid kit (Qiagen, Studio City, CA, USA).

2.1.4. Rapid amplification of cDNA ends (RACE)

The template cDNA was amplified by 3′ RACE in a volume of 50 μl with a commercially available kit (3′ RACE System for Rapid Amplification of cDNA Ends: Life Technologies, Rockville, TX, USA), using 1 unit of Taq DNA polymerase (Sigma, St Louis, MO, USA). PCR amplifications were performed by 35 cycles of denaturation (98°C, 20 sec), annealing (60°C, 1 min), extension (72°C, 90 sec) and polymerization (72°C, 10 min) with the gene specific primer described above. The resulting product was prepared and cloned in the same manner as described above.

2.1.5. Nucleotide sequence determination

The PCR products cloned into plasmids were sequenced by the dideoxy chain termination method using an ABI Prism BigDye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of both DNA strands of the cloned fragments were determined.

2.2. Subjects

2.2.1. Healthy dogs

For the healthy dogs, 2 mongrels and 2 beagles were used, and they included 3 spayed females and 1 castrated male. These dogs were kept for experimental purposes in my laboratory. Their ages ranged from 4 to 13 years (median: 7.5 years). No gross skin lesions were observed in these dogs.

2.2.2. Dogs with AD

Diagnosis of AD was made according to Willemse's criteria for clinical diagnosis of canine AD (Willemse, 1986). All of the dogs with AD had compatible historical and clinical findings of AD including seasonal or recurrent and chronic pruritus with superficial pyoderma. Dogs with other skin diseases causing pruritus such as infection of parasites, bacteria or fungi were excluded based on routine dermatologic

examinations and a therapeutic trial of antibiotics. Food hypersensitivity was excluded when there was no history of change in clinical signs against change of foods. If food hypersensitivity was suspected from clinical history, food elimination test was performed with a commercial prescription diet (Hill's Z/d ultra) or rice-based homemade diet with boiled chicken meat for 8 weeks. In the present study, however, there were no dogs showing improvement of clinical signs by the food elimination. Ten dogs were diagnosed as AD and included in this study. Breeds, ages and ages of onset are listed in Table 1. The breeds of the dogs with AD varied, and included Shiba Inu (3 cases), German shepherd (2), French bulldog (1), miniature Pinscher (1), West Highland White Terrier (1), and mongrel (2). The dogs with AD consisted of 5 males (1 intact and 4 castrated) and 5 females (1 intact and 4 spayed). The ages varied from 2 to 13 years (median: 5.7 years) at the time of examination. The first onset of clinical signs was before the age of 4 years in 9 of the 10 dogs. Cases 7 and 9 showed the onset at the age of 11 and 7 years, respectively.

2.2.3. Intradermal testing

Intradermal testing was performed in the dogs with AD as reported previously (Masuda et al., 2000). Allergen extracts used in this study were purchased from a commercial supplier (Greer Laboratories, Lenoir, NC, USA). In the testing, 8 groups of allergens were used: HDM (house dust mites; *Dermatophagoides farinae* and *D. pteronyssinus*), arthropods (cockroach, housefly, mosquito, etc.), cat epithelia, foods (wheat, rice, beef,

etc.), molds (*Curvularia spicifera, Penisillium camemberti*, etc.), grasses (Kentucky blue, orchard, redtop, etc.), weeds (cocklebur, lambs quarter, rough pigweed, etc.), and trees (Japanese cedar, white ash, red birch, etc.). Positive allergens were defined when the size of a reactive wheal was equal to or larger than that against histamine solution (0.05 ml of 0.0275 mg/ml). Positive allergens in each dog with AD were recorded (Table 1).

2.2.4. IgE testing

In vitro allergen-specific IgE testing was carried out using 2 commercially available kits (Topscreen test and Immunodot test kits, CMG Laboratories, Fribourg, Switzerland) as described previously (Masuda et al., 2000). Mixtures of allergens such as outdoor, indoor, 2 foods, and mold allergen groups, were used for the screening assay of 5 allergen groups (e.g., indoor, outdoor, foods, molds), while the Immunodot test was used to detect IgE specific to 24 individual antigens in each allergen group (e.g. house dust mites, storage mites, grasses, fleas). Allergens showing positive reactions were recorded (Table 1).

2.3. Procedure of skin biopsy

Skin biopisy was carried out in both dogs with AD and healthy dogs using a 4-mm disposable punch biopsy apparatus (DERMAPUNCH®, Nipro Medical Industries Ltd.,

Tokyo, Japan) under local anesthesia 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 (Dormicum®, Roche, Basel, Switzerland) at a dose of 0.3 mg/kg. Of the skin samples from the lesional skin of the dogs with AD, 6 were biopsied from the ventral abdomen, 3 were taken from the lateral femur and 1 was collected from the back. All of the skin samples from the healthy dogs were collected from the ventral abdomen. Gross findings of the lesional skin were consistent in the various dogs and consisted of lichenification and hair loss. In each dog with AD, a pair of skin samples was taken from the lesional skin; one was used for extraction of total RNA and the other was used for histological examination. One skin sample was obtained from non-lesional skin for extraction of total RNA. In each healthy dog, two skin samples were collected from the ventral abdomen for the extraction of RNA and histological examination, respectively. All skin samples for extraction of total RNA were immediately snap-frozen in liquid nitrogen and stored at –80°C until use for total RNA extraction.

2.4. Quantification of mRNA expression of cytokines using a real-time sequence detection system

To investigate the association of the expression of cytokines with the expression of TARC mRNA, the mRNA expression of IL-1β, IL-4, IFN-γ and TNF-α in the skin

samples was quantified using a quantitative real-time sequence detection system with a commercially available kit (TaqMan™ Gold RT-PCR Kit, Applied Biosystems, Foster City, CA, USA). To quantify the mRNA levels of canine IL-1β, IL-4, IFN-γ, TNF-α and β-actin (internal reference), a primer and probe pair for each gene was prepared and selected using Primer Express software (Applied Biosystems, Foster City, CA, USA) based on the reported nucleotide sequences. Sequences of the primer and probe pairs for each cytokine used in this study are shown in Table 2. All internal probes were labeled at the 5′ end with a reporter dye (6-carboxyfluorescein, FAM) and the 3′ end with a quencher dye (6-carboxytetramethyl-rhodamine, TAMRA) according to the manufacturer's instructions (Japan Bio-Service, Saitama, Japan). Using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), the RT-PCR reaction and measurement of the fluorescence were carried out in a 25 μL volume of the reaction solution containing 1 × TaqMan Buffer A, 5.5 mM $MgCl_2$, 300 μM dA/dC/dGTP, 600 μM dUTP, 0.625 U AmpliTaq Gold DNA Polymerase, 6.25 U MuLV Reverse Transcriptase, 10 U of RNase Inhibitor, 100 nM primers (forward and reverse), 100 nM TaqMan probe, and 0.1 μg of total RNA. The TaqMan RT-PCR reaction consisted of RT (48°C, 30 min), deactivation of MuLV and activation of AmpliTaq Gold DNA polymerase (95°C, 10 min) and 40 cycles of denaturation (95°C, 15 sec), annealing (60°C, 1 min). A comparative CT (threshold cycle) method was used for quantification of each cytokine. For each sample, the CT values for the target amplicon (cytokines) and the calibrator (canine β-actin) were determined to report the relative transcription of the amplicon cDNA against calibrator cDNA, respectively.

The CT values of the calibrator was subtracted from the CT values of the target cytokine (ΔCT) to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT step. All samples were examined in duplicate and the mean value of ΔCT was calculated. The amount of mRNA for each targeted gene was calculated by $2^{-\Delta CT}$, resulting in evaluation of the samples as an *n*-fold difference relative to β-actin.

2.6. Statistical analysis

Statistical analysis was performed by using the JMP IN 3.0 program (SAS institute inc., Cary, NC, USA). Two-way analysis of variance (ANOVA) was used to test for a significant difference in the $2^{-\Delta CT}$ value of each cytokine among the lesional and the non-lesional skin samples of the dogs with AD and the normal skin samples of the healthy dogs. If a significant difference was found by ANOVA, Tukey's Honestly Significant Difference test was performed to determine which pair showed the difference. Statistical significance was defined as $P<0.05$.

3. Results

3.1 Molecular cloning of canine TARC

By combining the sequences of the 5′, central and 3′ overlapping DNA fragments obtained in this study, a linear sequence corresponding to canine TARC cDNA was obtained (Figure 1), which was 467 bp long and contained an entire open reading frame of canine TARC cDNA encoding 99 amino acid residues (GenBank/EMBL/DDBJ accession number AB054642). The canine TARC cDNA was shown to have 77.5%, 67.4% and 68.5% amino acid sequence similarities with human (Imai et al., 1996b), mouse (Lieberam and Forster, 1999) and rat (GenBank/EMBL/DDBJ accession number AF312687) TARC cDNAs, respectively (Figure 2). The canine TARC cDNA was shown to have four cysteine residues including two adjacent cysteines characteristic to CC chemokine family (Figure 2).

3.2 Evaluation of TARC mRNA in normal dog tissues

Distinct bands of 300 bp derived from canine TARC mRNA were detected in thymus and spleen, whereas relatively faint bands of the same size were found in heart, lung and lymph node (Figure 3). Expression of TARC mRNA was not detected by RT-PCR in liver, kidney, adrenal gland and small intestine as well as in skin.

3.3. Evaluation of TARC mRNA expression in skin samples

A distinct band of 300 bp derived from canine TARC mRNA was detected in all samples from the lesional skin of the dogs with AD (Figure 4), whereas it was not detected in any of the samples from the non-lesional skin of the dogs with AD or the normal skin of the healthy dogs (data not shown). The intensity of the band was almost equal to that of GAPDH mRNA in all of the cases except for case 2, in which the band of TARC mRNA was more intense than that of GAPDH mRNA.

3.4. Quantification of mRNA expression of inflammatory cytokines using the real-time sequence detection system

Expression of mRNAs for IL-1β, IFN-γ and TNF-α mRNA was observed in all skin samples (Figure 5), while IL-4 mRNA was not detected (data not shown). Expression of IL-4 mRNA was detected in PBMC stimulated with mitogen (data not shown), indicating that the lack of IL-4 mRNA in the skin samples was not due to poor sensitivity of the assay system employed in this study. The ΔCT value for IL-1β in the lesional skin of the dogs with AD ranged from 6.28 to 14.08, with a mean of 11.32, while the value in the non-lesional skin varied from 11.96 to 19.67, with a mean of 14.38, among the dogs examined in this study. Among the healthy dogs, the ΔCT value of IL-1β varied between 11.02 and 15.05 with a mean of 12.77. When the $2^{-\Delta CT}$ values were calculated from these data and compared among the sample groups, the 2^{-}

$^{\Delta CT}$ values of IL-1β in the lesional skin were significantly higher than those in the non-lesional skin of the dogs with AD and the normal skin of the healthy dogs ($P<0.05$). Similarly, for mRNA expression of IFN-γ, the ΔCT values varied from 11.18 to 15.73 and the mean value was 13.77 in the samples of the lesional skin. The ΔCT values of IFN-γ mRNA in the non-lesional skin of the dogs with AD and the normal skin of the healthy dogs ranged between 12.73 and 17.52, with a mean of 15.39, and between 14.41 and 17.55, with a mean of 15.41, respectively. The statistical analysis of the $2^{-\Delta CT}$ value among the sample groups showed that the mRNA expression of IFN-γ in the lesional skin was significantly higher than those in the non-lesional skin of the dogs with AD and the normal skin of the healthy dogs ($P<0.05$). The range of ΔCT value for TNF-α mRNA in the lesional skin of the dogs with AD was between 9.55 and 12.55, and the mean value was 10.88. The ΔCT values for TNF-α mRNA were varied between 10.34 and 17.14, with a mean of 12.95 in the non-lesional skin and between 10.66 and 13.99, with a mean of 12.08 in the normal skin. As in the case of the mRNA expression of IL-1β and IFN-γ, a significant difference was found in the groups in terms of $2^{-\Delta CT}$ value for expression of TNF-α mRNA, with significantly higher in the lesional skin than in the non-lesional skin and the normal skin ($P<0.05$).

4. Discussion

The nucleotide sequence reported in this study contained the entire open reading frame of canine TARC cDNA. The amino acid sequence encoded by canine TARC cDNA had four cysteine residues including two adjacent cysteines characteristic to CC chemokine family. In man, Northern blot analysis of TARC mRNA revealed that its high level expression in thymus and its low level expression in lung, colon and small intestine (Imai et al., 1996b). In this study, RT-PCR showed that the TARC mRNA was detected not only in thymus and lung but also in spleen, heart and lymph node. Unlike in man, expression of TARC mRNA was not detected in colon and small intestine in the dog in this study. It is conceivable that TARC mRNA is expressed in thymus and lung in general. But further study is required to know which tissue or organ expresses TARC in normal condition. Moreover, species difference of the expression of TARC in various tissues should be further investigated.

The present study mainly aimed to investigate whether there was an association of TARC with the pathogenesis of canine AD. The results in this study clearly indicated that TARC was selectively expressed in the lesional skin from the dogs with AD, but not in the non-lesional skin from the dogs with AD or in the normal skin of the healthy dogs. In this study histological analysis showed that perivascular infiltration of lymphocytes in the dermis in the lesional skin of the dogs with AD. In a previous study using dogs with AD, lymphocytes infiltrating lesional skin turned out to be CD4+ cells, suggesting

that helper T lymphocytes play an important role in the development of canine AD (Sinke et al., 1997). Although polarization toward either Th1 or Th2 for these CD4+ cells has not been clearly demonstrated in dogs with AD, the present results suggest that TARC may play an essential role in inducing infiltration of these CD4+ cells into lesional skin. However, the expression of CCR4, a receptor for TARC, on the lymphocytes in the lesional skin must be examined in order to understand whether there is an association between TARC expression and lymphocyte infiltration in the lesional skin in AD.

The expression levels of IL-1β, IFN-γ and TNF-α mRNA in the lesional skin were higher than those in the non-lesional skin and the normal skin, and the elevated expression was also correlated with TARC expression. A previous study using murine keratinocytes showed that IL-1β, IFN-γ and TNF-α were strong inducers of TARC expression (Vestergaard et al., 2001). The present results also demonstrated that TARC expression was highly correlated with the expression of these cytokines in the lesional skin of the dogs with AD. In the lesional skin of dogs with AD, therefore, it is thought that TARC production would be maintained by cytokines such as IL-1β, IFN-γ and TNF-α, resulting in a vicious cycle of AD. However, the source of these cytokines was not examined in this study. Immunohistochemical analysis will be needed to identify the cells producing these cytokines in the lesional skin of dogs with AD.

I initially assumed that the expression of TARC in the lesional skin of the dogs with AD would be correlated with IL-4 expression, because it is known that IL-4 is mainly produced by Th2 cells and one major biological function of TARC is to initiate migration of Th2 cells (Imai et al., 1999). In a previous study of dogs with AD, mRNA expression of IL-4 was detected in the lesional skin (Nuttall et al., 2002a; Olivry et al., 1999). However, IL-4 mRNA was not detected in any of the skin samples from the dogs examined in this study. As a possible explanation for this, it can be considered that the pattern of cytokine production would be dynamically altered depending on the duration of the skin lesion. The current concept of AD in humans indicates that IFN-γ plays a more important role than IL-4, especially in development of chronic skin lesions in AD (Grewe et al., 1998; Leung and Soter, 2001), suggesting a regional alteration of the cytokine pattern from Th2 to Th1 in the lesions as the disease progresses. In a study of dogs with AD, the expression of both IL-4 and IFN-γ was detected in only one-fourth of the samples examined because the authors collected samples from animals with either initial or chronic skin lesions (Olivry et al., 1999). In the present study, all the dogs with AD had been affected for more than 1 year, and thus the skin lesions would have become chronic. This might have resulted in continuous expression of IFN-γ mRNA, instead of IL-4 mRNA, in the lesional skin at this point in the disease process. The sequential observation of IL-4 mRNA expression from acute to chronic phase in lesional skin would give us a chance to detect the time point of the alteration of the cytokine pattern in the lesions. Further studies will be necessary to understand the alterations of IL-4 production in the lesional skin of dogs

with AD.

In this study, the expression of mRNA was measured in whole skin samples. It is still unclear what kinds of cells in the lesional skin would be a major source of TARC and the cytokines examined in this study. In humans, it was reported that TARC production was detected in keratinocytes in the lesional skin (Kakinuma et al., 2001). Infiltrating lymphocytes and proliferating keratinocytes were shown to produce cytokines such as IFN-γ and TNF-α, which induce TARC production by keratinocytes in human patients with AD (Leung and Soter, 2001; Pastore et al., 1998; Vestergaard et al., 2001). Acanthosis observed in the lesional skin might indicate that keratinocytes are in a stage of active proliferation, possibly resulting in increased production of TARC. Since the cells contributing to the development of atopic skin lesion must be associated with each other in a complex manner in dogs with AD, it will be necessary to examine the production of chemokines and cytokines from each cell in the lesion independently. Immunohistochemical analysis will be helpful for detecting the cells producing TARC and the cytokines in the lesional skin. Further analysis of the cells in the lesions will be necessary to clarify the pathogenesis of the development of atopic lesions.

Since I did not evaluate TARC expression in other inflammatory skin diseases besides AD, it is not clear whether TARC is associated with other inflammatory skin diseases. In humans with AD, TARC expression was detected in lesional skin with psoriasis, though the expression was weak compared with that in AD (Kakinuma et al., 2001).

Furthermore, it was reported that the serum TARC level in humans with AD was higher than that in patients with psoriasis (Kakinuma et al., 2001). These results suggest that TARC is strongly expressed in the lesional skin of AD. Quantitative analysis with real-time sequence detection system will be necessary to compare the levels of TARC mRNA in atopic and non-atopic skin lesions in dogs.

In conclusion, I performed molecular cloning of canine TARC cDNA and found its mRNA was expressed not only in thymus and lung but also in spleen, heart and lymph node of normal dog tissue. Furthermore, it was demonstrated that TARC expression was highly specific in lesional skin of dogs with AD. Moreover, the expression of inflammatory cytokines including IL-1β, IFN-γ and TNF-α were correlated with TARC expression. The present results strongly suggest that TARC may be one of the important chemokines in the pathogenesis of canine AD, as in human AD, and could be used as a target chemokine for a novel clinical marker and immunotherapy.

Chapter 2

Molecular cloning of CC chemokine receptor 4 (CCR4) and its mRNA expression in dogs with atopic dermatitis

Abstract

CC chemokine receptor 4 (CCR4) is a G protein-coupled seven transmembrane receptor that is selectively expressed on Th2 cells and plays an important role in the trafficking of Th2 cells into inflammatory sites. In this study, a full-length canine CCR4 cDNA was cloned and characterized in order to examine the potential role of CCR4 in allergic responses that produce skin lesions in canine atopic dermatitis (AD). The canine CCR4 cDNA reported in this study contained an open reading frame of 1083 nucleotides encoding 360 amino acids. The predicted amino acid sequence of canine CCR4 showed 91.9, 85.3 and 84.5% similarity with those of the human, mouse and guinea pig counterparts, respectively. Expression of CCR4 mRNA was detected in various tissues including thymus, spleen, heart, small intestine and lymph node. Furthermore, it was found that CCR4 mRNA was preferentially expressed in lesional skin of dogs with AD, together with the mRNA of thymus and activation-regulated chemokine (TARC), which is a ligand for CCR4. The present study demonstrates that CCR4 contributes strongly to the immunopathogenesis of canine AD.

1. Introduction

Atopic dermatitis (AD) is a chronic and recurrent inflammatory skin disease associated with infiltration of antigen-specific T lymphocytes into inflammatory lesions of the skin in humans (Leung and Soter, 2001). Among these lymphocytes, Th2 cells play an important role in the initiation of allergic inflammation by releasing various types of cytokines such as IL-4, IL-5 and IL-13 (Grewe et al., 1998; Kimura et al., 1999). These cytokines are known to play important roles in the differentiation and activation of inflammatory cells including eosinophils, basophils and mast cells (Leung and Soter, 2001). In human AD, antigen-specific Th2 cells are frequently present in the peripheral blood (Kimura et al., 1999), resulting in their infiltration into the skin and the development of atopic lesions (Hamid et al., 1996; Thepen et al., 1996).

Chemokines are important regulators of the selective migration of leukocytes expressing specific chemokine receptors on the cell surface. Th1 cells predominantly express chemokine receptors CCR5 and CXCR3 (Bonecchi et al., 1998; Loetscher et al., 1998; Sallusto et al., 1998), whereas Th2 cells preferentially express CCR3 (Sallusto et al., 1997), CCR4 (Imai et al., 1999) and CCR8 (Zingoni et al., 1998). Of the chemokine receptors, CCR4 has been shown to be strongly expressed on Th2 cells (Lloyd et al., 2000) which are attracted by ligands for CCR4, thymus and activation-regulated chemokine (TARC) and macrophage derived chemokine (MDC) (Imai et al., 1999). In human AD, it has been reported that the number of CD4+ T cells expressing CCR4 is

increased in peripheral blood mononuclear cells (PBMCs) (Nakatani et al., 2001), and there is an increased level of serum TARC (Kakinuma et al., 2001). These findings suggest that chemotaxis of cells expressing CCR4 is induced by TARC, resulting in the selective migration of Th2 cells into lesional skin of human AD.

Recently, canine AD has been recognized as an important allergic disease in small animal practice. Since canine AD shows many clinical features similar to those of human AD, the immunopathogenesis of AD in dogs is likely to be comparable to that of AD in humans in terms of the cell types involved in skin lesions. An immunohistochemical study revealed that predominantly CD4+ T cells were found in the lesional skin of canine AD (Sinke et al., 1997). It was also reported that transcription of the IL-4 gene was frequently detected in the lesional skin of canine AD (Nuttall et al., 2002a; Olivry et al., 1999). It was found that TARC, a ligand for CCR4, was selectively expressed in lesional skin of canine AD, but not in non-lesional or normal skin as shown in Chapter 1. These results suggest that Th2 cells may also be involved in the immunopathogenesis of canine AD, as they are in human AD. Due to a lack of available markers for Th2 cells in dogs, however, the involvement of Th2 cells has not been directly proven in canine AD.

In the present study, in order to improve the understanding of the immunopathogenesis of canine AD, I cloned canine CCR4 and examined its expression in various normal canine tissues and in lesional and non-lesional skin of dogs with AD.

2. Materials and Methods

2.1. Molecular cloning of canine CCR4 and its expression in various normal canine tissues, lesional and non-lesional skin of dogs with AD

2 1.1. Preparation of cDNAs

Total RNAs were extracted from various tissues of a normal dog kept for experimental purposes and from the skin of dogs with AD by using the acid guanidium-phenol-chloroform (AGPC) method with RNAzol (Biotecx, Houston, TX, USA). For the molecular cloning of canine CCR4 cDNA, total RNA extracted from the thymus of a normal dog was used. Reverse transcription of the total RNA was performed to generate single-strand cDNAs for use as templates in PCR with an RNA PCR kit (PERKIN ELMER, Branchburg, NJ, USA).

2.1.2. PCR primers

PCR primers were prepared based on the sequences conserved between human and mouse CCR4 cDNAs: h0S (5′-GGAGCCTGTAGAGTTAAAAA-3′, nt -19 to 0 in human CCR4 cDNA, (Power et al., 1995)), h1S (5′-AAGCCTTGCACCAAAGAAGG-3′, nt 79 to 99 in human CCR4 cDNA) and h1R (5′-ATGGTGGACTGCGTGTAAGA-3′, nt 1056 to 1036 in human CCR4 cDNA). These primers were used to amplify

canine CCR4 cDNA fragments from the dog thymus cDNA template. Primers specific to the canine CCR4 sequence were prepared based on the sequence of the canine CCR4 fragment obtained in this study: c1S (5′-GCATGCAGTGTTCTCCCTGA-3′, nt 419 to 439 in canine CCR4 cDNA, GenBank/EMBL/DDBJ accession number AB080188), c2S (5′-AGGCCTTTTATTCAGCACCT-3′, nt 512 to 532 in canine CCR4 cDNA), c3S (5′-TACCTGGTGGGCTTTTACAGTGGCATCTTC-3′, nt 348 to 378 in canine CCR4 cDNA) and c1R (5′-GCCAGGGTCTCTGTGGCCTGAATAGCGTAG-3′, nt 878 to 848 in canine CCR4 cDNA). These primers were used to amplify the 5′ and 3′ regions of canine CCR4 cDNA and to evaluate CCR4 expression in various tissues of the normal dog and in skin from dogs with AD. In order to evaluate TARC expression in the skin of dogs with AD, the following primers were prepared: cTS1 (5′-ATGATTCCCTTGAAGATGCT-3′, nt 1-20 in canine TARC cDNA, GenBank/EMBL/DDBJ accession number AB054642) and cTR1 (5′-TCAGGACTCTTGGGGCCCTC-3′, nt 300-281 in canine TARC cDNA). As an internal control, canine GAPDH cDNA was amplified using the following primer pair: cGS (5′-CTCATGACCACAGTCCATGC-3′, nt 514-533 in canine GAPDH cDNA, GenBank/EMBL/DDBJ accession number AB038240) and cGR (5′-TGAGCTTGACAAAGTGGTCA-3′, nt 925-906 in canine GAPDH cDNA).

2.1.3 Polymerase chain reaction (PCR) amplification

The template cDNA was amplified by PCR. The reaction was performed in a volume

of 50 μl with an RNA PCR Kit (PERKIN ELMER, Branchburg, NJ, USA) containing a pair of primers (1.5 μl each). PCR amplifications were performed by 35 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min) and polymerization (72°C, 7 min). The PCR products were electrophoresed on a 1.5% agarose gel and extracted from the gel. These DNA fragments were cloned into a plasmid vector using the pGEM-T Easy Vector system (Promega, Madison, WI, USA). *Escherichia coli* INVαF′ (Invitrogen, San Diego, CA, USA) cells were transformed with the ligation mixture and plated onto 2×TY agar plates containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (36 μg/ml) and isopropyl β-D-galactoside (40 μg/ml). Plasmid DNAs were prepared with a QIAGEN plasmid kit (Qiagen, Studio City, CA, USA).

2.1.4. Rapid amplification of cDNA ends (RACE)

The template cDNA was amplified by 3′ RACE in a volume of 50 μl with a commercially available kit (3′ RACE System for Rapid Amplification of cDNA Ends: Life Technologies, Rockville, TX), USA, using 1 unit of Taq DNA polymerase (Sigma, St Louis, MO). PCR amplifications were performed by 35 cycles of denaturation (98°C, 20 sec), annealing (60°C, 1 min), extension (72°C, 90 sec) and polymerization (72°C, 10 min) with primers specific to canine CCR4 cDNA. Using 1 μl of the products amplified by 3′ RACE, a nested PCR was performed in the same manner as the first PCR except that the annealing temperature was 65°C. The resulting product was prepared and cloned in the same manner as described above.

2.1.5. Nucleotide sequence determination

The PCR products cloned into plasmids were sequenced by the dideoxy chain termination method using an ABI Prism BigDye Primer Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of both DNA strands of the cloned fragments were determined.

2.2. Dogs with AD

2.2.1. Subjects

Clinical diagnosis of AD was made in a total of 8 dogs showing chronic dermatitis with pruritus according to Willemse's criteria (Willemse, 1986). The clinical signs in all the dogs consisted of non-seasonal, recurrent, chronic inflammation of the skin which was frequently accompanied by superficial pyoderma. Dogs with other pruritic skin diseases, such as parasitic, bacterial or fungial infections, were excluded based on routine dermatological examinations and a therapeutic trial of antibiotics. Food hypersensitivity was excluded when there was no history of change in clinical signs after dietary change. If food hypersensitivity was suspected from clinical history, a food elimination test was performed with a commercial prescription diet (Hill's Z/d ultra) or a homemade rice-based diet with boiled chicken meat for 8 weeks. In the

present study, however, no dogs showed improvement of clinical signs upon food elimination. The breeds, ages and ages at the first onset of clinical signs are listed in Table 3.

2.2.2 Intradermal testing

Intradermal testing was performed in the dogs with AD as reported previously (Masuda et al., 2000). Allergen extracts used in this study were purchased from a commercial supplier (Greer Laboratories, Lenoir, NC, USA). Japanese cedar antigen was prepared by the method previously reported (Yasueda et al., 1983). For skin testing, 8 groups of allergens were used: HDM (house dust mites; *Dermatophagoides farinae* and *D. pteronyssinus*), arthropods (cockroach, housefly, mosquito, etc.), cat epithelia, foods (wheat, rice, beef, etc.), molds (*Curvularia spicifera, Penicillium camemberti*, etc.), grasses (Kentucky blue, orchard, redtop, etc.), weeds (cocklebur, lambs quarter, rough pigweed, etc.), and trees (Japanese cedar, white ash, red birch, etc.). Positive reactions were defined when the size of a reactive wheal was equal to or larger to that resulting from 0.05 ml of histamine solution at a concentration of 0.0275 mg/ml. Positive reactions in each dog with AD were recorded (Table 3).

2.2.3. IgE testing

In vitro allergen-specific IgE testing was carried out using 2 commercially available kits

(Topscreen test and Immunodot test kits, CMG Laboratories, Fribourg, Switzerland) as described previously (Masuda et al., 2000). Mixtures of allergens such as outdoor, indoor, 2 foods, and mold allergen groups, were used for the screening assay of 5 allergen groups (e.g., indoor, outdoor, foods, molds), while the Immunodot test was used to detect IgE specific to 24 individual antigens in each allergen group (e.g. house dust mites, storage mites, grasses, fleas). Serum samples from the dogs were added to nitrocellulose strips each containing an allergen mixture. After incubation, peroxidase conjugated monoclonal anti-canine IgE antibody and substrate solution were applied to the strips for colorimetric detection. The intensity of the color was measured using a densitometer. Optical values greater than 1.0 in the Topscreen and 2.0 in the Immunodot test were considered positive for the allergen group or specific allergen, respectively. Allergens showing positive reactions were recorded (Table 3).

2.2.4. Skin biopsies

Skin biopsies were carried out using a 4-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 (Dormicum®, 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. Of the skin samples from the lesional skin of the dogs with AD, 5 were biopsied from the ventral abdomen and 3 were taken from the lateral thigh. Gross findings of the lesional skin were generally consistent among the dogs and included lichenification and hair loss. All skin samples were immediately snap-frozen in liquid nitrogen and stored at –80°C until used for the extraction of total RNA.

3. Results

3.1. Molecular cloning and sequencing of canine CCR4 cDNA

With primers h1S and h1R, the PCR generated a single DNA fragment of about 950 bp, covering the central region of canine CCR4. Based on the sequence of a clone of this fragment, canine CCR4-specific primers c1S, c2S, c3S and c1R were prepared. The PCR amplification with primers h0S and c1R yielded a single DNA fragment of 900 bp that included the 5′ region of canine CCR4. To amplify the 3′ region of canine CCR4, the RACE method was employed with primer c1S. Subsequently, a nested PCR was conducted with the RACE product, using primer c2S. The nested PCR generated a single fragment of about 1.5 kbp that included the 3′ region of canine CCR4.

By combining the sequences of the overlapping 5′, central and 3′ DNA fragments obtained in this study, a linear sequence corresponding to canine CCR4 cDNA was obtained (Figure 6). This sequence was 1083 bp long and contained the entire open reading frame of canine TARC cDNA encoding 360 amino acid residues (GenBank/EMBL/DDBJ accession number AB080188). As in the CCR4 counterparts in other species, the three potential N-linked glycosylation sites were all conserved. The deduced amino acid sequence of canine CCR4 cDNA cloned in this study was shown to have 91.9, 85.3 and 84.5% similarity with those of the human, mouse and guinea pig counterparts, respectively (Figure 7).

3.2. Expression of CCR4 mRNA in various normal canine tissues

To examine the expression of CCR4 cDNA in various normal canine tissues, reverse transcription-polymerase chain reaction (RT-PCR) was performed by using primers c3S and c1R. Distinct 532-bp bands derived from canine CCR4 mRNA were detected in spleen, thymus, heart, small intestine and lymph node (Figure 8). Expression of CCR4 mRNA was not detected in liver, lung, colon, kidney or skin.

3.3. Expression of TARC and CCR4 mRNA in the lesional and non-lesional skin of dogs with AD

RT-PCR was performed to evaluate the expression of TARC and CCR4 mRNAs in lesional and non-lesional skin of the dogs with AD. Distinct bands of 300 bp for TARC and 532 bp for CCR4 were detected in all samples from the lesional skin of the dogs with AD (Figure 8), except in case 1, in which the intensity of the band for CCR4 was relatively weak compared with that of GAPDH mRNA (Figure 9). TARC mRNA was not detected in the non-lesional skin of any of dogs with AD, whereas weak expression of CCR4 mRNA was found in cases 1, 2 and 6 (Figure 9).

4. Discussion

The nucleotide sequence reported in this study contained the entire open reading frame of canine CCR4 cDNA. The amino acid sequence encoded by canine CCR4 cDNA showed high sequence similarity with those of its human, mouse and guinea pig homologues. Three potential N-linked glycosylation sites were encoded by canine CCR4 cDNA, as is generally similar to the case for CCR4 of other species (Hoogewerf et al., 1996; Jopling et al., 2002; Power et al., 1995). Based on the high similarity among the canine, human, mouse and guinea pig genes, it can be plausibly assumed that the biological properties of canine CCR4 are similar to those of CCR4 from the other species.

In humans, Northern blot analysis revealed high expression of CCR4 mRNA in thymus and peripheral blood leukocytes and low expression in spleen (Power et al., 1995). In the present study CCR4 mRNA was detected not only in the thymus and spleen but also in the heart, small intestine and lymph node by RT-PCR. It is conceivable that CCR4 mRNA is expressed in thymus, spleen, heart and lymph node in general because these tissues constitutively express TARC as shown in Chapter 1, which is a major biological ligand for CCR4. In this study, CCR4 mRNA was also detected in the small intestine, although the expression was relatively weak compared with that in other tissues. Initially, CCR4 expression in the small intestine was not expected because expression of TARC mRNA was not detected in the small intestine as shown in Chapter 1 and a recent

study in humans reported that CCR4 protein was not detected in any intestinal segment, including the jejunum, ileum and colon (Kunkel et al., 2002). To clarify the biological function of CCR4 in dogs, further studies employing methods such as Northern blot analysis or immunohistochemical analysis will be required.

I showed that TARC mRNA was selectively expressed in lesional skin but not in non-lesional skin of dogs with AD in Chapter 1. Consistent with the distribution of TARC mRNA expression, it was demonstrated that CCR4 mRNA expression was preferentially detected in lesional skin rather than non-lesional skin in this study. This suggests that CCR4-positive cells, which are most likely Th2 cells, may be recruited by TARC produced from skin lesions in dogs, as has been shown in humans. Recent studies have demonstrated that the number of CD4+ cells expressing CCR4 is increased in PBMCs obtained from humans with AD (Nakatani et al., 2001) in accordance with the elevated level of TARC protein (Kakinuma et al., 2001). Likewise, it is highly plausible that CCR4 may be a good clinical marker for the diagnosis and disease severity of AD in dogs.

In contrast to TARC mRNA expression, however, the present results showed that CCR4 mRNA was weakly expressed in some of the samples from non-lesional skin. This was most likely due to the incidental detection of the CCR4 mRNA of circulating lymphocytes that may be present in various normal tissues.

Another possible explanation for the weak expression of CCR4 in non-lesional skin is that a small number of CCR4-positive cells might have already infiltrated into non-lesional skin samples considered normal based on gross findings at the examination. TARC mRNA was not expressed in the non-lesional skin examined in this study, however, MDC, another ligand for CCR4, may have been expressed and acted as an attractant for CCR4. In NC/Nga mice, which afford an animal model for AD, MDC was found to be constitutively expressed not only in lesional skin but also in non-lesional skin, although the level of expression of its mRNA in lesional skin was significantly higher than that in non-lesional skin (Vestergaard et al., 1999). Thus, it is possible that the weak expression of CCR4 mRNA may have been associated with expression of MDC in non-lesional skin of dogs with AD in the present study. Although the involvement of MDC in the pathogenesis of dogs with AD remains to be studied, MDC may also regulate the trafficking of lymphocytes expressing CCR4. To clarify the clinical relevance of the expression of CCR4 mRNA in non-lesional skin, however, further studies employing quantitative PCR or immunohistological analysis using monoclonal antibodies will be necessary.

In conclusion, I cloned canine CCR4 cDNA and evaluated its expression in normal dog tissues and in lesional and non-lesional skin of dogs with AD. Canine CCR4 is highly homologus to CCR4 of humans, mice and guinea pigs with respect to amino acid similarity, tissue distribution and association with the expression of TARC in canine AD. The similarities of the CCR4 gene between dogs and humans suggest that the biological

function of canine CCR4 is similar to that of human CCR4, and CCR4 plays important roles in the immunopathogenesis of canine AD.

Chapter 3

Increase of CCR4 positive cells in the peripheral CD4+ cells in dogs with atopic dermatitis and experimentally sensitized with Japanese ceder pollen

Abstract

Since dogs frequently develop allergic disease, similar to those in humans, dogs may be a possible animal model for allergy in humans. In human atopic dermatitis (AD), CC chemokine receptor 4 (CCR4) was shown to play an important role to develop allergic inflammation of AD, however, an association between allergic reaction and CCR4 has not been well understood in dogs. To examine CCR4 expression in peripheral blood CD4+ cells in dogs with AD and experimentally sensitized with Japanese ceder pollen, peripheral blood mononuclear cells (PBMCs) were isolated from 17 dogs with AD. Proportions of CCR4+ cells in peripheral blood CD4+ cells (CCR4/CD4) were evaluated by flowcytometry and compared with those in 10 healthy dogs. Similarly, in dogs which were experimentally sensitized to Japanese ceder pollen antigen, the proportions of CCR4/CD4 were examined before and after the sensitization. The proportions of CCR4/CD4 in dogs with AD were 40.3 ± 3.3%, significantly higher than those in normal dogs (23.6 ± 4.3%) ($P < 0.01$). In the experimentally sensitized dogs, the proportions of CCR4/CD4 were 25.4 ± 2.6% before the sensitization and they significantly increased up to 29 ± 2.9% after the sensitization ($P < 0.01$). The proportions of CCR4+ cells in peripheral blood CD4+ cells were measured in dogs with allergic conditions. The present study indicated that CCR4+ cells may be involved in the pathogenesis of allergy in dogs. In addition, dogs can be recognized as a suitable animal model for humans with allergic diseases.

1. Introduction

Atopic dermatitis (AD) is known as a common inflammatory skin disease in humans, which is mediated by allergic reactions. In immunopathogenesis of human AD, Th2-type cyokines such as IL-4, IL-5 and IL-13 were shown to play essential roles to initiate and maintain allergic inflammations (Grewe et al., 1998). In lesional skin of human AD, selective infiltration of allergen-specific Th2 lymphocytes was shown to be a characteristic finding (Thepen et al., 1996). Not only in lesional skin but also in peripheral blood, the number of Th2 cells was shown to increase in human AD (Nakazawa et al., 1997). These studies suggest that Th2 cells that increase in peripheral blood in patients with AD will migrate into inflammatory sites, finally resulting in development of atopic lesions.

Chemokines are expressed from various types of leukocytes and regulate the specific or selective cell trafficking (Zlotnik and Yoshie, 2000). Specific expression profile of chemokine receptor was shown in two different subsets of helper T-cells, Th1 and Th2 cells (Bonecchi et al., 1998). In terms of specific chemokine receptors, it has been shown that Th1 cells selectively express CXCR3 (Sallusto et al., 1998) and CCR5 (Loetscher et al., 1998), Th2 cells express CCR3 (Sallusto et al., 1997) and CCR4 (Imai et al., 1999). Thus, the different pattern in expression of chemokine receptors between Th1 and Th2 cells can cause selective infiltration or migration of the cells into inflammatory sites, depending on chemokines produced in allergic lesion. In human

AD, it was found that CCR4 expression on CD4+ cells was up-regulated (Okazaki et al., 2002) and correlated with the disease severity (Wakugawa et al., 2001). In lesional skin of human AD, CCR4+ cells were found to be predominantly infiltrated (Nakatani et al., 2001), which could be involved in production of a functional ligand for CCR4, thymus and activation regulated chemokine (TARC/CCL17), in the lesion. Plasma TARC level was increased in human AD and correlated with disease severerity (Kakinuma et al., 2001). In lesional skin of human AD, keratinocytes were the major cell source of TARC production (Vestergaard et al., 2000a). In HuCaT cells, a cell line of keratinocytes, TARC production was up-regulated by stimulation with inflammatory cytokines incuding IL-1β, IFN-γ and TNF-α mRNA (Vestergaard et al., 2001). These results suggested that CCR4 and TARC play an essential role in immunopathogenesis of AD in humans in terms of development of allergic lesion.

It has been proposed that NC/Nga mice can be a useful animal model for human AD (Vestergaard et al., 2000b) because the mice develop skin lesion accompanied with an overproduction of IgE when kept under conventional conditions (Matsuda et al., 1997). The overproduction of IgE is most likely attributed to presence of Th2 dominant immune responses which may be due to deficiency of Th1 immune responses (Habu et al., 2001). Interestingly, it was indicated that the mice did not develop skin lesion under specific pathogen-free (Suto et al., 1999), suggesting that some environmental factors may be important to initiate allergic inflammation, although it was not identified yet. As to involvement of chemokine in development of skin lesion in NC/Nga mice, a

recent study showed that TARC was found to be produced from keratinocytes in lesional skin together with lesional expression of CCR4 mRNA (Vestergaard et al., 1999). In this model, however, macrophage-derived chemokine (MDC/CCL22), another ligand for CCR4, was expressed not only in lesional skin but also in non-lesional skin (Vulcano et al., 2001), indicating that the pathogenesis of skin lesion in the mice might be different from that in human AD in terms of regulation of cell infiltration by chemotaxis. Furthermore, expression of CCR4 in peripheral blood has not been investigated in NC/Nga mice.

Canine AD shares a number of clinical features with human AD (Marsella and Olivry, 2003). House dust mite allergen, which is the most common environmental allergen in humans, has been identified as the most important allergen in canine AD (Hill and DeBoer, 2001; Hillier et al., 2000; Masuda et al., 2000; Noli et al., 1996; Nuttall et al., 2001; Nuttall et al., 2002b; Vollset, 1985). It has been speculated that predisposing genetic factor may exist in dogs with AD with a high incidence of the disease in certain breeds and families (Sousa and Marsella, 2001). Histological analysis reveled that CD4+ cells were predominantly infiltrated in lesional skin of canine AD (Sinke et al., 1997). Recent studies indicated that IL-4 mRNA was preferentially expressed in lesional skin of dogs with AD (Nuttall et al., 2002a; Olivry et al., 1999). In lesional skin of canine AD, expressions of TARC and CCR4 mRNA were detected in conjunction with the expression of inflammatory cytokines including IL-1β, IFN-γ and TNF-α as shown in Chapters 1 and 2. These studies indicate that immunopathogenesis

of canine AD may be similar with that of human AD, suggesting that canine AD can be recognized as an animal model with spontaneous allergy. Furthermore, dogs experimentally sensitized to allergens were shown to have Th2-skewed response, similar to those in humans with allergy (Fujiwara et al., 2003; Yamashita et al., 2000).

In this study, to clarify an association between allergic reaction and CCR4 expression, proportion of CCR4+ cells in peripheral blood CD4+ cells was investigated in dogs with AD and experimentally sensitized with Japanese ceder pollen.

2. Materials and Methods

2.1. Healthy dogs

Ten healthy dogs kept for experimental purposes were used to obtain control samples. All of the dogs were female and the age ranged from 1 to 2 years old. These dogs showed no clinical signs of allergic diseases and no physical signs of skin diseases.

2.2. Diagnosis of AD

Seventeen dogs referred to the Veterinary Medical Center of the University of Tokyo were diagnosed as AD according to Willemse's criteria for clinical diagnosis of canine AD (Willemse, 1986). All of the dogs with AD had seasonal or nonseasonal chronic pruritus with or without superficial pyoderma in the regions of axilla, thigh and/or ventral abdomen. Dogs with other skin diseases causing pruritus such as infection of parasites, bacteria or fungi were excluded based on routine dermatologic examinations and a therapeutic trial of antibiotics. Food hypersensitivity was excluded when there was no history of change in clinical signs after change of diets. If food hypersensitivity was suspected from the clinical history, food elimination test was performed with a commercial prescription diet using hydrolyzed proteins (Hill's Z/d ultra, Hill's Pet Nutrition Inc., Topeka, KS, USA) or rice-based homemade diet with boiled chicken meat in a period of at least 8 weeks. In the 17 dogs in this study, there

was no dog showing improvement of clinical signs by the food elimination.

Intradermal testing was performed in the dogs with AD as reported previously (Masuda et al., 2000). Allergen extracts used in this study were purchased from a commercial supplier (Greer Laboratories, Lenoir, NC, USA). In the testing, 8 groups of allergens were used: house dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*), arthropods (cockroach, housefly, mosquito, etc.), cat epithelia, foods (wheat, rice, beef, etc.), molds (*Curvularia spicifera, Penisillium camemberti*, etc.), grasses (Kentucky blue, orchard, redtop, etc.), weeds (cocklebur, lambs quarter, rough pigweed, etc.), and trees (Japanese cedar, white ash, red birch, etc.). Most of allergens were used at a concentration of 1000 PNU after being diluted with sterile diluent (0.9% sodium chloride and 0.4% phenol). The diluent was also used as a negative control. Histamine phosphate (0.05 ml of 0.0275 mg/ml) was used as a positive control. The hair coat on the lateral thorax was clipped and 0.05 ml of each allergen solution was injected intradermally using a skin test syringe with a 26-gauge needle. Diameter of wheals were graded as follows: equal to or greater than the diameter of the positive control; +++, equal to or greater than the mean diameter of the positive and negative control; ++, larger than the diameter of the negative control but small than the mean diameter the mean diameter of the positive and negative control; +, equal to or smaller than the diameter of the negative control, -. Positive allergens were defined when the size of a reactive wheal was graded as more than ++.

In vitro allergen-specific IgE testing was carried out using 2 commercially available kits (Topscreen test and Immunodot test kits, CMG Laboratories, Fribourg, Switzerland) as described previously (Masuda et al., 2000). Mixtures of allergens such as outdoor, indoor, 2 foods, and mold allergen groups, were used for the screening assay of 5 allergen groups (e.g., indoor, outdoor, foods, molds), while the Immunodot test was used to detect IgE specific to 24 individual antigens in each allergen group (e.g. house dust mites, storage mites, grasses, fleas). Amounts of antigen-specific IgE were expressed by laboratory unit (LU) ranging from 1 to 100 (0 as negative) based on standard titration curves of reference standard (a pooled serum). Positive allergens were noted when measurable amount of antigen-specific IgE was present in the sera.

2.3. Experimental sensitization with Japanese ceder (Cryptomeria japonica, CJ) pollen antigen

Seven healthy dogs kept for experimental purposes were used for experimental sensitization with Japanese ceder (*Cryptomeria japonica*, CJ) pollen antigen under a protocol approved by the Institutional Animal Care and Use Committee at the University of Tokyo. The dogs included 5 female and 2 male beagles and the age ranged from 1 to 3 years old. These dogs did not have any clinical signs of allergic diseases and any physical signs of skin diseases. The sensitization was carried out as described previously (Yamashita et al., 2000). Briefly, the dogs were injected subcutaneously with 100 μg of the CJ pollen antigen conjugated with 20 mg alum for

each injection. The injection was performed twice at 2 weeks intervals. Intradermal testing and *in vitro* allergen-specific IgE testing were then performed to confirm successful sensitization in these dogs. Blood samples were collected from the dogs before and 1 week after the second injection.

2.4. Detection of canine CCR4 mRNA by RT-PCR

Total RNA was extracted from two canine lymphoid tumor cell lines, CL-1 (Momoi et al., 1997) and GL-1 (Nakaichi et al., 1996), by a commercially available kit (SV Total RNA Isolation System: Promega, Madison, WI, USA) according to the manufacturer's instructions. To examine CCR4 mRNA expression in the canine lymphoid cell lines, reverse transcription-polymerase chain reaction (RT-PCR) was carried out with primers specific to canine CCR4 (Forward, 5′-TACCTGGTGGGCTTTTACAGTGGCATCTTC-3′, nucleotides (nt) 348-378 in canine CCR4 cDNA, GenBank/EMBL/DDBJ accession number AB080188; Reverse, 5′-GCCAGGGTCTCTGTGGCCTGAATAGCGTAG-3′, nt 878-848) or canine GAPDH (Forward, 5′-CTCATGACCACAGTCCATGC-3′, nt 514-533 in canine GAPDH cDNA, GenBank/EMBL/DDBJ accession number AB038240; Reverse, 5′-TGAGCTTGACAAAGTGGTCA-3′, nt 925-906), using a commercially available kit (RNA PCR Kit: PERKIN ELMER, Branchburg, NJ, USA).

2.5. Cross-reactivity of anti-human CCR4 monoclonal antibody with canine CCR4

Anti-human CCR4 monoclonal antibody (BD PharMingen, San Diego, CA, USA) was used to detect canine CCR4 by a flow cytometer (FACSCalibur, Becton Dickinson, Mountain View, CA, USA). At first, cross-reactivity of the anti-human CCR4 monoclonal antibody to canine CCR4 was evaluated using canine lymphoid cell lines (CL-1 and GL-1). The cells were incubated with anti-human CCR4 monoclonal antibody or an appropriate isotype (purified mouse IgG1, BD PharMingen, SanDiego, CA, USA) as a negative control in washing buffer (PBS with 2% FBS) at 4°C for 30 min. The cells were then stained with PE-conjugated anti-mouse IgG1 monoclonal antibodies (BD PharMingen, SanDiego, CA, USA) in washing buffer at 4°C for 30 min. The fluorescence intensities were examined by the flow cytometer.

2.6. Proportion of CCR4/CD4 in PBMCs

Heparinized whole blood samples were obtained from the dogs. Each sample was diluted with an equal volume of PBS and then layered on Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway). Following centrifugation at 350 × g at room temperature for 45 min, a layer of PBMCs was collected and washed twice with washing buffer. The PBMCs were incubated with anti-canine CD4 (COSMO BIO Co. Ltd., Tokyo, Japan) and anti-human CCR4 monoclonal antibodies in washing buffer at 4°C for 30 min. Antibodies with the appropriate isotypes (purified rat IgG2a and mouse IgG1, BD PharMingen) were used as negative controls. The PBMCs were then stained with FITC-conjugated anti-rat IgG2a and PE-conjugated anti-mouse IgG1 monoclonal

antibodies (BD PharMingen, SanDiego, CA, USA) in washing buffer at 4°C for 30 min. After gating the fraction of lymphocytes, proportions of CCR4/CD4 were determined by the flow cytometer.

2.7. Statistical analysis

Paired *t*-test was used to compare the proportions of CD4/CCR4 between pre and post sensitization. Two sample *t*-test was used to compare the proportions of CCR4/CD4 between healthy dogs and dogs with AD at α level of 0.05. All the statistical analysis was performed using JMP Version 5 (SAS Institute Inc., Cary, NC, USA)

3. Results

3.1. Dogs with AD

Seventeen dogs diagnosed with AD based on the clinical diagnostic diagnostic criteria proposed by Willemse (Willemse, 1986). The dogs with AD consisted of 11 males (5 neutered) and 6 females (3 neutered) and the age ranged from 1 to 9 years old (5.0 ± 2.8 years old). All of the dogs with AD had various degrees of pruritic skin lesions such as erythema, hair loss, papules, and lichenification on ventral abdomen and showed positive results to *Dermatophagoides farinae* and *D. pteronyssinus* which were the most common environmental allergen for AD in Japan (Masuda et al., 2000) in intradermal testing and *in vitro* allergen-specific IgE testing. Some dogs also showed positive results to other allergens such as storage mites, grasses and trees on these allergy tests.

3.2. Dogs experimentally sensitized to CJ pollen antigen

The results of intradermal testing and *in vitro* allergen-specific IgE testing revealed that all of the dogs experimentally sensitized to CJ pollen antigen showed positive results only to CJ pollen antigen (Table 4). No clinical signs were induced in these dogs after the sensitization.

3.3. Cross-reactivity of anti-human CCR4 monoclonal antibody to canine CCR4

The results of RT-PCR analysis showed that CCR4 mRNA was expressed in CL-1 cells but not in GL-1 cells (Figure 10a). The cross-reactivity of the anti-human CCR4 monoclonal antibody to canine CCR4 was then examined using these cell lines. The results of flowcytometric analysis using the anti-human CCR4 monoclonal antibody revealed that CL-1 cells expressing mRNA of CCR4 were positively stained, whereas GL-1 cells without expression of CCR4 mRNA were negative (Figure 10b).

3.4. Proportion of CCR4/CD4 in PBMCs

In canine PBMCs, most of CCR4+ cells were found in CD4+ cells as shown in humans (Okazaki et al., 2002) (Figure 11a). In healthy dogs and dogs with AD, proportions of CCR4/CD4 (average ± SEM) were 23.6 ± 4.3% and 40.3 ± 3.3%, respectively (Figure 11b). The proportions of CCR4/CD4 in dogs with AD were significantly higher than those in healthy dogs ($P < 0.01$). Proportions of CCR4/CD4 increased after the experimental sensitization with CJ pollen antigen (Figure 11c) and its percentages were (25.4 ± 2.6%) and (29.8 ± 2.9%) before and after the sensitization, respectively. The rate of CCR4/CD4 was significantly different between the pre and post sensitization ($P < 0.01$).

4. Discussion

Chemokines are important regulators for selective recruitment of inflammatory cells into sites of inflammation (Zlotnik and Yoshie, 2000). It can be hypothesized that infiltration of Th2 cells from the peripheral blood into atopic lesion is regulated by a specific pair of chemokine and chemokine receptor in AD. Studies in human AD demonstrated the increase of CCR4+ cells in peripheral blood (Okazaki et al., 2002; Uchida et al., 2002; Wakugawa et al., 2001) and lesional skin (Nakatani et al., 2001; Vestergaard et al., 2000a). TARC, a bilogical ligand of CCR4, was also found to be selectively expressed in lesional skin of humans with AD (Kakinuma et al., 2001). These results suggest that peripheral CCR4+ cells can be recruited into lesional skin by chemotaxis with lesional production of TARC in AD, finally resulting in the development of skin lesions in human AD. In this study, both spontaneously and experimentally induced allergic reaction could cause increased number of CCR4+ cells in peripheral CD4+ cells. This may suggest that CCR4+ cells can increase in number as a consequence of Th2 type reaction, which will be attracted to lesions to develop clinical signs as shown in human AD. Thus, it could be considered that an association between allergic reaction and recruitment of cells by the chemokine was further emphasized.

A canine lymphoid cell lines, CL-1, expressing CCR4 mRNA was used to evaluate whether the anti-human CCR4 monoclonal antibody cross-reacted with canine CCR4.

It was confirmed that CL-1 cells expressed functional CCR4 on their cell surface, which was demonstrated by transwell chemotaxis assay with recombinant canine TARC expressed by *E. coli* as shown in Chapter 4. Therefore, it can be considered that CL-1 is a suitable cell line to examine cross-reactivity of anti-human CCR4 monoclonal antibody with canine CCR4. In fact, the flowcytometric analysis in this study demonstrated that the anti-human CCR4 monoclonal antibody bound to CL-1 cells, but not to GL-1 cells negative for CCR4 mRNA. The high level of sequence homology of CCR4 among humans (Power et al., 1995), dogs (GenBank/EMBL/DDBJ accession number AB080188), mice (Hoogewerf et al., 1996) and guinea pig (Jopling et al., 2002) can also support the possible cross-reactivity of the monoclonal antibody to CCR4 in these species. As reported previously, anti-human CCR4 monocloncal antibodies cross-reacted with stable transfectant cells expressing guinea pig CCR4 (Jopling et al., 2002). The transfectant also showed chemotactic activity toward the human and murine form of CCR4 ligands, MDC and TARC (Jopling et al., 2002). These results further emphasize that CCR4 is highly conserved among different species in terms of the antigenecity and biological function, indicating that the anti-human CCR4 monoclonal antibody used in this study reacted with canine CCR4.

Since canine AD shares many clinical and pathological features with human AD (Marsella and Olivry, 2003), dogs can be considered as an appropriate animal model for human AD. In this study, therefore, CCR4 expression on CD4+ cells was evaluated in dogs with AD in order to further demonstrate similarities on involvement of chemokines

in immunopathogenesis of AD between dogs and humans. The present results clearly indicated that the proportions of CCR4/CD4 in dogs with AD were higher than those in normal dogs. In addition, I further demonstrated that CCR4 expression was induced by allergic stimuli, using experimental allergy model with dogs sensitized to CJ pollen antigen. Previous studies indicated that the dogs experimentally sensitized to CJ pollen antigen had increased serum allergen-specific IgE and IL-4 mRNA expression in PBMC (Yamashita et al., 2000). Furthermore, the level of IL-4 mRNA was increased in PBMC from dogs sensitized with CJ pollen antigen (Fujiwara et al., 2003). These results also support the fact that the dogs experimentally sensitized with CJ pollen antigen can be used for an animal model with allergic inflammation.

Recent studies proposed that a regulation of the chemotaxis can be a new therapeutic strategy for allergic disease (Kawasaki et al., 2001; Wakugawa et al., 2002; Yoneyama et al., 1998). In a study of murine model of bacteria-induced hepatic failure, it was shown that CCR4+ cells were infiltrated by chemotaxis mediated by TARC produced in hepatic lesion and the cell infiltration was inhibited by administration of anti-TARC monoclonal antibody (Yoneyama et al., 1998). Another study also indicated that a neutraizing monoclonal antibody to TARC suppresses the allergic inflammation in a murine model with allergen-induced asthma (Kawasaki et al., 2001). None of the clinical studies on suppression of chemotaxis has been carried out in humans with allergic diseases, however, these results strongly suggest that intervention of chemotaxis may be effective to reduce allergic inflammation in AD or bronchial asthma. To

evaluate clinical efficacy of chemokine-targeted therapy in human AD, therefore, a suitable animal model with spontaneously occuring AD should be useful in advance of clinical trials in humans. NC/Nga mice may be used as animal model for this purpose, however, the profile of chemokine expression was not identical to that of humans. In this murine model, the expression of MDC mRNA was shown in the in non-lesional skin (Vestergaard et al., 1999). Furthermore, the number of peripheral CCR4+ cells was not examined in these mice. From the results of the present study, therefore, I propose that it should be beneficial to use dogs as an animal model for allergic diseases such as AD in order to evaluate the clinical efficacy of chemokine-targeting therapy.

In conclusion, together with the findings in Chapter 1 and 2 in this thesis showing lesional expression of TARC and CCR4 mRNA, the present study demonstrate that involvement of chemkines in immunopathogenesis of canine AD is similar to that of human AD, strongly indicating canine AD is an appropriate animal model for human AD.

Chapter 4

Production of a monoclonal antibody to canine TARC and its detection in lesional skin of dogs with atopic dermatitis

Abstract

A monoclonal antibody directed to canine thymus and activation-regulated chemokine (TARC/CCL17) was developed to examine the association of TARC with immunopathogenesis of canine atopic dermatitis (AD). Recombinant canine TARC was prepared by *E. coli* expression system. Results of transwell chemotaxis assay demonstrated that the recombinant canine TARC showed chemotactic activity for canine lymphoid cells expressing CC chemokine receptor 4 (CCR4). Mice were then immunized with the recombinant canine TARC to obtain monoclonal antibodies. Among the monoclonal antibodies obtained in this study, a monoclonal antibody (CTA-1) was found to react with both of recombinant and authentic canine TARC in ELISA and flowcytometric assay, respectively. Immunohistochemical analysis using the monoclonal antibody, CTA-1, demonstrated that keratinocytes were a major cell source for TARC production in lesional skin of dogs with AD.

1. Introduction

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases in dogs. Dogs with AD have severe pruritus and develop skin lesions showing erythema, hair loss, hyperpigmentation and lichenification at ventral abdomen, thigh or axilla. An immunohistochemical study revealed that CD4+ cells were predominantly infiltrated in lesional skin of dogs with AD (Sinke et al., 1997), suggesting that CD4+ cells may play an important role in immunopathogenesis of canine AD. In acute skin lesion of human AD, it has been shown that infiltrated CD4+ cells produce Th2-type cytokines such as IL-4, IL-5 and IL-13 that conceivably initiate and maintain allergic inflammation (Leung and Soter, 2001). Recent studies in dogs demonstrated that expression of IL-4 mRNA was up-regulated in lesional skin of dogs with AD (Nuttall et al., 2002a; Olivry et al., 1999). These results suggest that infiltrated CD4+ cells in atopic lesion in dogs may be conmposed of Th2 cells to develop skin lesion in canine AD similar to human AD.

Chemokines are known to be important molecules to regulate trafficking of leukocytes (Zlotnik and Yoshie, 2000). Previous studies indicated that CC chemokine receptor 4 (CCR4) was selectively expressed on Th2 cell in humans (Imai et al., 1999) and mice (Andrew et al., 2001), implying that CCR4 may play important roles in selective infiltration of Th2 cells in atopic skin lesion. It was reported that CCR4+ cells were predominantly infiltrated in atopic lesion of humans (Nakatani et al., 2001), which was

most likely induced by a biological ligand for CCR4, thymus and activation-regulated chemokine (TARC/CCL17). In humans with AD (Kakinuma et al., 2001; Zheng et al., 2003) and NC/Nga mice (Vestergaard et al., 1999), keratinocytes distributed in lesional skin were a major cell source for TARC production. These results suggest that the association between TARC and CCR4 can be involved in the recruitment of Th2 cells into atopic lesions.

Findings obtained in Chapter 1 and 2 in this thesis indicated that mRNA of both TARC and CCR4 were selectively expressed in lesional skin of dogs with AD but not in non-lesional or normal skin. Furthermore, it was also found that the number of CCR4+ cells was increased in peripheral CD4+ cells from dogs with AD (Chapter 3). These results imply that chemokines may also play important roles in immunopathogenesis of canine AD, similar to that of human AD.

In this study a monoclonal antibody to canine TARC was developed in order to further understand the association of chemokine with the pathogenesis of canine AD.

2. Materials and methods

2.1 Preparation of recombinant canine TARC

A cDNA fragment encoding mature form of canine TARC was amplified from canine thymus cDNA by PCR using a forward primer (5′-ACCGAATTCGCTCGAGGCACCAACGTGGGCCGG-3′, nucleotides (nt) 70-93 in canine TARC cDNA, GenBank/EMBL/DDBJ accession number AB054642; underlined sequence is *Eco*RI adaptor) and a reverse primer (5′-AGGGTCGACTCAGGACTCTTGGGGCCCTCCCTT-3′, nt 300-277; underlined sequence is *Sal*I adaptor) with a commercially available kit (RNA PCR Kit, Applied Biosystems, Foster City, CA, USA). After digestion with *Eco*RI and *Sal*I, the TARC cDNA was ligated into *Eco*RI-*Sal*I sites of pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ, USA) to express TARC as a fusion protein with glutathione S-transferase (GST), using a commercially available kit (DNA Ligation kit, TAKARA BIO INC., Shiga, Japan). The expression vector, pGEX-TARC, was prepared in *Escerichia coli* (*E. coli*) competent cells (Top10: Invitrogen Corp, Carlsbad, CA, USA) and purified using a commercially available kit (Qiagen Endotoxin Maxi kit, Qiagen, Chatsworth, CA, USA). The plasmid was then introduced into *E. coli* strain BL21 (Novagen Inc., Madison, WI) and incubated in Luria-Bertani Medium (LB media) at 37°C overnight. One milliliter of this culture was further expanded to 1 liter at 37°C for 90 min followed by expression induction with isopropyl-β-D-thiogalactopyranoside

(1mM) for 4 hours. The cells were then pelleted and suspended in PBS for a sonication. Solublized bacterial suspensions were centrifuged at 10,000 g at 4°C for 10 min and the supernatant was rotated with Glutathione Sephalose (Amersham Biosciences, Piscataway, NJ, USA) at 4°C overnight. The fusion protein combined with Glutathione Sepharose was then separated from the supernatant using a Glutathione Sepahrose 4B column (Amersham Biosciences, Piscataway, NJ, USA). The fusion protein was incubated with thrombin (100 U/ml, Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 16 hours in order to separate a complex of GST-Glutathione Sepharose from the fusion protein. Recombinant TARC protein was then purified by a gel infiltration chromatography (Superrose12HR10/30, Amersham Biosciences, Piscataway, NJ, USA). The protein concentration of the purified recombinant TARC was determined by BCA protein assay (Pierce Biotechnology, Rockford, IL, USA) and the purity was examined by SDS-PAGE (Gradient gel 10-20%). Sequence analysis of NH_2-terminal of the recombinant TARC was performed on a protein sequencer (HP G1005A Protein sequencing System, Hewlett Packard, Palo Alto, CA, USA).

2.2 Transwell chemotaxis assay

In order to evaluate chemotactic activity of the recombinant canine TARC, transwell chemotaxis assay was performed using 24-well microchemotaxis chamber with 5-μm pores (Corning Coster Corporation, Cambridge, MA, USA) as described previously

(Imai et al., 1996b). Briefly, the recombinant TARC was diluted in RPMI 1640 (Sigma Chemical Co., Ltd., St. Louis, MO, USA) with 1% bovine serum albumin (BSA, Sigma Chemical Co., Ltd, USA) at various concentrations from 0.01 to 500nM, and 600 μl of the diluted solution was placed in the lower chamber. Three lymphoid cell lines were used for the chemotaxis assay: two canine lymphoid cell lines with and without expression of CCR4 (CL-1 and GL-1, respectively) (described in Chapter 3) and one human lymphoid cell line expressing human CCR4 (Hut 102 (Imai et al., 1997)). The cells were re-suspended in RPMI 1640 with 1% BSA at a density of 1.0×10^7 cells/ml and 125 μl was placed in the upper chamber. The chamber was incubated at 37°C for 3 hours in humidified atmosphere of 5% CO^2. After the incubation, the membrane filters were removed from the chamber and stained with Diff-Quik (International Reagent Corporation, Hyogo, Japan). Migrated cells were counted in 10 high-power fields (× 800) that were randomly selected. The assay was repeated 5 times in separate experiments.

2.3 Production of monoclonal antibodies

BALB-c mice of 10 weeks of age were immunized with 100 μg of canine recombinant TARC emulsified with an equal volume of incomplete Freund's adjuvant (IFA, Beckton Dickson Diagnostic Systems, Sparks, MD, USA). The immunization was carried out 4 times with a 2-week interval. Three days after the last immunization without IFA, the mice were sacrificed by cervical dislocation and spleen was removed to collect spleen

cells. The spleen cells were then fused with myeloma cells and maintained in RPMI 1640 with 10% FBS and 2% HAT (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) as described previously (Kohler and Milstein, 1975). The culture supernatant was assayed for the binding to recombinant canine TARC, using ELISA. Clones showing reactivity more than 0.5 optical density (OD) units in ELISA were subjected to sub-cloning by limiting dilution. A single clone (CTA-1) that showed the highest reactivity to canine TARC in the ELISA was chosen from 7 clones obtained. The CTA-1 was re-suspended in PBS and 1 ml (10^7 cells/ml) was injected intraperitonealy to BALB-c mice which was pre-treated with 2, 6, 10, 14- tetramethyl pentadecan (Sigma Chemical Co., Ltd, USA). Ascites was collected from the mice 10 days after the injection of CTA-1. In order to purify CTA-1, the ascites was eluted in a Protein G Sepharose column (Amersham Biosciences, Piscataway, NJ, USA) followed by a lyophilization in a freeze dryer (EYELA FD-81, Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

2.4 ELISA

ELISA was used to examine a sensitivity of the reactivity of CTA-1 to recombinant canine TARC. CTA-1 was diluted 500 times with PBS and 100 μl of the antibody solution was immobilized on each well of a 96-well microplate (F96 Maxisoap 439454, Nalge Nunc International, Rochester, NY, USA) at 37°C for 1 hour. One hundred microlitter of 1% BSA was then placed in each well and incubated at 37°C for 1 hour for blocking. After washing each well with 0.05% Tween 20 in PBS, 50 μl of the

recombinant canine TARC which had been serially diluted with distilled water at various concentrations from 0.12 to 7.5 μg/ml was placed in each well and incubated at 37°C for 1 hour. After washing each well 5 times with 0.05% Tween 20 in PBS, biotin-conjugated CTA-1 was diluted 250 times with 1% BSA in PBS and reacted at 37°C for 1 hour in each well. To detect CTA-1 bound to recombinant canine TARC, HRP-conjugated streptavidin (Prozyme, SanLeandro, CA, USA) diluted with 1% BSA in PBS was placed into each well for a reaction at 37°C for 1 hour. After washing, the plates were developed using 2.2-azino-bis[3-ethilbenzothiazoline-6-sulfonate] (ABTS , Sigma Chemical Co., Ltd, USA) and incubated at 37°C for 10 min. Reaction was terminated with 0.32% sodium fluoride. Optical density was measured at 414 nm.

2.5 Specificity of the monoclonal antibody (CTA-1) to authentic canine TARC

2.5.1 Evaluation of TARC mRNA expression on canine lymphoid cell lines

Total RNA was extracted from two cells of canine lymphoid cell lines, CL-1 (Momoi et al., 1997) and GL-1 (Nakaichi et al., 1996), by a commercially available kit (SV Total RNA Isolation System: Promega, Madison, WI, USA) according to the manufacturer's instructions. To examine TARC mRNA expression in these cell lines, RT-PCR was carried out with primers specific to canine TARC (Forward, 5′-ATGATTCCCTTGAAGATGCT -3′, nt 1-300 in canine TARC cDNA, GenBank/EMBL/DDBJ accession number AB054642; Reverse, 5′-

TCAGGACTCTTGGGGCCCTC -3′, nt 300-281) or canine GAPDH (Forward, 5′-CTCATGACCACAGTCCATGC-3′, nt 514-533 in canine GAPDH cDNA, GenBank/EMBL/DDBJ accession number AB038240; Reverse, 5′-TGAGCTTGACAAAGTGGTCA-3′, nt 925-906), using a commercially available kit (RNA PCR Kit: Applied Biosystems, Foster City, CA, USA). The PCR products were electrophoresed through a 3% agarose gel and stained with ethidium bromide for visualization.

2.5.2 Flowcytometric analysis

Specificity of CTA-1 to authentic canine TARC was examined by a flowcytometer (FACSCalibur, Becton Dickinson, Mountain View, CA, USA), using CL-1 and GL-1. The cells were fixed with Cytofix/Cytoperm™ solution (BD PharMingen, San Diego, CA, USA) at 4°C for 20 min. After washing the cells with Perm/Wash buffer™ (BD PharMingen), the cells were stained with CTA-1 or an appropriate isotype (purified mouse IgG1, BD PharMingen, San Diego, CA, USA) as a negative control in Perm/Wash™ buffer at 4°C for 30 min followed by staining with FITC-conjugated anti-mouse IgG monoclonal antibody (Jackson ImmunoResearch, West Grove, PA, USA) in washing buffer (PBS with 2% FBS) at 4°C for 30 min. The fluorescence intensities were examined by a flow cytometer.

2.6 Detection of TARC in lesional skin of dogs with AD

2.6.1 Dogs with AD

Five dogs diagnosed with AD according to Willemse's criteria for clinical diagnosis of canine AD (Willemse, 1986) at Veterinary Medical Center of the University of Tokyo were used in this study. The dogs with AD consisted of 3 female (2 neutered) and 2 males (1 neutered) and the age ranged from 2 to 6 years old (4.3 ± 1.8). Diagnosis of AD was made All of the dogs with AD had seasonal or nonseasonal chronic pruritus with or without superficial pyoderma in the regions of axilla, thigh and ventral abdomen. Positive allergens were identified based on the results from allergy tests including intradermal testing and *in vitro* allergen-specific IgE testing as reported previously (Masuda et al., 2000). Dogs with other skin diseases causing pruritus such as infection of parasites, bacteria or fungi were excluded based on routine dermatologic examinations and a therapeutic trial of antibiotics. Food hypersensitivity was excluded when there was no history of change in clinical signs after change of diets. If food hypersensitivity was suspected from the clinical history, food elimination test was performed with a commercial prescription diet using hydrolyzed proteins (Hill's Z/d ultra, Hill's Pet Nutrition Inc., Topeka, KS, USA) or rice-based homemade diet with boiled chicken meat at least 8 weeks. In the present study, there was no dog showing improvement of clinical signs by the food elimination.

2.6.2 Procedure of skin biopsy

Immunohistochemical staining was performed in lesional skin of 5 dogs with AD and the normal skin of 5 healthy dogs (age ranged from 1 to 2 yeas, all female beagle) which were kept for experimental purposes under a protocol approved by the Institutional Animal Care and Use Committee at the University of Tokyo. All of the healthy dogs showed no clinical signs of allergic diseases and no physical signs of skin diseases. Skin biopsies were carried out using a 4-mm disposable punch biopsy apparatus (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 (Dormicum®, Roche, Basel, Switzerland) at a dose of 0.3 mg/kg. All of the skin samples were biopsied from the ventral abdomen. The lesional skin samples were collected from chronic skin lesions showing lichenification or pigmentation. The skin samples were immediately embedded in OCT compound (Tissue-Tek, Baxter Diagnostics Inc., McGaw Park, IL, USA) and frozen in liquid nitrogen. The samples were stored at –80°C until use for immunohistochemical staining.

2.6.3 Immunohistochemistry

Skin tissue sections of 5 μm in thickness were prepared using a cryostat and air-dried at room temperature for 1 hour followed by fixtation in acetone at 4°C for 10 min. A pair

of serial sections from each skin sample were then incubated with either CTA-1 or appropriate isotype antibody (Purified mouse IgG, Jackson ImmunoResearch, West Grove, PA, USA) at 4°C overnight. The primary antibody was detected by use of an ABC-AP kit (Vectascan ABC-AP method; Vector Laboratories, Burlingame, CA, USA) followed by incubation with levamisole (Vector Laboratories, Burlingame, CA, USA) in order to block endogenous alkaline phosphatase according to the manufacturer's instructions. The sections were then stained with hematoxilin (Gill's formula; Vector Laboratories, Burlingame, CA, USA) for nuclear counter stain.

3. Results

3.1 SDS-PAGE analysis of the recombinant canine TARC

In SDS-PAGE analysis, the recombinant canine TARC expressed by *E. coli* was detected as a band with molecular weight of approximately 10kDa (Figure 12a). Results of amino acid sequence analysis demonstrated that the NH_2 terminus of the recombinant canine TARC started at Ala-24 of the predicted sequence and the first seven amino acid sequences were identical between determined and deduced sequences (Figure 12b).

3.2 Chemotactic activity of the recombinant canine TARC for canine and human lymphoid cells

Using canine (CL-1 and GL-1) and human (Hut 102) lymphoid cell lines, chemotactic activity of the recombinant canine TARC was assessed for their migration across a membrane in a chemotaxis chamber. Cells of a human lymphoid cell line, Hut 102, which were reported to show chemotaxis by recombinant human TARC (Imai et al., 1996a), responded to the recombinant canine TARC in a dose-depended manner showing a maximum effect at the concentration of 100 nM (Figure 13). A similar chemotactic activity was confirmed in CL-1 cells with CCR4 expression but not in GL-1 cells with no detectable CCR4 expression (Figure 13).

3.3 Reactivity of the monoclonal antibody to recombinant canine TARC

In ELISA, it was found that CTA-1 recognized the recombinant canine TARC at the concentrations ranging from 0.12 to 7.5 μg/ml. The values of O.D. were proportionally increased with the concentration of the recombinant canine TARC in a linear manner. The linearity was expressed by an equation ($Y= -0.0140764X^2+ 0.1371079X+ 0.5145161$, $R^2=0.997$, $P<0.0001$) (Figure 14).

3.4 Specificity of the monoclonal antibody to authentic canine TARC expressed in canine lymphoid cell line

The result of RT-PCR analysis showed that TARC mRNA was expressed in CL-1 cells but not in GL-1 cells (Figure 15a). The reactivity of CTA-1 to authentic canine TARC was then examined using these cell lines. The results of flowcytometric analysis using CTA-1 revealed that CL-1 cells expressing TARC mRNA were positively reacted with the antibody, whereas GL-1 cells without detectable TARC mRNA expression were shown to be negative (Figure 15b).

3.5 Detection of TARC in lesional skin of the dogs with AD

The samples of lesional skin were obtained from 5 dogs diagnosed with AD. All the

samples were collected from ventral abdomen where the lesions were characterized as chronic skin lesions showing lichenification, hyperpigmentation or hair loss. The samples of normal skin were also obtained from ventral abdomen in 5 healthy dogs. Immunohistochemical analysis using CTA-1, a monoclonal antibody detected to canine TARC, revealed that one third to half of the keratinocytes in the epidermis were positively stained in all lesional skin samples of the dogs with AD (Figure 16a, b), whereas no keratinocytes were stained in the normal skin samples (Figure 16c).

4. Discussion

In this study, I prepared recombinant canine TARC protein by *E.coli* expression system and produced an anti-canine TARC monoclonal antibody in order to examine the association of chemokine with pathogenesis of canine AD. The present study clearly indicated that the recombinant canine TARC induced chemotaxis for the cells of human (Hut 102) and canine (CL-1) lymphoid cell lines, which had been known to have CCR4 expression (Imai et al., 1997). The recombinant canine TARC, however, had no chemotactic activity for the cells of another canine lymphoid cell line (GL-1) without detectable expression of CCR4. A similar chemotactic activity was confirmed for Hut 102 and CL-1 cells when human recombinant TARC was used (data not shown). These results demonstrated that biological function of TARC was conserved between dogs and humans, and the function was defineded as a regulation of trafficking of CCR4+ cells (Imai et al., 1997). These results further suggest that canine TARC may be a pivotal chemokine in the pathogenesis of canine AD as reported in human AD.

By immunization of the recominant canine TARC, an anti-canine TARC monoclonal antibody was produced in this study. As an anti-canine TARC monoclonal antibody in this study, CTA-1 was chosen because it showed the highest reactivity to canine TARC in the preliminary ELISA screen. The present study showed that the CTA-1 reacted to recombinant and endogeneous canine TARC protein in ELISA and flowcytometric analysis, respectively. Initially, ELISA using CTA-1 was developed for the purpose of

the quantification of the plasma concentration of TARC protein in dogs, however, the sensitivity of the ELISA system was not enough to measure the concentration of TARC in dog plasma. In humans with AD, it was reported that plasma TARC level was correlated with disease activity (Kakinuma et al., 2001), suggesting that the measurement of plasma TARC level can be used for diagnostic marker for AD. Further effort is necessary to improve the ELSA system for canine TARC to allow the quantification of plasma TARC level in dogs, which may be useful to diagnose and evaluate disease severity of canine AD.

It was reported that TARC was strongly expressed in lesional skin of NC/Nga mice (Vestergaard et al., 1999), suggesting that TARC may play an important role in the pathogenesis of AD in the mouse strain. In atopic lesion, keratinocytes were reported as a major cell source of TARC production in humans (Kakinuma et al., 2001; Zheng et al., 2003) and mice (Vestergaard et al., 1999). Findings obtained in Chapter 1 indicated that TARC mRNA was selectively expressed in lesional skin of dogs in conjunction with the expression of inflammatory cytokines such as IL-1β, IFN- γ and TNF-α. These cytokines were known to induce TARC production in cells of a keratinocyte cell line, HaCaT (Vestergaard et al., 2001). Therefore, it was assumed that a pattern of TARC production in canine AD might be similar to human AD. The present study clearly indicated that a major source of the cells to produce TARC was keratinocytes in epidermis of the lesional skin of dogs with AD.

Infiltration of Th2 cells in atopic lesion is a noticeable finding in humans with AD (Leung and Soter, 2001). Similarly, recent studies demonstrated that expression of IL-4 mRNA was up-regulated in lesional skin of dogs with AD, suggesting the association of Th2 cells with the pathogenesis of canine AD (Nuttall et al., 2002a; Olivry et al., 1999). The infiltration of Th2 cells can be regulated by chemotaxis possibly mediated by the interaction between TARC and CCR4 in canine AD. Therefore, it is conceivable that infiltration of Th2 cells into atopic lesion can be suppressed by regulation of chemotaxis. In a study of murine model of bacteria-induced hepatic failure, it was shown that infiltration of CCR4+ cells in hepatic lesion mediated by TARC was inhibited by administration of a monoclonal antibody to TARC (Yoneyama et al., 1998). Another study also indicated that use of a neutralizing monoclonal antibody to TARC suppressed allergic inflammation in murine model with allergen-induced asthma (Kawasaki et al., 2001). These studies further indicate that chemokine-targeting therapy may be an effective therapeutic strategy for allergic diseases.

In conclusion, the present study suggests that TARC is involved in the pathogenesis of canine AD similar to human AD, which is supported by the selective expression of TARC in keratinocytes in the lesional skin of dogs with AD. Thus, TARC can be considered as an appropriate molecular target for development of novel diagnostic or therapeutic strategy for canine AD.

Conclusion

Chemokines are expressed from various types of leukocytes and regulate the specific or selective cell trafficking (Zlotnik and Yoshie, 2000). Specific expression profile of chemokine receptors was shown in two different subsets of helper T-cells, Th1 and Th2 cells (Bonecchi et al., 1998). In terms of specific chemokine receptors, it has been shown that Th1 cells selectively express CXCR3 (Sallusto et al., 1998) and CCR5 (Loetscher et al., 1998), Th2 cells express CCR3 (Sallusto et al., 1997) and CCR4 (Imai et al., 1999). Thus, CCR4 may play important roles in selective infiltration of Th2 cells in the atopic skin lesion, which is induced by lesional expression of TARC. In human AD, it was found that CCR4 expression on CD4+ cells was up-regulated (Okazaki et al., 2002) and correlated with the disease severity (Wakugawa et al., 2001). In the lesional skin of human AD, CCR4+ cells were found to be predominantly infiltrated (Nakatani et al., 2001), which could be involved in production of a functional ligand for CCR4, thymus and activation regulated chemokine (TARC/CCL17), in the lesion. Plasma TARC level was increased in human AD and correlated with disease severity (Kakinuma et al., 2001). These results suggested that CCR4 and TARC play an essential role in immunopathogenesis of AD in humans in terms of development of allergic lesion. However, it has not been investigated whether these chemokines is involved in immunopathogenesis of canine AD. In the present study, therefore, I carried out a series of studies to clarify the association of TARC and CCR4 in immunopathogenesis of canine AD.

In Chapter 1, I cloned canine TARC cDNA from canine thymus by reverse

transcription-polymerase chain reaction (RT-PCR) with rapid amplification of cDNA ends (RACE) method. The canine TARC clone contained a full-length open reading frame encoding 99 amino acids and included four cysteine residues characteristic to CC chemokine family. The canine TARC cDNA showed 77.5%, 67.4%, and 68.5% amino acid sequence similarity with human, mouse and rat homologues, respectively. Expression of TARC mRNA was detected not only in thymus but also in spleen, lymph node, lung and heart of the various normal dog tissues examined. Furthermore, TARC mRNA was found to be selectively expressed in lesional skin of the dogs with AD, but not in non-lesional skin of the dogs with AD or the normal skin of the healthy dogs. The expression levels of IL-1β, IFN-γ and TNF-α in the lesional skin were also significantly higher than those in the non-lesional skin of the dogs with AD. However, IL-4 mRNA was not detected in any of the skin samples in this study. The present results suggest that TARC and inflammatory cytokines such as IL-1β, IFN-γ and TNF-α may play roles in the pathogenesis of canine AD as well as that of human AD.

In Chapter 2, a full-length canine CCR4 cDNA was cloned and characterized in order to examine the potential role of CCR4 in allergic responses that produce skin lesions in canine atopic dermatitis (AD). The canine CCR4 cDNA reported in this study contained an open reading frame of 1083 nucleotides encoding 360 amino acids. The predicted amino acid sequence of canine CCR4 showed 91.9, 85.3 and 84.5% similarity with those of the human, mouse and guinea pig counterparts, respectively. Expression of CCR4 mRNA was detected in various tissues including thymus, spleen, heart, small

intestine and lymph node. Furthermore, it was found that CCR4 mRNA was preferentially expressed in lesional skin of the dogs with AD, together with the mRNA of thymus and activation-regulated chemokine (TARC), which is a ligand for CCR4.

In Chapter 3, PBMCs were isolated from 17 dogs with AD to examine CCR4 expression in peripheral blood CD4+ cells in dogs with AD and experimentally sensitized with Japanese ceder pollen. Proportions of CCR4+ cells in peripheral blood CD4+ cells (CCR4/CD4) were evaluated by flowcytometry and compared with those in 10 healthy dogs. Similarly, in dogs which were experimentally sensitized to Japanese ceder pollen antigen, the proportions of CCR4/CD4 were examined before and after the sensitization. The proportions of CCR4/CD4 in dogs with AD were 40.3 ± 3.3%, significantly higher than those in normal dogs (23.6 ± 4.3%) ($P < 0.01$). In the experimentally sensitized dogs, the proportions of CCR4/CD4 were 25.4 ± 2.6% before the sensitization and they significantly increased up to 29 ± 2.9% after the sensitization ($P < 0.01$). The results in Chapter 3 indicated that CCR4+ cells may be involved in the pathogenesis of allergy in dogs.

In Chapter 4, A monoclonal antibody to canine thymus and activation-regulated chemokine (TARC/CCL17) was developed to examine the association of TARC with immunopathogenesis of canine AD. Among the monoclonal antibodies obtained in this study, a monoclonal antibody (CTA-1) was found to react with both of recombinant and endogenous canine TARC in ELISA and flowcytometric assay, respectively.

Immunohistochemical analysis using the monoclonal antibody, CTA-1, demonstrated that keratinocytes were major cells for TARC production in the lesional skin of dogs with AD.

From the results obtained in a series of studies in this thesis, I conclude that TARC and CCR4 are associated with immunopathogenesis of canine AD, similar to that in human AD. The similarity of chemokine expression in AD between dogs and humans emphasizes that canine AD can be used as a useful animal model for human AD. Furthermore, it is conceivable that these chemokines can be appropriate targets to develop novel diagnostic or therapeutic strategies for canine AD. The present study will provide a fundamental knowledge for further understanding of the immunopathogenesis in allergic diseases.

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Table 1. Cases with atopic dermatitis and the results in allergy tests (Chapter 1)

No.	Breed	Age(Y)	Onset (Y)	Sex	IDT	IgE	Lesions for skin biopsy
1	Mongrel	6	2	M	DF, DP, Flea	HDM, SM, Grasses, Flea	Ventral abdomen
2	Shiba Inu	5	2	SF	DF, DP	HDM, SM	Ventral abdomen
3	West Highland White Terrier	5	1	SF	DF, DP	HDM, SM, Grasses	Lateral femur
4	German Shepard	3	2	F	DF	HDM	Ventral abdomen
5	German Shepard	2	1	SF	DF	HDM, SM	Lateral femur
6	French Bulldog	4	2	M	DF, DP	HDM	Back
7	Mongrel	13	11	M	DF, DP	HDM	Lateral femur
8	Shiba Inu	7	3	M	Grasses	HDM	Ventral abdomen
9	Miniature Pinscher	8	7	M	DF, DP, Milk	HDM	Ventral abdomen
10	Shiba Inu	4	3	SF	DF	HDM, SM, Flea	Ventral abdomen

IDT: intradermal testing, IgE: allergen-specific serum IgE test, M: male, F: female, SF: spayed female, CM: castrated male, DF:*Dermatophagoides farinae*, DP: *D. pteronyssinus*, HDM: house dust mites (mixture of DF and DP), SM: Storage mites, Grasses: 7 grass mix (Kentucky blue, orchard, redtop, timothy, sweet vernal, meadow fescue and perennial rye)

Table 2. Sequences of primers and probes for quantitative real-time sequence detection system

Cytokine	Primer	Sequence (5′-3′)	Probe	Probe sequence (5′-3′)
IL-1β	cIL1 2s	TCTCCCACCAGCTCTGTAACAA	ciL-1	CTGAGGCATTTCGTGTCAGTCATTGTAGCTT
	cIL1 2r	GCAGGGCTTCTTCAGCTTCTC		
IL-4	cIL4 1s	CATCCTCACAGCGAGAAACG	ciL-4	CATGGAGCTGACTGTCAAGGACGTCTTCA
	cIL4 1r	CCTTATCGCTTGTGTTCTTTGGA		
IFN-γ	cIFN-g 1s	GCGCAAGGCGATAAATGAAC	ciFN-g	TGATGAATGATCTCTCACCAAGATCCAACC
	cIFN-g 1r	CTGACTCCTTTTCCGCTTCCT		
TNF-α	cTNF-a 2s	GAGCCGACGTGCCAATG	cTNF-a	CGTGGAGCTGACAGACAACCAGCTG
	cTNF-a 2r	CAACCCATCTGACGGCACTA		
β-actin	cB-ac 2s	TGACCCTGAAGTACCCCATTG	cB-Ac	ATCGTCACCAACTGGGACGACATGG
	cB-ac 2r	GTTGTAGAAGGTGTGGTGCCAG		

Table 3. Cases with atopic dermatitis and the results in allergy tests (Chapter 2)

No.	Breed	Age(Y)	Onset (Y)	Sex	IDT	IgE	Lesions for skin biopsy
1	Shiba Inu	5	2	SF	DF, DP	HDM, SM	Ventral abdomen
2	West Highland White Terrier	5	1	SF	DF, DP	HDM, SM, Grasses	Lateral thigh
3	German Shepard	3	2	F	DF	HDM	Ventral abdomen
4	German Shepard	2	1	SF	DF	HDM, SM	Lateral thigh
5	Mongrel	13	11	M	DF, DP	HDM	Lateral thigh
6	Shiba Inu	7	3	M	Grasses	HDM	Ventral abdomen
7	Miniature Pinscher	8	7	M	DF, DP, Milk	HDM	Ventral abdomen
8	Shiba Inu	4	3	SF	DF	HDM, SM, Flea	Ventral abdomen

IDT: intradermal testing, IgE: allergen-specific serum IgE test, M: male, F: female, SF: spayed female, DF: *Dermatophagoides farinae*, DP: *D. pteronyssinus*, HDM: house dust mites (mixture of DF and DP), SM: Storage mites, Grasses: 7 grass mix (Kentucky blue, orchard, redtop, timothy, sweet vernal, meadow fescue and perennial rye)

Table 4. Results on allergy tests in dogs experimentally sensitized to CJ pollen antigen

Dog number	Intradermal testing	*In vitro* allergen-specific IgE testing (LU)
Dog 1	+++	40
Dog 2	+++	18
Dog 3	+++	33
Dog 4	+++	39
Dog 5	+++	51
Dog 6	+++	62
Dog 7	+++	48

LU: laboratory unit

Figure Legends

Figure 1. Nucleotide and deduced amino acid sequences of canine TARC cDNA (GenBank/EMBL/DDBJ accession number AB054642). The numerals above each line refer to the nucleotide positions. The amino acid sequence with underline indicates the putative signal peptide. The asterisk after the amino acid sequence shows the position of the termination codon.

Figure 2. Comparison of the deduced amino acid sequence of canine TARC cDNA with those of human, mouse and rat homologues. Asterisks indicate identities with amino acids of canine TARC sequence. Numerals on the right ends of each line show the total numbers of amino acid residues. Conserved cysteine residues are boxed.

Figure 3. Detection of TARC mRNA in various normal dog tissues. TARC mRNA (upper lanes) and GAPDH mRNA (lower lanes) were detected by RT-PCR using primers specific to canine TARC and GAPDH cDNAs. Lane 1, thymus; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, spleen; lane 6, kidney; lane 7, adrenal gland; lane 8, colon; lane 9, small intestine; lane 10, lymph node; lane 11, skin.

Figure 4. Detection of TARC mRNA in the lesional skin of the dogs with AD. TARC (upper lanes) and GAPDH (lower lanes) mRNA were detected by RT-PCR using primers specific to canine TARC and GAPDH cDNAs. Lane number accords with

case number examined in the present study.

Figure 5. Levels of mRNA expressions of the cytokines (a) IL-1β, (b) IFN-γ and (c) TNF-α in the lesional (n=10) and the non-lesional skin (n=10) of the dogs with AD and the normal skin (n=4) of the healthy dogs. The amount of mRNA for each targeted gene was calculated by $2^{-\Delta CT}$, resulting in evaluation of the samples as an n-fold difference relative to β-actin. Error bars represent SEM. *($P<0.05$)

Figure 6. Nucleotide and deduced amino acid sequence of canine CCR4 cDNA (GenBank/EMBL/DDBJ accession number AB080188). The numerals above each line refer to the nucleotide positions. The putative transmembrane domains are underlined and three potential N-linked glycosylation sites are boxed. The asterisk after the amino acid sequence shows the position of the termination codon.

Figure 7. Comparison of the deduced amino acid sequence of canine CCR4 cDNA with those of the human, mouse and guinea pig homologues. Asterisks indicate identity with the amino acids of the canine CCR4 sequence. Numerals at the right ends of each line show the total number of amino acid residues. Dashes indicate gaps introduced for maximal alignment.

Figure 8. Detection of CCR4 mRNA in various tissues. CCR4 mRNA (upper lanes) and GAPDH mRNA (lower lanes) were detected by RT-PCR using primers specific to

canine CCR4 and GAPDH cDNAs (CCR4, c3S and c1R; GAPDH, cGS and cGR). Lane1, spleen; lane 2, thymus; lane 3, liver; lane 4, lung; lane 5, skin; lane 6, heart; lane 7, colon; lane 8, small intestine; lane 9, lymph node; lane 10, kidney.

Figure 9. Detection of TARC and CCR4 mRNAs in the lesional and non-lesional skin of dogs with AD (a, lesional; b, non-lesional). TARC mRNA (upper lanes), CCR4 mRNA (middle lanes) and GAPDH mRNA (lower lanes) were detected by RT-PCR using primers specific to canine TARC, CCR4 and GAPDH cDNAs, respectively (TARC, cTS1 and cTR1; CCR4, c3S and c1R; GAPDH, cGS and cGR). Lane numbers accord with the case numbers.

Figure 10. Cross-reactivity of anti-human CCR4 monoclonal antibody with canine CCR4. (a) RT-PCR analysis for expression of CCR4 and GAPDH mRNAs in canine lymphoid cell lines (CL-1 and GL-1). (b) Flowcyometric analysis of CCR4 expression in CL-1 and GL-1 cells. Histogram shows the staining intensity of isotype (shaded area) and CCR4 (open area) monoclonal antibodies.

Figure 11. CCR4 expression in PBMCs from dogs. (a) A representative result on the expression of CCR4 in CD4+ cells in the PBMC from dogs with atopic dermatitis (AD). Expression of CCR4 in the lymphocyte fraction as gated (left) was examined by double staining with anti-CD4 and anti-CCR4 monoclonal antibodies (right). (b) Proportions of CCR4+ cells in CD4+ cells in the PBMC from healthy dogs and dogs with AD. (c)

Changes (pre- and post-sensitization) of the proportions of CCR4+ cells in CD4+ cells in dogs experimentally sensitized to CJ pollen antigen.

Figure 12. Purification of recombinant canine TARC protein expressed by *E. coli* expression system. a) SDS-PAGE (10-20% gradient gel) analysis of the purified recombinant canine TARC protein. b) Comparison of the N-terminal sequence of the purified recombinant canine TARC with the deduced sequence from the canine TARC cDNA. Asterisk indicates no amino acid detected.

Figure 13. Chemotactic activity of the recombinant canine TARC. Cells of a human lymphoid cell line (Hut 102) and two canine lymphoid cell lines (CL-1 and GL-1) were analyzed for their migration in response to the indicated concentration of the recombinant TARC. Each point represents mean ± SD of the results from 5 separate assays.

Figure 14. Sensitivity of the anti-canine TARC monoclonal antibody (CTA-1) to the recombinant canine TARC in ELISA. Optical density (OD) was measured at 414 nm.

Figure 15. Specificity of the anti-canine TARC monoclonal antibody (CTA-1) to the authentic canine TARC. a) RT-PCR analysis for the TARC mRNA expression in the cells of two canine lymphoid cell lines (CL-1 and GL-1). b) Flowcytometric detection of authentic canine TARC in CL-1 and GL-1 cells. Histogram shows the staining

intensities by isotype antibody (shaded area) and anti-canine TARC antibody (open area).

Figure 16. A represenative result of the immunohistochemical analysis for the expression of TARC in skin biopsies. a) Immunohistochemical staining with the anti-canine TARC monoclonal antibody (CTA-1) in a lesional skin section from a dog with AD; original magnification: ×200. b) A section stained with the isotype control (purified mouse IgG) from a lesional skin of a dog with AD; original magnification: ×200. c) A section with stained with CTA-1 from normal skin of a healthy dog; original magnification: ×200. Immunoreactive TARC is visualized in red, and nuclei are counter stained with hematoxylin (blue).

```
        10         20         30         40         50         60
ATGATTCCCTTGAAGATGCTGCTCCTGGTCACGCTCCTCCTGGGGGCTTCTCTGCAGGTC
 M  I  P  L  K  M  L  L  L  V  T  L  L  L  G  A  S  L  Q  V

        70         80         90        100        110        120
ACCCATGCAGCTCGAGGCACCAACGTGGGCCGGGAGTGCTGCCTAGAGTACTTCAAAGGA
 T  H  A  A  R  G  T  N  V  G  R  E  C  C  L  E  Y  F  K  G

       130        140        150        160        170        180
GCCATTCCTATCAGCAGGCTGACAAGGTGGTACAAGACTTCAGGGGAGTGTCCCAAGGAT
 A  I  P  I  S  R  L  T  R  W  Y  K  T  S  G  E  C  P  K  D

       190        200        210        220        230        240
GCCATCGTGTTTGTAACTGTCCAAGGCAAGTCCATCTGTTCCGACCCCAAGGACAAGAGG
 A  I  V  F  V  T  V  Q  G  K  S  I  C  S  D  P  K  D  K  R

       250        260        270        280        290        300
GTGAAGAAGGCGGTCAGATATTTACAAAGAACCTGGAAGGGAGGGCCCCAAGAGTCCTGA
 V  K  K  A  V  R  Y  L  Q  R  T  W  K  G  G  P  Q  E  S  *

       310        320        330        340        350        360
TTCCTGCCTGGATCATTTGGAGACCTCCACCCTCAGCGTTTGCTACCCCAACCTCCAGCT

       370        380        390        400        410        420
GCTGGAGTCCAGTGGAGGCCTCCAAGGACAAAGGTGAACCCTGTCCCCTCTTCTGGATGG

       430        440        450        460
AACCATGGCACGAAAGAGCCCATTAAAGTCTGTCTCTTTGTACTTT
```

Figure 1

```
Dog     MIPLKMLLLVTLLLGASLQVTHAARGTNVGRECCLEYFKGAIPISRLTRWYKTSGECPKD 60
Human   *A*****A**********HI****G**********LRK*KT**Q**ED*SR* 60
Mouse   *RS*Q****AA****TF**HAR***A*********D********RK*VS*****V**SR* 60
Rat     *MS*Q****AA****T***HAS***A*********D********RK*VT*FR**V****** 60

Dog     AIVFVTVQGKSICSDPKDKRVKKAVRYLQRTWKGGPQES 99
Human   *********RA******NN****N**K***SLERS 94
Mouse   ****L*****L**A*****H****I*LVKNPRP 93
Rat     ****E****RL**T*****H****I*H*KNQRL 93
```

Figure 2

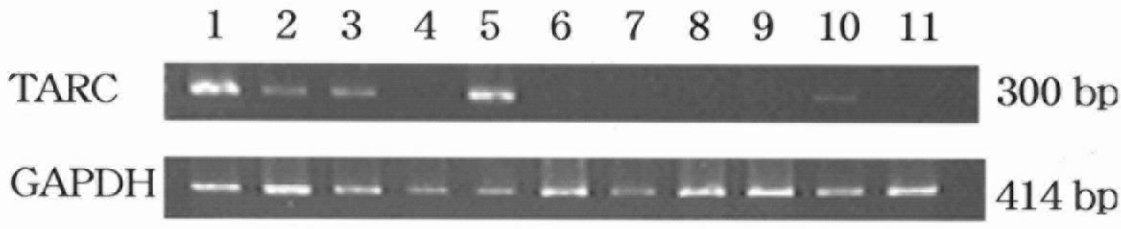


Figure 3

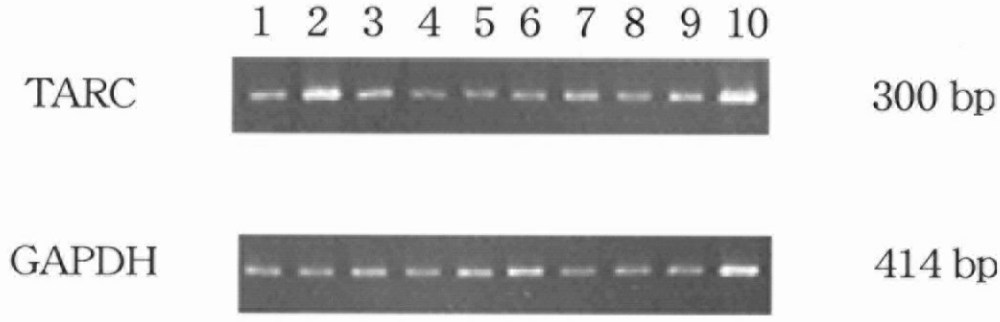
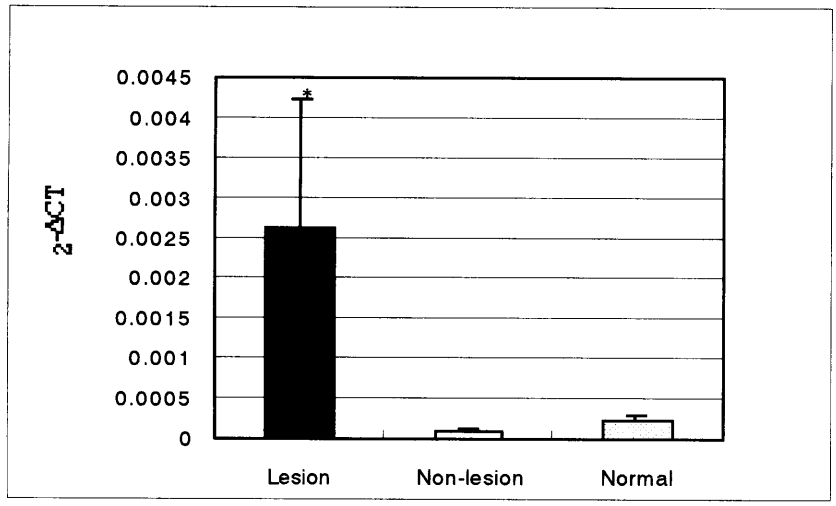
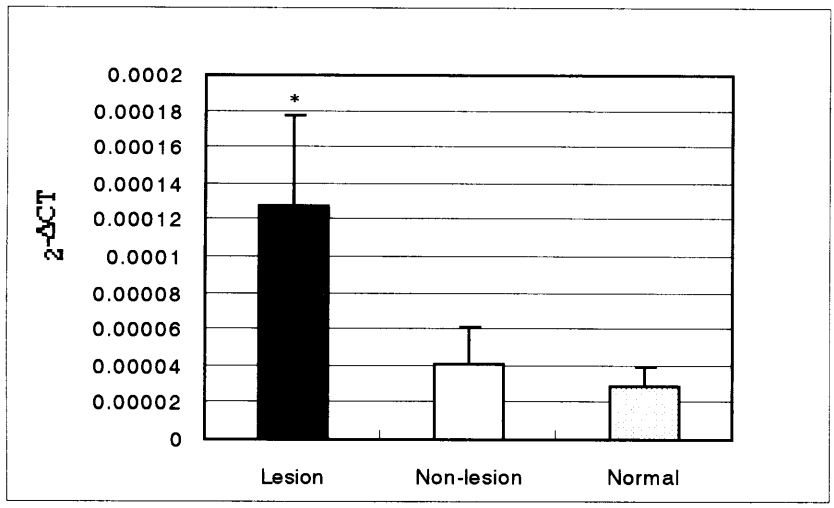


Figure 4

(a)


0.0045
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
2-ΔCT
*
Lesion
Non-lesion
Normal

(b)


0.0002
0.00018
0.00016
0.00014
0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
2-ΔCT
*
Lesion
Non-lesion
Normal

(c)

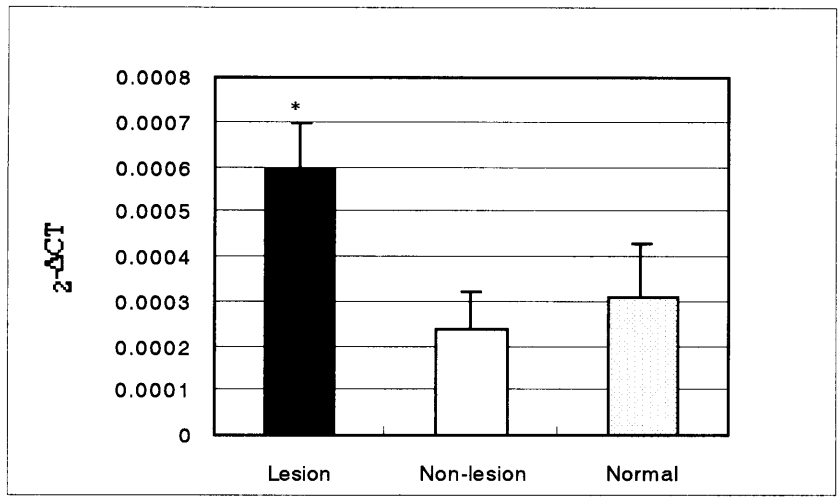

0.0008
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
2-ΔCT
*
Lesion
Non-lesion
Normal

Figure 5

```
ATGAATCCCACAGATATAGCAGACACCACCTTGGATGAAAGCATCTATAATAATTACTAT     60
 M  N  P  T  D  I  A  D  T  T  L  D  E  S  I  Y  N  N  Y  Y      20

CTCTACGAAAACATCCCTAAGCCTTGCACCAAAGAAGGCATCAAGGCATTTGGGGAGCTC     120
 L  Y  E  N  I  P  K  P  C  T  K  E  G  I  K  A  F  G  E  L      40

TTCCTGCCCCCTCTCTACTCCTTGGTCTTCCTGTTTGGTCTCCTTGGAAACTCTGTGGTG     180
 F  L  P  P  L  Y  S  L  V  F  L  F  G  L  L  G  N  S  V  V      60

GTTGTGGTCCTGTTCAAGTACAAGAGGCTCAAATCCATGACTGACGTGTACCTGCTCAAC     240
 V  V  V  L  F  K  Y  K  R  L  K  S  M  T  D  V  Y  L  L  N      80

CTTGCCATCTCGGACCTGCTCTTCGTGCTCTCTCTCCCCTTCTGGGGCTACTATGCTGCA     300
 L  A  I  S  D  L  L  F  V  L  S  L  P  F  W  G  Y  Y  A  A      100

GACCAGTGGGTTTTTGGACTAGGTCTCTGCAAGATTATTTCCTGGATGTACCTGGTGGGC     360
 D  Q  W  V  F  G  L  G  L  C  K  I  I  S  W  M  Y  L  V  G      120

TTTTACAGTGGCATCTTCTTCATCATGCTCATGAGCATCGACAGATACCTGGCAATTGTG     420
 F  Y  S  G  I  F  F  I  M  L  M  S  I  D  R  Y  L  A  I  V      140

CATGCAGTGTTCTCCCTGAGGGCGAGGACCTTGACGTATGGGGTCATCACTAGCTTGGCC     480
 H  A  V  F  S  L  R  A  R  T  L  T  Y  G  V  I  T  S  L  A      160

ACGTGGTCTGTGGCTGTCCTGGCCTCTCTTCCAGGCCTTTTATTCAGCACCTGTTATACC     540
 T  W  S  V  A  V  L  A  S  L  P  G  L  L  F  S  T  C  Y  T      180

GAGCGCAACCATACCTACTGCAAAACCAAGTACTCCCGCAACTCTACAAGGTGGAAGGTG     600
 E  R  N  H  T  Y  C  K  T  K  Y  S  R  N  S  T  R  W  K  V      200

CTGAGCTCCCTGGAGATCAACATTCTGGGATTGGTGATTCCCTTGGGCACCATGCTGTTC     660
 L  S  S  L  E  I  N  I  L  G  L  V  I  P  L  G  T  M  L  F      220

TGCTACTCCATGATCATCAGGACACTGCAGCACTGCAAAAATGAGAAGAAGAGCAAAGCA     720
 C  Y  S  M  I  I  R  T  L  Q  H  C  K  N  E  K  K  S  K  A      240

GTGAGGATGGTCTTTGCCGTGGTGGCCCTCTTTCTCGGGTTCTGGGCGCCTTACAATGTG     780
 V  R  M  V  F  A  V  V  A  L  F  L  G  F  W  A  P  Y  N  V      260

GTGCTCTTCCTGGAGACTCTGGTGGAACTGGAGGTCCTTCAGGACTGCACCTTTGAAAGG     840
 V  L  F  L  E  T  L  V  E  L  E  V  L  Q  D  C  T  F  E  R      280

CACCTGGACTACGCTATTCAGGCCACAGAGACCCTGGCTTTCGTTCACTGCTGCCTTAAT     900
 H  L  D  Y  A  I  Q  A  T  E  T  L  A  F  V  H  C  C  L  N      300

CCCGTCATCTACTTTTTCCTCGGGGAGAAATTCCGCAAGTATCTCGTGCAGCTCTTCAAA     960
 P  V  I  Y  F  F  L  G  E  K  F  R  K  Y  L  V  Q  L  F  K      320

ACCTGCAGGGGCCCTTTCATGCTCTGCCAATACTGTAGGCTCCTCCAAATGTACTCCCCC     1020
 T  C  R  G  P  F  M  L  C  Q  Y  C  R  L  L  Q  M  Y  S  P      340


GACACTCCCAGCTCGTCCTACACGCAGTCCACCGGGGATCACGATCTTCACGATGCTCTG     1080
 D  T  P  S  S  S  Y  T  Q  S  T  G  D  H  D  L  H  D  A  L      360

TAA                                                               1083
 *                                                                361
```

Figure 6

```
Dog           1:MNPTDIADTTLDESIYNNYYLYE-NIPKPCTKEGIKAFGELFLPPLYSLVFLFGLLGNSV 59
Human         1:****************S******-S*************************V******** 59
Mouse         1:**A*EVT***Q**TV**S**F**S-M*************V***********L**F**** 59
Guinea pig    1:***P*T****M*G***S***N**SSV*G****T*V************************ 60

Dog          60:VVVVLFKYKRLKSMTDVYLLNLAISDLLFVLSLPFWGYYAADQWVFGLGLCKIISWMYLV 119
Human        60:**L********R*****************F*********************M******* 119
Mouse        60:**L*************************************************V******* 119
Guinea pig   61:**L********R*************************************V**M***I*** 120

Dog         120:GFYSGIFFIMLMSIDRYLAIVHAVFSLRARTLTYGVITSLATWSVAVLASLPGLLFSTCY 179
Human       120:*********V*****************************************F*****F******* 179
Mouse       120:****************************K************I******F************ 179
Guinea pig  121:**********V************G***M*V**F***********A***F************ 180

Dog         180:TERNHTYCKTKYSRNSTRWKVLSSLEINILGLVIPLGTMLFCYSMIIRTLQHCKNEKKSK 239
Human       180:*************L***T*******************I*******************N* 239
Mouse       180:**H*******Q**V***T**********V***L****I***W***************NR 239
Guinea pig  181:*******S***R**A***T*******************I***************SK**N* 240

Dog         240:AVRMVFAVVALFLGFWAPYNVVLFLETLVELEVLQDCTFERHLDYAIQATETLAFVHCCL 299
Human       240:**K*I****V******T***I********************Y****************** 299
Mouse       240:****I*G**V******T*********************L**Y***********G*I**** 299
Guinea pig  241:**K*I****V******T***I****Y***********SL*KY**F*L********I**** 300

Dog         300:NPVIYFFLGEKFRKYLVQLFKTCRGPFMLCQYCRLLQMYSPDTPSSSYTQSTGDHDLHDA 359
Human       300:**I************IL********L*V*****G***I**A***********M******* 359
Mouse       300:***************IT***R*****LV**KH*DF**V**A*MS********V***FR** 359
Guinea pig  301:**I************I***********AP****A**RI**N**S*********M******G 360

Dog         360:L                                                           360
Human       360:*                                                           360
Mouse       360:*                                                           360
Guinea pig  361:*                                                           361
```

Figure 7

Figure 8

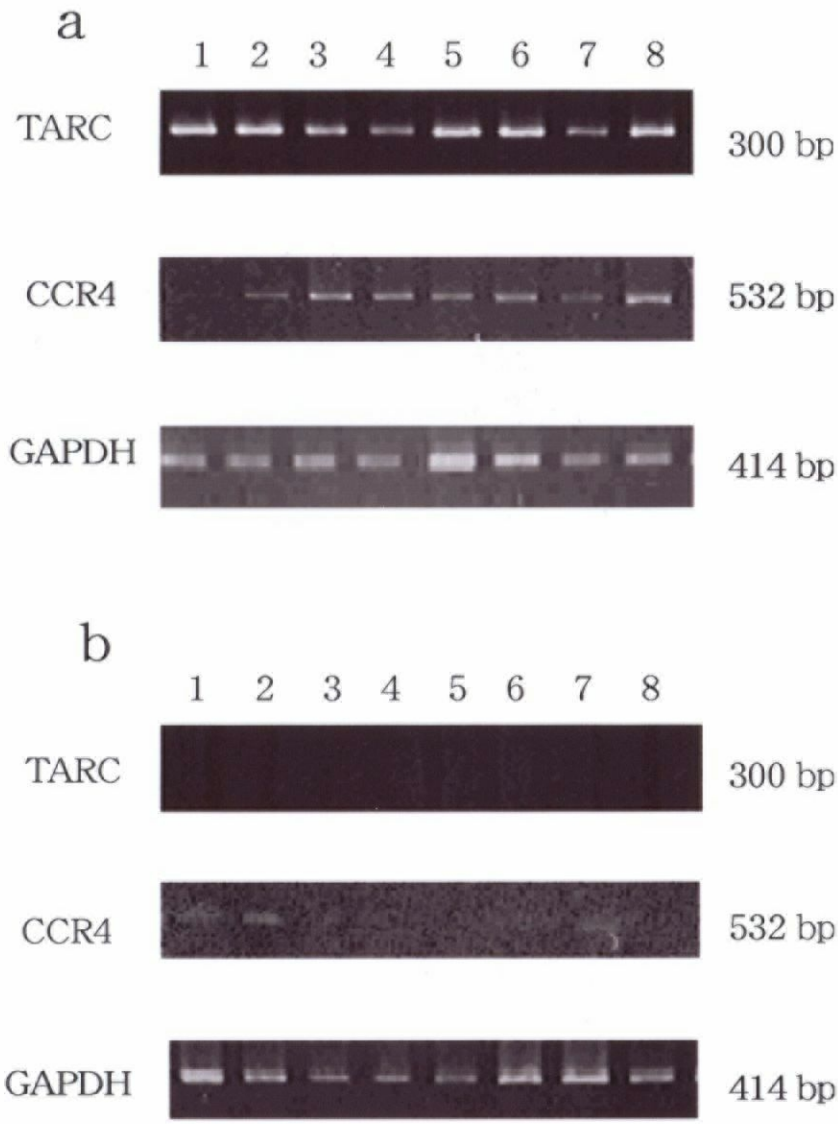
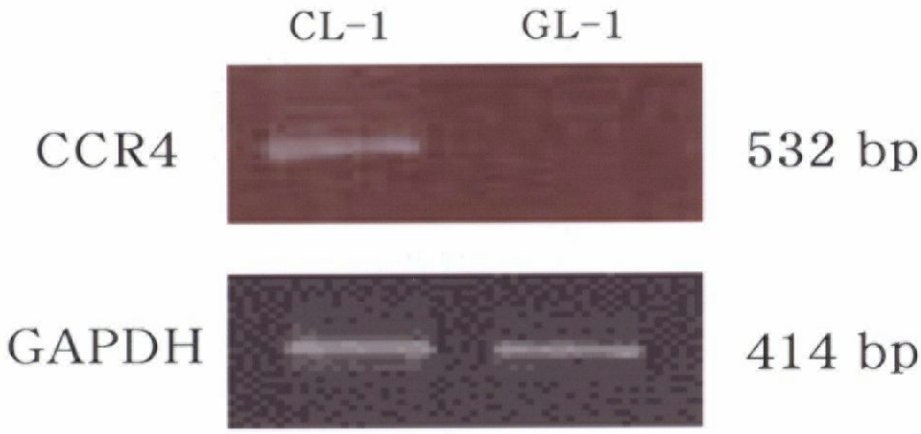

a
1 2 3 4 5 6 7 8
TARC
300 bp
CCR4
532 bp
GAPDH
414 bp
b
1 2 3 4 5 6 7 8
TARC
300 bp
CCR4
532 bp
GAPDH
414 bp

Figure 9

(a)


CL-1
GL-1
CCR4
532 bp
GAPDH
414 bp

(b)

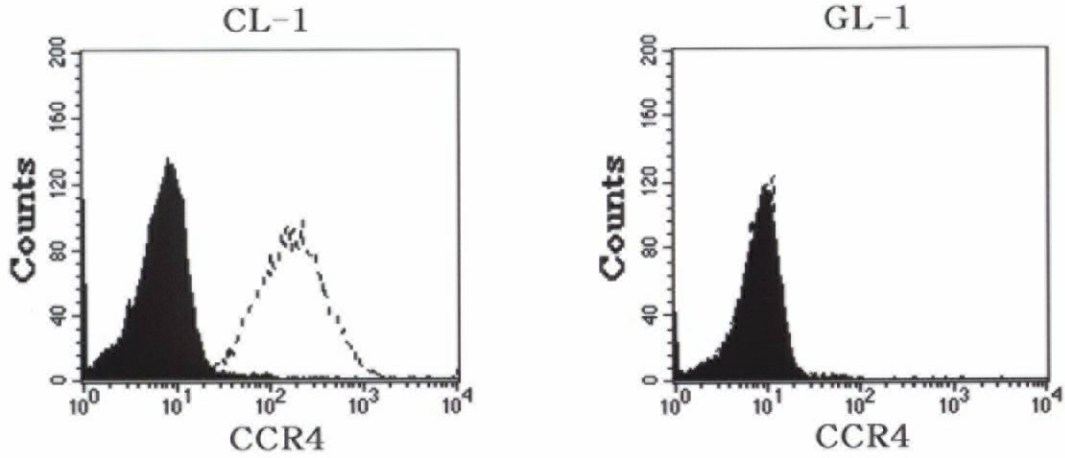

CL-1
GL-1
Counts
CCR4
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4

Figure 10

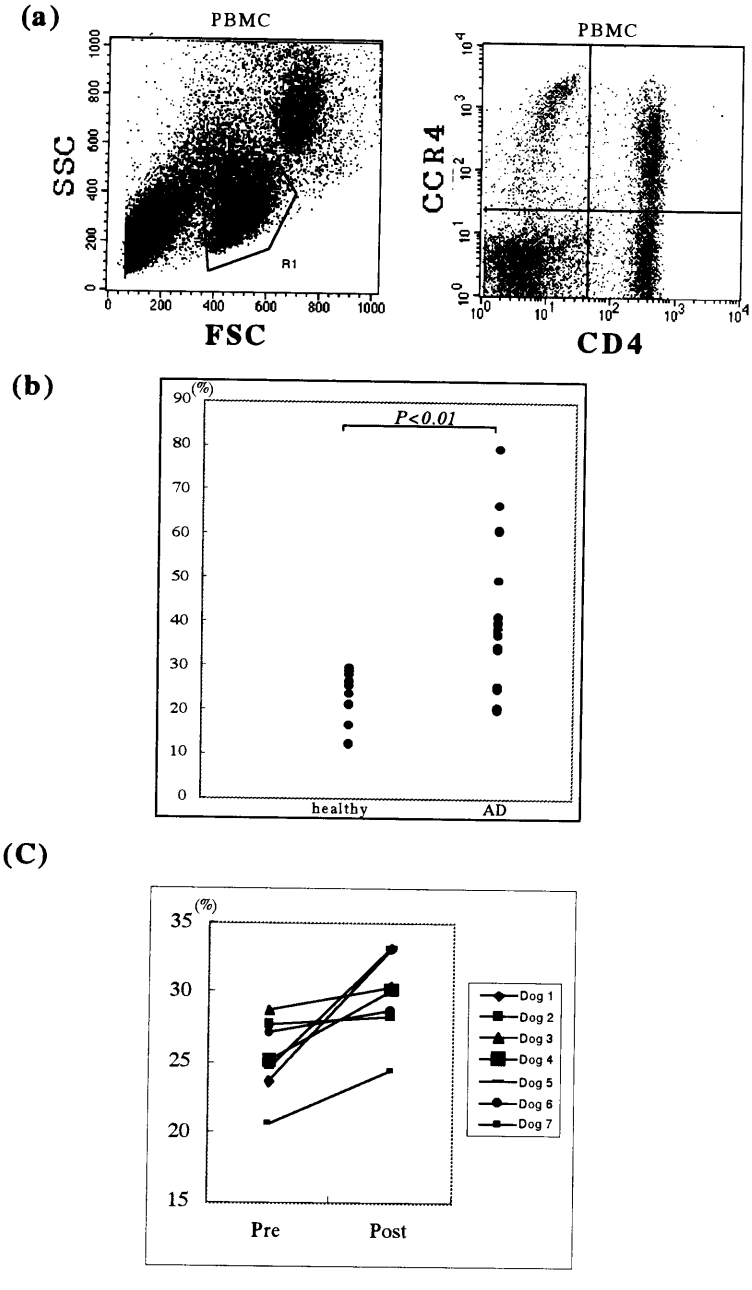
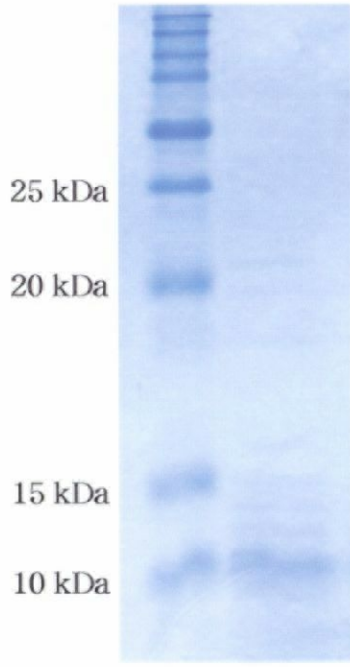

(a)
PBMC
SSC
FSC
R1
PBMC
CCR4
CD4
(b)
P<0.01
(%)
healthy
AD
(C)
(%)
Pre
Post
Dog 1
Dog 2
Dog 3
Dog 4
Dog 5
Dog 6
Dog 7

Figure 11

a)



b)

Deduced sequence: MIPLKMLLLVTLLLGASLQVTHAARGTNVGRECC
Determined sequence: ARGTNVGRE**

Figure 12

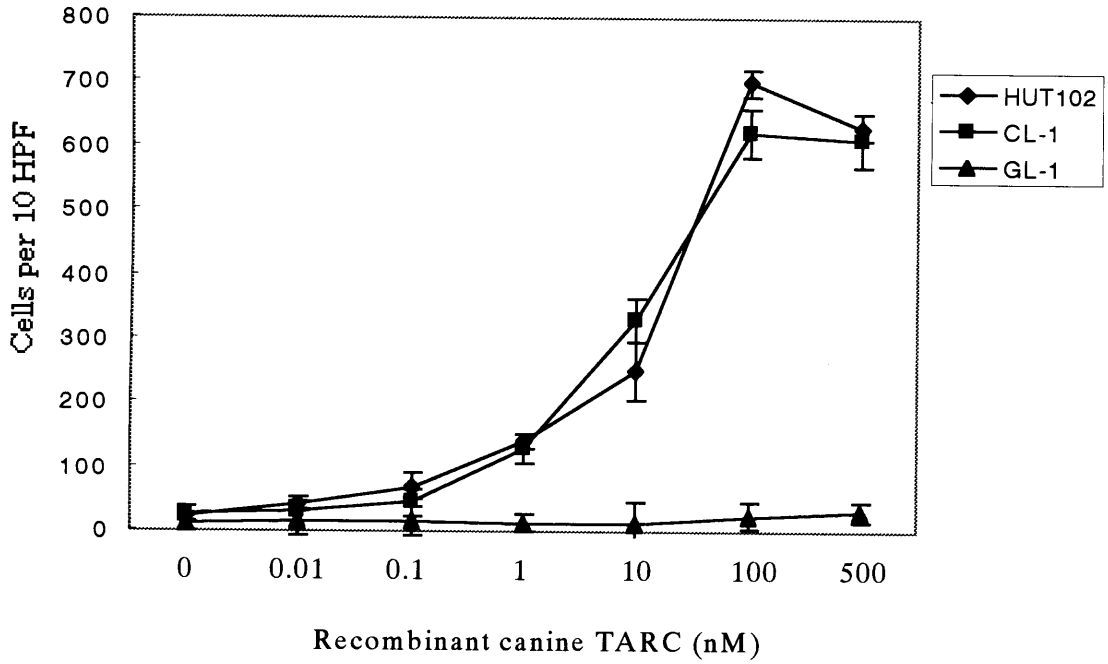

800
700
600
500
400
300
200
100
0
Cells per 10 HPF
0
0.01
0.1
1
10
100
500
Recombinant canine TARC (nM)
HUT102
CL-1
GL-1

Figure 13

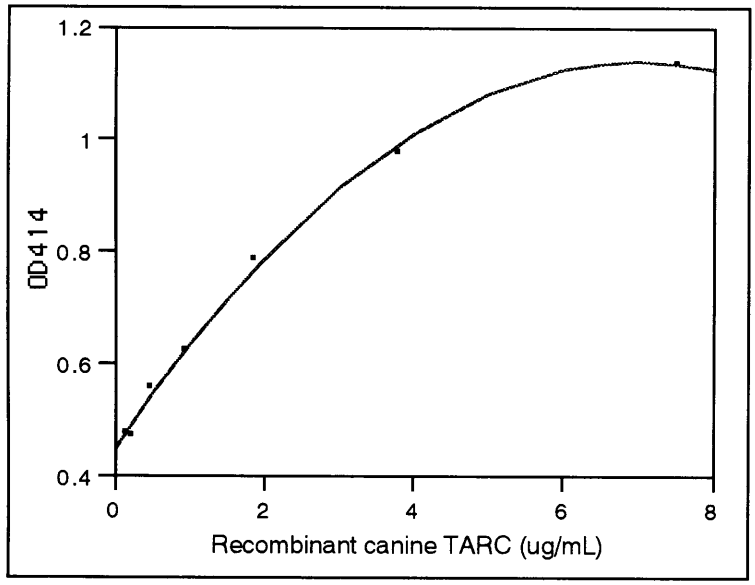
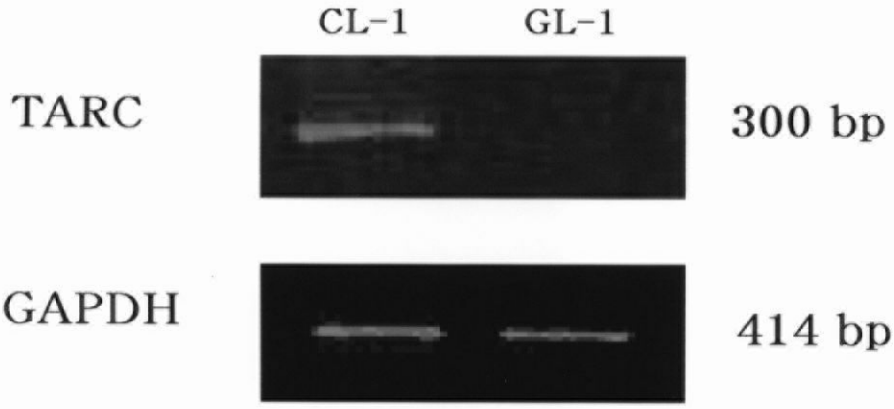


Figure 14

a)



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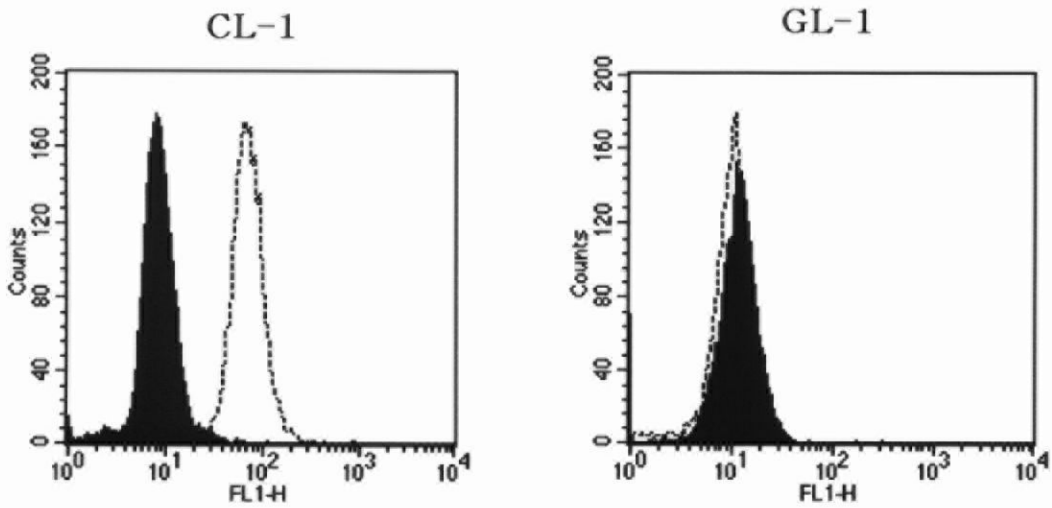
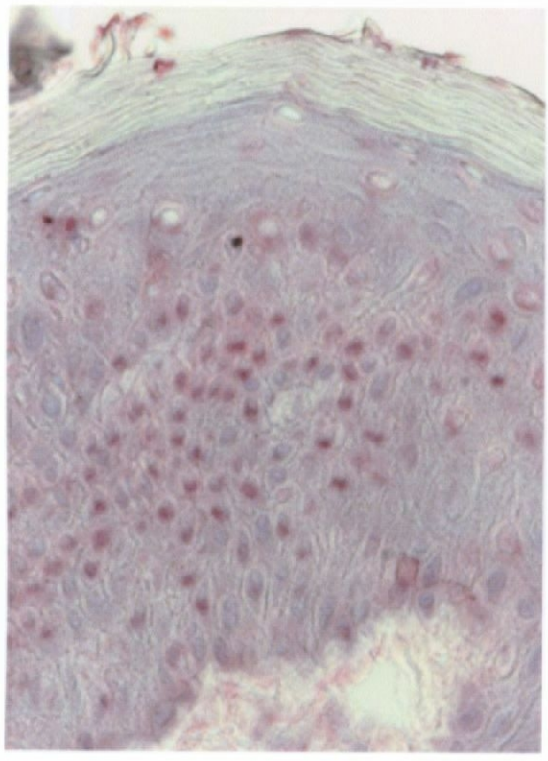


Figure 15

a)



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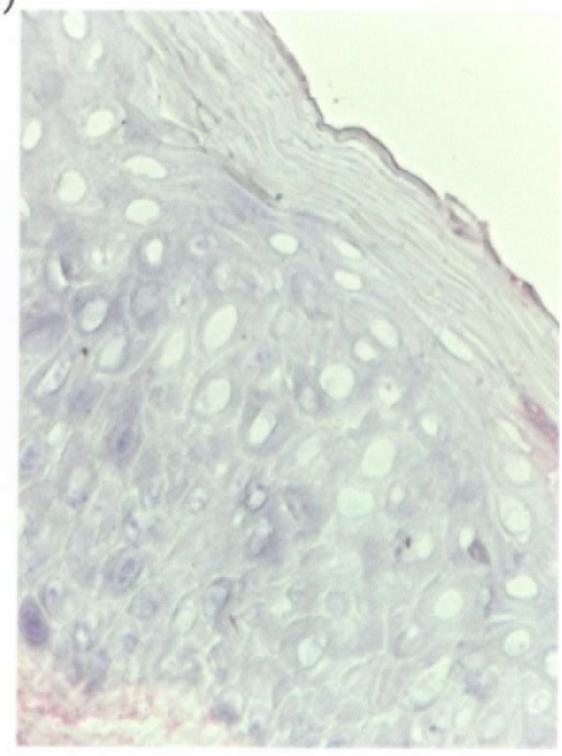

c)

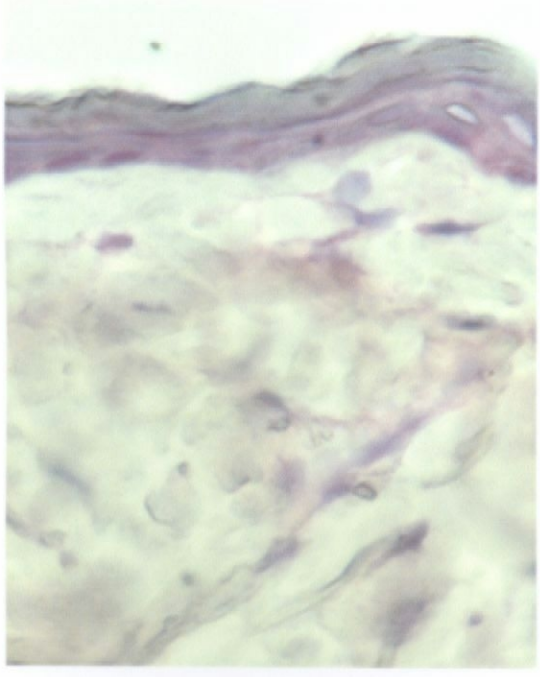

Figure 16