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SHORT COMMUNICATION

Intradermal injection of Hsp60 induces cytokine responses in canine atopic and healthy skin

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Abstract The purpose of this study was to investigate the immunoregulatory potential of Hsp60 in the skin of dogs with atopic dermatitis. Three dogs with chronic atopic dermatitis and four healthy dogs were injected intradermally with Hsp60 and phosphate-buffered saline. Biopsies were taken before testing from non-injected control skin, lesional and non-lesional atopic skin, and 48 and 72 h after injection. Analysis of cytokine messenger RNA was performed using quantitative real-time polymerase chain reaction. Forty-eight hours after Hsp60 injection, a rise in interleukin (IL)-10 was found (P=0.034) with the highest expression levels in non-lesional atopic and control skin. A rise of transforming growth factor beta (P=0.015) and IL-12p40 (P=0.017) was noticed 72 h after Hsp60 injection in control skin. No significant differences were observed for the expression of IL-4, IL-12p35, and interferon gamma. The results indicate that Hsp60 is able to induce cytokines of a regulatory and Th1 phenotype in the skin. Furthermore, this study seems to provide a first indication of deficient Hsp60 response in atopic dermatitis affected skin.

Keywords Hsp60 · Immunostimulation · Canine · Skin · Atopic dermatitis

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Introduction

Heat shock proteins (Hsp) are evolutionary conserved proteins present in all eukaryotic and prokaryotic cells. Microbial Hsp60 and Hsp70 are considered immunodominant antigens, which have immunomodulatory qualities, both at the level of innate defence mechanisms and at that of antigen-specific adaptive immunity. Current understanding of the initial phases of innate immune responsiveness attributes a significant role to Hsp in the signalling of the unprimed immune system, and based on cross-recognition of microbial and mammalian homologues, regulatory T cells are activated. Hsp have shown their immunomodulatory qualities of suppressing T-cell-mediated pathologies in models of experimental autoimmunity, such as type 1 diabetes and arthritis (Birk et al. 1996; Prakken et al. 2001). The effect has been shown to be mediated by Hsp-specific regulatory T cells that respond to mammalian Hsp and includes the production of IL-10 (Van Eden et al. 2005). Skewing of the immune system by Hsp may contribute to a setting in which allergic disorders are less likely to occur, possibly similar to the alleged effects of Bacille Calmette-Guérin vaccin of which Hsp60 is one of the dominant antigens (De Bruyn et al. 2000).

Atopic dermatitis (AD) is a chronic inflammatory skin disease in humans and dogs with comparable clinical features. The development of this disease is caused by allergen-specific and non-specific mechanisms. Previous studies of canine AD

have found a predominance of CD4+T cells in lesional skin (Olivry et al. 1997; Sinke et al. 1997), the presence of Th2-related cytokines [interleukin (IL)-4, IL-5, and IL-13] in acute lesional skin and of a Th1 type [interferon gamma (IFN-γ) and IL-18] in chronic lesional skin (Olivry et al. 1999; Sinke et al. 2002; Marsella et al. 2006). The prevalence of AD has increased in the last decades in humans (Bloomfield et al. 2006) and possibly in dogs as well. A likely explanation for this phenomenon was proposed by Bjorksten through the 'Microbial deprivation hypothesis' (Bjorksten 2004). Due to lack of infection with mycobacteria, oro-faecal infections or gut commensals and helminths, allergic manifestations are more likely to occur in genetically predisposed individuals (Bloomfield et al. 2006). The mechanism by which the reduced exposure to pathogenic and non-pathogenic microbes results in enhanced responses of Th2 cells might be due to a reduced production of Th1-polarizing cytokines (IL-12 and interferons) and immunosuppressive cytokines [IL-10 and transforming growth factor beta (TGF-β)], which are both produced in response to chronic microbial stimulation of the immune system (Romagnani 2004).

Management of AD in humans and dogs is directed at the elimination or avoidance of provoking or exacerbating factors such as allergens and infectious agents, the reduction of cutaneous inflammation and skin rehydration. Moreover, in dogs, current therapy commonly involves allergen-specific immunotherapy (Griffin and Hillier 2001) or symptomatic treatment with either corticosteroids (Olivry and Sousa 2001) or cyclosporin A (Olivry et al. 2002). Neither one of these therapies is guaranteed successful or without side effects. Therefore, a treatment that modulates the immune response towards regulatory T cells is an interesting perspective. More specifically, the

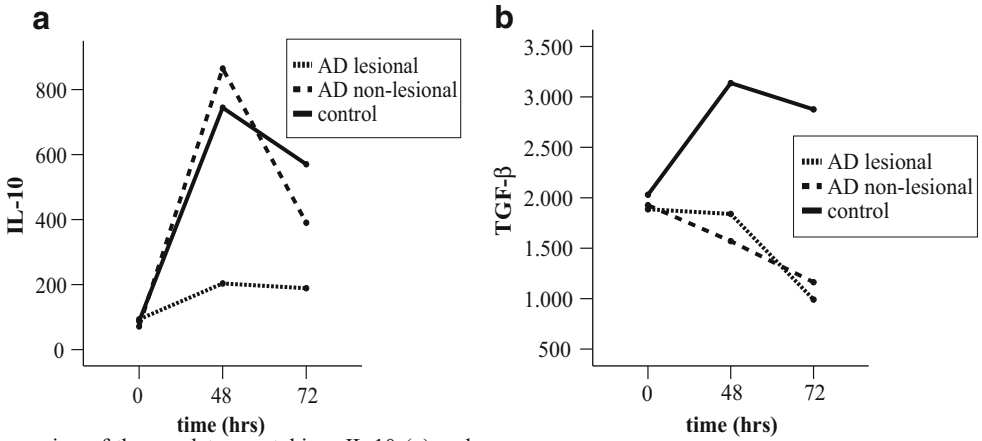

Fig. 1 Relative expression of the regulatory cytokines IL-10 (**a**) and TGF-β (**b**) over time: (0 h) and after (48 and 72 h) intradermal administration of Hsp60 in lesional atopic, non-lesional atopic and control skin. Cytokine expression was determined by quantitative real-time PCR at the messenger ribonucleic acid (mRNA) level from skin samples. Three 6-year-old experimental female Beagle dogs with chronic atopic dermatitis (*AD*) and four 2-year-old healthy, mixed breed control dogs (three females) were used. Recombinant human Hsp60 (0.1 ml of a 0.25 mg/ml solution containing <60 U endotoxin/mg) was injected intradermally in duplicate in lesional skin of AD dogs and in non-lesional skin of the thorax and/or abdomen of AD dogs and healthy dogs. PBS (0.1 ml) in duplicate was used as a control. Before Hsp60 injection, 6-mm punch biopsies were taken from non-injected lesional, non-lesional and control skin under local anesthesia with 2% lidocaine. At 48 and 72 h after injection of Hsp60 or PBS, skin biopsies were taken from intradermal injection sites under sedation with medetomidine (100 μg/kg IM). These samples were immediately snap-frozen and stored at −70°C until RNA isolation. Samples were disrupted and homogenized using an ultraturrax (T8, IKA® Labortechnik GmBH, Staufen, Germany). Total RNA was isolated according to the TRIzol manufacturers' instructions (Invitrogen, Breda, The Netherlands) with minor modifications described by Brinkhof et al. (2006). Spectrophotometric quantification of the RNA was performed by using Nanodrop ND-1000 (Isogen Life Science, Ijsselstein, The Netherlands). Quality of the purified RNA was checked by either agarose gel electrophoresis or the Agilent 2100 bioanalyzer (Agilent Technologies Netherlands, Amstelveen, The Netherlands). Good quality and intact RNA was indicated for all measurements. Isolated RNA was temporarily stored at −70°C before cDNA synthesis. The I-script cDNA synthesis kit (BioRad, Veenendaal, The Netherlands) was used for cDNA synthesis following the manufacturer's protocol. Quantative PCR was performed for a total of 6 cytokines (IL-4, IL-12p35, IL-12p40, IFN-γ, IL-10, and TGF-β) and four reference genes (Table 1) according to the method described by Brinkhof et al. (2006). The amount of the gene of interest and of the independent internal references was determined from the appropriate standard curve for each experimental sample. All samples were examined in duplicate. Data were considered valid if relative amounts of the internal reference genes were constant for a sample. Results were normalized according to the average amount of the internal references to generate relative expression levels (Spee et al. 2006). Results were expressed as relative expression levels and multiplied by an arbitrary factor (100.000). A multivariate analysis was performed using the general linear model to determine if there were significant differences in cytokine expression between the injection sites of Hsp60 and PBS for the different time slots given, between the two groups, for both lesional and non-lesional skin, for each cytokine. A P value<0.05 was considered statistically significant and analysis was performed using SPSS software (SPSS Benelux BV, Gorinchem, The Netherlands)

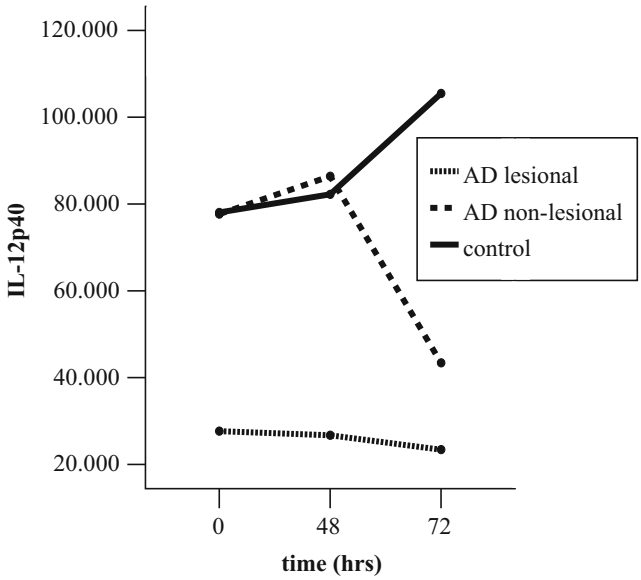

Fig. 2 Relative expression of IL-12p40 over time: before (0 h) and after (48 and 72 h) intradermal administration of Hsp60 in lesional atopic, non-lesional atopic and control skin

aim of this study was to evaluate the modulatory capabilities of Hsp60 as assessed by local cytokine production after intradermal injection of Hsp60 in healthy and AD dogs.

Results

Expression of IL-10 was significantly raised (P=0.034) at 48 h compared to base levels (T0) after stimulation with Hsp60 versus treatment with phosphate-buffered saline (PBS) as the negative control. Between lesional atopic, non-lesional atopic and control skin, no significant differences were found with respect to IL-10 production, although the highest expression was found in the latter two (Fig. 1a). The TGF-β expression was elevated in control skin only at 48 and 72 h post-Hsp60 injection. At 72 h after stimulation with Hsp60, this was significant (P= 0.015) compared to base level and PBS (Fig. 1b). No effect on the TGF-β expression was seen in lesional or non-lesional atopic skin. Compared with base levels and PBS, the expression of IL-12p40 was raised (P=0.017) at 72 h after stimulation with Hsp60 in control skin, whereas the expression in non-lesional atopic skin declined (Fig. 2). No differences were seen of the IL-12p40 expression in lesional atopic skin. Furthermore, treatment with Hsp60 had no effect on the expression levels of IL-4, IL-12p35, and IFN-γ. Until 72 h after intradermal injection of Hsp60, there were no local erythema or swelling noticed on the injection sites.

Discussion

In this study, the modulatory effect of Hsp60 in canine healthy and atopic skin was evaluated by measuring cytokine levels at 48 and 72 h after intradermal injection. On the cytokine level, a significant increase of the IL-12p40 expression at 72 h post-Hsp60 injection was seen in healthy canine skin. The (pro-)inflammatory cytokine IL-12 is composed of a p35 and p40 subunit and is a potent inducer of differentiation of Th1 cells (Trinchieri et al. 2003), thus possibly indicating a Th1-inducing effect of Hsp60 in healthy control skin but not atopic skin. Moreover, the TGF-β expression was increased by Hsp60 in healthy dog skin, at 48 and 72 h after stimulation but only significant at the latter. A previous study found a higher constitutive expression of TGF-β in healthy canine skin compared to atopic skin, which was thought to be associated with clinical tolerance (Nuttall et al. 2002). However, no difference in TGF-β expression was found in the unstimulated skin between atopic and control skin in the present study.

The cutaneous expression of IL-10 after Hsp60 injection showed a time-dependent increase in our study (both 48

Table 1 Cytokine and reference gene primers and optimal primer annealing temperatures (according to Brinkhof et al. 2006)

Cytokine	Primer sequence		Primer sequence		Tm (°C)
IL-4	Forward	ccaaagaacacaagcgataaggaa	Reverse	gtttgccatgctgctgaggtt	61
IL-10	Forward	cccgggctgagaaccacgac	Reverse	aaatgcgctcttcacctgctccac	63
IL-12p35	Forward	taatggatcccaagaggcag	Reverse	tcaagggaggatttctgtgg	62.5
IL-12p40	Forward	ggacgtttcacatgctggt	Reverse	ccactctgaccctctctgct	59
TGF-β	Forward	caaggatctgggctggaagtgga	Reverse	ccaggaccttgctgtactgcgtgt	65
IFN-γ	Forward	agcgcaaggcgataaatg	Reverse	gcggcctcgaaacagatt	55.8
Gusb	Forward	agacgcttccaagtacccc	Reverse	aggtgtggtgtagaggagcac	62
RPS5	Forward	tcactggtgagaaccccct	Reverse	cctgattcacacggcgtag	62.5
RPS19	Forward	ccttcctcaaaaagtctggg	Reverse	gttctcatcgtagggagcaag	61
RPL8	Forward	ccatgaatcctgtggagc	Reverse	gtagagggtttgccgatg	55

and 72 h) in control and non-lesional atopic skin in contrast to in lesional atopic skin. As the expression of TGF-β and IL-10 in lesional atopic skin is most likely time- and dose-dependent, the more as the expression of these cytokines at base level (T0) was similar in atopic (lesional and non-lesional) and control skin, it may be of interest to test serial dilutions of Hsp60 over a longer period of time in a future study. Alternatively, it might be of interest to look for other routes of administration, considering the 'microbial deprivation hypothesis' (Bloomfield et al. 2006). Therefore, it cannot be excluded that a chronic overexposure to Hsp60 of the gastrointestinal system could lead towards a reduction of Th2 cells and an up-regulation of regulatory T cells in lesional atopic skin.

IL-10 and TGF-β likely have a role in the regulation of the allergic inflammation, as in dogs with AD, a deterioration of clinical signs is associated with a decrease of regulatory cytokines levels in whole blood (Maeda et al. 2007). Together with our results, we believe that, although Hsp60 in the used concentration was unable to switch the immune response in atopic skin (lesional and non-lesional) equally as in control skin, there was a stimulating effect on the expression of in particular IL-10 in canine skin.

Self-Hsp cross-reactive T cells are thought to be present at the site of inflammation and are able to enhance the production of regulatory cytokines as IL-10 and TGF-β (Hauet-Broere et al. 2006; Wieten et al. 2007). Another interesting finding is that, in human atopic lesional skin, expression of Hsp70 and Hsp60 is more intensive than in normal healthy skin (Ghoreishi 2000). Under these circumstances, it may be expected to find the highest expression of the regulatory cytokines in canine lesional atopic skin after intradermal stimulation with Hsp60. In contrast, up-regulation of these cytokines expression was particularly found in healthy control skin. In non-lesional and lesional atopic skin, an up-regulation of only the IL-10 expression was found, which was low in the latter. This result is in accordance to the findings of Ricklin Gutzwiller et al. (2007). In the latter study, AD dogs were intradermally injected with a suspension of heat-killed *Mycobacterium vaccae*. Based on the reduction of clinical symptom scores and pruritus scores, this treatment was only found to be effective in dogs with mild to moderate but not severe AD. However, these authors did not follow the expression of cytokines in the skin over time.

In conclusion, the results of this study show that Hsp60 is able to induce cytokines of a regulatory and Th1 phenotype in healthy and partly in non-lesional atopic canine skin. This may indicate that Hsp interventions should be combined with, e.g. anti-inflammatory agents, to neutralize the dominance of pro-inflammatory cytokines. Moreover, the results may be explained by the route of administration, the single Hsp60 injection, the Hsp60 dose or possibly a suppression of the immune system. The results justify further research on the functional properties of Hsp60 and its clinical effect in dogs with AD.

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