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Evaluation of the expression of P-selectin, ICAM-1, and TNF-alpha in bacteria-free lesional skin of atopic dogs with low-to-mild inflammation

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Abstract

Canine atopic dermatitis (AD) is a pruritic skin condition that shares many clinical and pathophysiological features with its human counterpart. A major therapeutic challenge of AD is the control of the skin inflammatory process. A detailed knowledge of the pro-inflammatory molecules involved in cell recruitment in AD would allow for a better control of the disease. We thus have studied the protein expression of P-selectin, ICAM-1 and TNF- α in the lesional and non-lesional skin of atopic dogs that had been treated for bacterial infections. Despite a low-to-mild inflammatory process, P-selectin protein was clearly upregulated in the lesional skin areas when compared with non-lesional skin (four-fold average increase). This P-selectin upregulation was accompanied by signs of functional changes such as increased cell margination, and membrane-associated protein expression. Although the expression of ICAM-1 and TNF- α was not enhanced in the lesional *versus* the non-lesional skin, there was a trend towards a correlated upregulation of both molecules. Further studies will help elucidate the significance of the substantial overexpression of P-selectin in canine AD, in particular in a scenario where bacterial antigens are not contributing as pro-inflammatory stimuli.

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Keywords: Adhesion molecules; Atopic dermatitis; Cytokines; Inflammation

1. Introduction

Atopic dermatitis (AD) is a pruritic skin disease commonly recognised in humans and dogs. Human and canine AD share many features (Willemse, 1988; Griffin and DeBoer, 2001), and these similarities have advocated the use of dog systems as models for furthering into human AD (reviewed in de Mora et al., 2006). Although the precise immunological mechanisms of such cutaneous disorder are unclear, in both species a late phase reaction (LPR) with an underlying

Abbreviations: Ab, antibody; ABC, avidin/biotin complex; AD, atopic dermatitis; BOG, bacterial overgrowth; DAB, 3,3-diaminobenzidine; HPF, high power field; ICAM-1, intercellular adhesion molecule-1; OCT, Tissue-Tek[®] O.C.T. Compound; TNF- α , tumor necrosis factor- α

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inflammatory process develops. A key aim in AD research is to uncover the cellular and molecular elements that contribute to such persistent inflammation. Many groups have characterized the dermal inflammatory infiltrate of human (Leung, 1999), and canine (Olivry et al., 1997; Sinke et al., 1997) AD, but controversy arises as to what cells are primarily involved. Several pro-inflammatory molecules are potential candidates to coordinate this cell recruitment. Histamine appears to be increased in the skin of dogs (Nimmo Wilkie et al., 1990; de Mora et al., 1996), and humans (Ruzicka and Gluck, 1983; Sampson et al., 1989) with AD. This mediator is suggested to upregulate *in vitro* the expression of P-selectin (Jones et al., 1993). This might explain why P-selectin is one of the adhesion molecules associated to AD inflamed sites in human skin (Wakita et al., 1994; Barker, 1995; Jung et al., 1996), and also in experimental settings of *in vivo* murine IgE-mediated dermatitis (de Mora et al., 1998). A report from Chenier and Dore (1998) described increased protein levels of P-selectin in different inflammatory canine skin diseases, and found a positive correlation with the amount of infiltrating cells. However, no data on its expression pattern in canine AD were provided. In addition, high levels of free and membrane-associated intercellular adhesion molecule-1 (ICAM-1) have been found in human patients with atopic asthma and dermatitis (Barker, 1995; Jung et al., 1996). This molecule participates in the adhesion phase of the migration process, i.e. subsequent to the selectins-induced rolling. Olivry et al. (1997) suggested neo-expression of this adhesion molecule in the epidermal basal layer, and upregulation in endothelial cells in lesional skin samples of dogs with AD. A major upregulator of ICAM-1 is the pro-inflammatory cytokine tumor necrosis factor (TNF)- α , which has been largely associated to clinical and experimental allergy (de Vries et al., 1998; Junghans et al., 1998). At

the mRNA level, canine TNF- α is overexpressed in the lesional skin of dogs (Olivry et al., 1999; Maeda et al., 2002; Nuttall et al., 2002), and humans (Grewe et al., 1998; Leung, 1999) with AD, but a complementary analysis of this cytokine's protein expression in lesional and non-lesional skin of dogs with AD is also of interest.

In order to assess the expression of the pro-inflammatory proteins P-selectin, ICAM-1 and TNF- α in the skin of atopic dogs, lesional and non-lesional biopsies were collected from selected atopic dogs that had been managed for dermal infections.

2. Materials and methods

2.1. Atopic dogs inclusion criteria

Six mixed-breed atopic dogs were selected from patients of the Veterinary Hospital at the *Universidad Autónoma de Barcelona*. Diagnosis was based on the presence of at least three major and three minor features (see legend under Table 1 for a list of the diagnostic criteria) as proposed by Willemse (1986). The presence of elevated allergen-specific IgE levels in serum (Allercept, Univet, Spain) was always among the minor features of the selected animals. All dogs were fed an 8-week hypoallergenic Purina restriction diet to rule out adverse food reactions. They underwent also flea control and were treated, if needed, for yeast and/or fungi infections. A cytological examination to detect *Malassezia*, bacterial overgrowth (BOG), and/or superficial bacterial infection (through the scraping technique), was carried out in each dog. BOG was considered positive if under the scotch test, the number of cocci per 100 $\times$ microscope field (immersion objective) exceeded 10. As for the dermatological examination, most of the animals (five out of six) had superficial staphylococcal pyoderma at the first visit. They were treated with Cephalexin (Rilexine 300, Virbac, 1 tablet/20 kg b.i.d.)

Table 1

Summary of the most relevant data about the clinical and the histopathological diagnostic of the six selected atopic dogs

Dog ref.	Diagnostic tests			Skin biopsy analysis	
	Allercept ^a	Major features ^b	Minor features ^c	Level of inflammation	Hyperkeratosis and follicular keratosis
1	15	a, b, c, e	f, h, j	Low	Hyperplasic perivascular dermatitis
2	2	a, b, c, e	f, h, j	Low	Hyperplasic perivascular dermatitis
3	3	a, b, c, e	f, g, j	Medium	Hyperplasic perivascular dermatitis
4	24	a, b, c,	f, h, j	Medium	Hyperplasic perivascular dermatitis
5	4	a, d	h, j	Medium	Hyperplasic perivascular dermatitis
6	4	a, c	h, i, j	Medium	Hyperplasic perivascular dermatitis

^a Number of allergens towards which specific IgE was detected.

^b a: pruritus, b: facial and/or digital involvement, c: chronic dermatitis, d: history of atopy, e: breed predilection.

^c f: signs before 3 years g: facial erythema and chelitis, h: superficial pyoderma, i: hyperhydrosis and j: IgE (allercept+).

given orally for 2 weeks, and the presence of bacteria was reassessed dermatologically right before taking the biopsy. If no further infection was detected after the antibiotic treatment, the dog was included in the study. The skin samples were therefore obtained from atopic dogs with no secondary infections. Any treatment was withdrawn for at least 3 weeks prior to the skin biopsy collection. All procedures were approved and certified by written consent by the owner.

2.2. Skin sample collection and processing

Eight millimeter punch biopsy samples were collected from the atopic dogs under local anaesthesia with 2% lidocaine. Two skin biopsies were performed in each dog: one from a lesional site, and one from a non-lesional site. Each fresh biopsy specimen was divided into two pieces. One half was fixed in 10% formalin, and processed under a paraffin-embedding protocol both, for a routine histopathological diagnostic study, and for paraffin- and formalin-resistant immunolabelling (P-selectin and TNF- α). The other half was embedded in Tissue-Tek[®] O.C.T. Compound (OCT), snap-frozen in cold isopentane (cooled in N₂ liquid), and stored at -80°C until processing for ICAM-1 immunohistochemistry since the available primary

antibody (Ab) does not bind to ICAM-1 on formalin-fixed and paraffin-embedded sections.

2.3. Immunohistochemistry

Immunolabelling for P-selectin, ICAM-1 and TNF- α was performed on skin sections following an indirect Avidin/Biotin Complex (ABC) method (Pierce, Rockford, IL, USA). Briefly, after overnight labelling at 4°C with the corresponding primary Ab, a biotinylated secondary Ab was added (2 h, RT) followed by the incubation with an enzyme-labeled ABC. A negative control was done to account for the unspecific reaction (see a typical image of the isotype matched control in Fig. 1d). The details of each labelling are as follows.

P-selectin was assessed on paraffin sections using the anti-canine P-selectin MD3 Ab (Doré et al., 1993) kindly donated by Dr. Monique Doré (Université de Montréal, Québec, Canada) diluted 1/10, and a secondary biotinylated horse-antimouse IgG (Vector, Burlingame, CA, USA) antibody followed by ABC complexed to horseradish peroxidase (Pierce). A metal enhanced 3,3-diaminobenzidine (DAB) from Pierce was used as a substrate. A 0.1% protease pre-treatment (8 min, 37°C) was shown to help retrieve the Ag before the incubation with the primary Ab, and prior to the

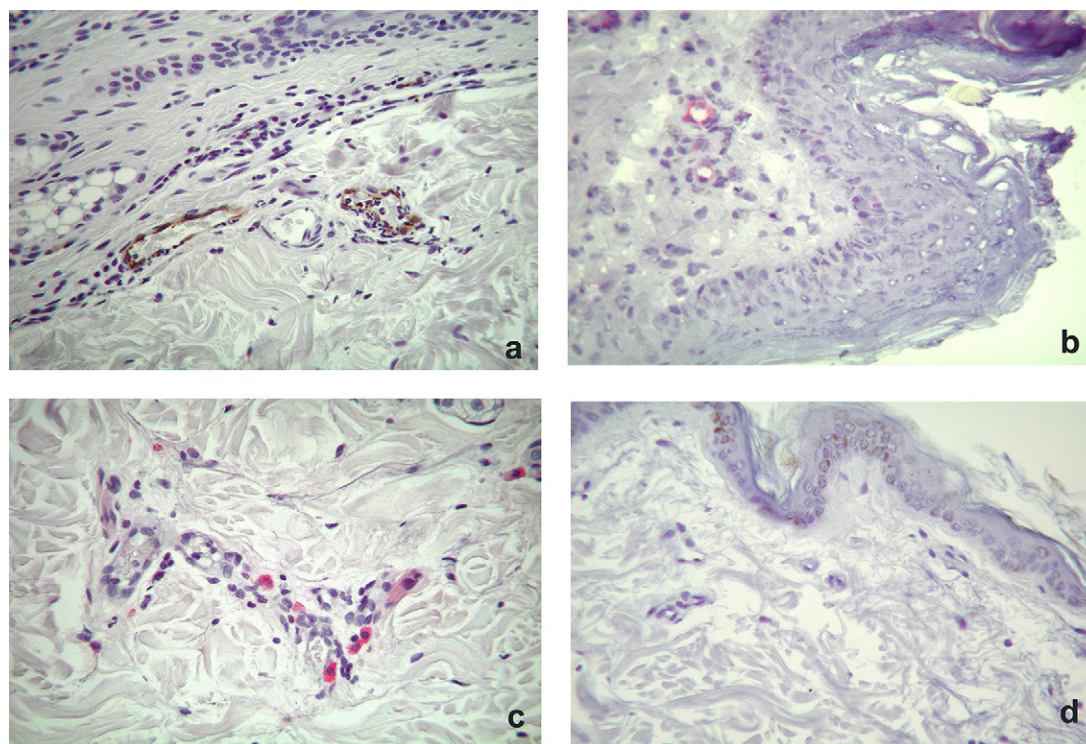


Fig. 1. Canine skin immunolabelling for (a) P-selectin, (b) ICAM-1, (c) TNF- α , and (d) typical image of the isotype negative control (20 $\times$).

labelling, endogenous peroxidase was quenched by incubating the slides in 1% hydrogen peroxide in methanol for 30 min. A parallel incubation with a primary antibody matched isotype (mouse IgG₁) was used as a control.

The expression of ICAM-1 was assessed on OCT frozen sections. One microgram per millilitre of the anti-canine ICAM-1 CL18/1D8 mouse monoclonal Ab (Smith et al., 1991) kindly donated by Dr. M.L. Entman (Baylor College of Medicine, Houston, TX, USA) was used followed by a biotinylated horse-antimouse IgG (Vector). A mouse IgG₁ was used as a negative control. The ABC was complexed to alkaline phosphatase (Pierce), and the substrate used to measure the enzyme's activity was the chromogen Fast Red-Naphtol AS-MX-levamisol from Sigma (St. Louis, MO, USA).

The immunolabelling for canine TNF- α was performed on paraffin sections. The primary Ab used was a polyclonal anti-human TNF- α (IP-300, Genzyme, Cambridge, MA, USA) diluted 1/500, an Ab proven to also bind to the canine molecule (Pickvance et al., 1993; Grone et al., 2000). The secondary Ab was a goat anti-rabbit IgG (Vector), and the ABC was complexed to alkaline phosphatase. As for ICAM-1, its activity was evaluated with Fast Red Naphtol AS-MX-levamisol from Sigma. In order to further unmask the antigen, a digestion with 0.1% protease was done before the incubation with the primary Ab. The signal specificity was double checked with two batches of the anti-TNF- α Ab, and with negative controls.

2.4. Molecules quantitation

The quantitation of the expression of the tested pro-inflammatory molecules was done blind by two independent observers. There were no differences between the results obtained by both observers, and therefore their counting was averaged.

The quantitative data were expressed as the number of dermal positive cells (TNF- α), or vessels (P-selectin and ICAM-1), per 40 $\times$ high power field (HPF). A full microscope HPF corresponded to a so-called dermis area. The positive elements (cells or vessels) of three different dermis areas randomly selected in each section were counted (duplicate counting per section). The mean of the three counted areas was calculated.

2.5. Expression and distribution of antigen, and cell margination

In addition to counting P-selectin and ICAM-1 positive vessels, and TNF- α expressing cells, the

presence of cell margination and the distribution of each molecule (tissue and/or cellular location) were assessed blind. To evaluate these parameters three HPF per dermal zone in each biopsy were examined (three HPF in the upper dermis, three HPF in the mid dermis, and three HPF in the deep dermis). This was done briefly as follows. Cell margination was only assessed in P-selectin stained vessels. The rate of P-selectin positive vessels revealing margination was assessed in relation to the total number of stained (positive) vessels. To examine either the membrane and/or the cytoplasm location of the P-selectin Ag, 10 vessels randomly selected in each section were examined with the immersion objective (100 $\times$).

2.6. Statistics

The statistical analysis was done using a Student's *t*-test for paired data when comparing lesional with non-lesional samples (with a level of significance $\alpha = 0.05$)

3. Results

3.1. Atopic dogs' clinical data

The most relevant clinical features of the selected dogs are summarized in Table 1. The age range of the atopic dogs was 1–5 years old. Of the six selected dogs, three were females and three males. Skin lesions included erythema and alopecia. All the atopics had increased levels of specific IgE against house dust mites, and many of them (five out of six) had also increased concentrations of IgE towards other environmental allergens.

3.2. Skin histopathology

In the epidermis, lesional skin samples of atopic dogs did not show cellular exocytosis, but showed hyperplasia and hyperkeratosis. Additionally, a low-to-mild dermal perivascular inflammatory infiltrate compatible with AD was seen in the lesional skin from the six selected atopics (see Table 1). The infiltrate was located in the superficial and/or mid dermis and, as for the haematoxylin–eosin staining, was composed by mast cells and a mix of neutrophils, eosinophils, lymphocytes and plasma cells.

3.3. Pro-inflammatory molecules expression

3.3.1. P-selectin

Immunohistochemistry for canine P-selectin revealed a clear labelling of the endothelial cells.

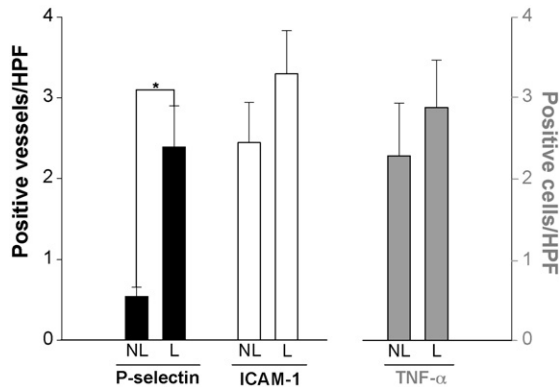


Fig. 2. Level of expression of P-selectin (black bars), ICAM-1 (white bars) and TNF- α (grey bars) protein in lesional (L) and non-lesional (NL) skin samples of atopic dogs. The plots represent the number of positive elements (either vessels or cells) per high power field (HPF), level of statistical significance = 0.05.

Positive vessels were mostly venules and capillaries (Fig. 1a). P-selectin expression was quantitated in vessels from the lesional and the non-lesional skin of atopic dogs and the results are depicted in Fig. 2 (black bars). The number of P-selectin positive vessels in the lesional atopic skin was over four-fold the number counted in the non-lesional area (2.4 ± 0.5 vessels/HPF *versus* 0.5 ± 0.1 vessels/HPF). P-selectin was particularly overexpressed in the upper and the mid dermis. These data were consistent for every single atopic dog i.e. the expression of P-selectin was individually upregulated in the lesional skin from the six selected animals. The average difference was statistically significant ($p < 0.05$) as for a paired Student's *t*-test.

As for the Ag cellular location, 40% of the positive vessels showed a mixed labelling pattern (membrane and cytoplasm expression) in the lesional areas *versus* 12% in the non-lesional skin. In addition, we observed a four-fold increased neutrophils margination associated to the P-selectin expressing vessels, in lesional (32%) *versus* non-lesional skin (8%).

3.3.2. ICAM-1

The ICAM-1 labelling was mostly observed in endothelial cells (Fig. 1b) but also in some keratinocytes, and mast cells of the lesional and non-lesional skin samples. The number of positive vessels for ICAM-1 in the lesional samples was statistically similar to the non-lesional ones (3.3 ± 0.5 *versus* 2.4 ± 0.5) as shown in Fig. 2 (white bars). Although the individual data are not shown, in four out of six dogs, a higher number of ICAM-1 positive vessels was counted in the individual lesional sites (*versus* non-lesionals). This observation

was particularly clear in the upper and the mid *versus* the deep dermis. Only in the lesional samples we also observed that the sweat glandular epithelium was slightly positive for ICAM-1.

3.3.3. TNF- α

The TNF- α labelling in the skin biopsies of the selected atopic dogs was mainly observed in mast cells at any dermal location (Fig. 1c). The expression of TNF- α was statistically similar in the lesional and non-lesional skin despite the finding that the counted number of positive cells was higher (on average, and in four out of six dogs) than in the non-lesional ones (2.9 ± 0.6 *versus* 2.3 ± 0.7) (Fig. 2, grey bars).

4. Discussion

Histological features of AD were found in all lesional samples of the selected atopic dogs, matching findings previously reported for humans (Leung, 1999) and dogs (Willemse, 1986; Olivry et al., 1997; Scott et al., 2001). However, unexpectedly, inflammation was clearly low-to-mild in the selected atopic dogs when compared both with literature data, and with our own historical experience collecting and assessing biopsies from atopic dogs for routine clinical diagnostic. Particularly, there was no cellular exocytosis in the epidermis in any of the six selected atopics. Exocytosis is a feature usually observed in regular AD biopsies. The low inflammatory infiltrate was an interesting observation that might be attributable to the absence of a concomitant bacterial presence in the skin of the selected atopic dogs, and, if so, suggests the relevance of bacterial superantigens as enhancers of pro-inflammatory signals in canine AD as shown in human AD (Leung and Bieber, 2003). We could therefore tentatively suggest both, the need to evaluate the bacterial burden when interpreting signs of inflammation in canine AD, and the potential clinical beneficial impact of antibacterial treatment to this condition.

Despite the low inflammation we have shown that the endothelial cells, primarily in capillaries and venules, from the atopic dogs' lesional skin, express significantly higher P-selectin protein (four-fold) than the non-lesional skin, similarly to what had been reported in humans (Jung et al., 1996). P-selectin is usually stored in endothelial cells in the Weibel–Palade bodies, and is translocated to the membrane after stimulation. When present in both locations, immunolabelling uncovers a mixed membrane and cytoplasmatic cellular pattern. In our study, we could clearly correlate the presence of a mixed pattern with both the higher number of positive

vessels within the lesional area – as opposed to only cytoplasmic P-selectin in the non-lesional skin – and with an increased cell margination (four-fold), that most probably reflects the cellular rolling induced by the selectin family of adhesion molecules (Chenier and Dore, 1998). Overall, these data show that functional P-selectin is over-expressed in the lesional skin of atopic dogs, suggesting that this adhesion molecule might contribute to the initial recruitment of inflammatory cells in canine AD.

As for ICAM-1, it is important to highlight that, although not significant, the counted number of stained vessels was higher for most of the individual lesional *versus* non-lesional skin samples, bringing the idea of a trend towards an increased expression of this molecule in canine AD. If so, these results add a quantitative perspective to previous studies with atopic dogs (Olivry et al., 1997), and parallel observations in human lesional and non-lesional samples (Jung et al., 1996). We found a very similar trend when evaluating TNF- α protein expression. While the cytokine's mRNA had been shown to be increased in skin samples of atopic dogs in earlier studies (Olivry et al., 1999; Maeda et al., 2002; Nuttall et al., 2002), under our experimental conditions, we cannot conclude that this is translated into protein overexpression. Both, the technical approach, or the sampling of uninfected skin lesions could explain such difference. In addition, our data, in agreement with literature background results (Ackermann and Harvima, 1998), confirm that TNF- α positive cells in AD skin are, at least partly, identified as mast cells morphology- and location-wise. Contrary to Olivry et al. (2003), we did not find TNF- α positive keratinocytes in lesional skin. Yet surprising, this could be attributable to the used immunolabelling techniques (notably a different primary Ab). The correlation between the expression of ICAM-1 and TNF- α in lesional *versus* non-lesional sites could be uncovering a tight functional association between both molecules in AD. This is supported by our previous finding that mast cells-induced ICAM-1 upregulation in canine endothelial cells is mostly attributable to TNF- α (Torres et al., 2002), as also shown in human endothelial cells (Meng et al., 1995). As discussed earlier, the fact that we can only suggest a trend towards increased expression of ICAM-1 and TNF- α , and not actual statistical differences, could be caused by the removal of contributing pro-inflammatory stimuli through the removal of bacterial superantigens.

In addition to endothelial cells, we observed low-to-mild ICAM-1 labelling in the epidermal basal layer in the lesional and the non-lesional areas of the atopic dogs, and in some mast cells from the AD lesions.

Although mast cells ICAM-1 labelling had not been previously reported in skin samples of dogs with AD, it has been previously described in mast cell cultures (Weber et al., 1997). Interestingly, we also observed ICAM-1 signal in the basal area of the sweat glands epithelium only in the lesional AD samples. A similar observation to the epithelium of the eccrine sweat gland of humans in which positivity for different adhesion molecules had already been reported (Hale et al., 1995; Mori et al., 1995; Dietz et al., 1996). Additional experiments need to be performed to establish the relevance of such findings to AD.

In conclusion, despite the removal of a strong pro-inflammatory stimulus such as bacterial superantigens, unlike ICAM-1 and TNF- α , P-selectin is substantially upregulated in the lesional skin of atopic dogs. Given the resemblance between the pathogenesis of canine and human AD, a further assessment of the kinetics of P-selectin *in vivo* in spontaneous or experimental canine AD, will contribute to elucidate its significance to such condition.

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