Topical heat shock protein 70 prevents imiquimod-induced psoriasis-like inflammation in mice

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Received: 15 December 2017 / Revised: 14 March 2018 / Accepted: 15 March 2018 / Published online: 3 April 2018
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Abstract

Psoriasis is a chronic inflammatory skin disease with systemic manifestations and potential genetic etiology. The newest treatments utilize antibodies against one of several cytokines known to underlie the inflammatory signaling molecules that produce the skin and systemic symptoms. However, these agents must be regularly injected, and they may compromise the normal responses of the immune system. Furthermore, they do not address the causes of the abnormal immunoregulatory responses of the disease because the etiology is not yet completely understood. In this short-term treatment study, the potential anti-inflammatory activity of an alfalfa-derived Hsp70-containing skin cream (aHsp70) was tested on imiquimod (IMQ)-induced psoriasis-like lesions in wild-type mice. Treatment of the mice with the aHsp70 skin cream simultaneously with the imiquimod application mitigated the induction of psoriatic-like lesions and correlated with altered expression of various skin cytokines.

Keywords Hsp70 · Alfalfa · Psoriasis · Skin cream · Anti-inflammatory

Introduction

Psoriasis is a chronic inflammatory skin disorder that affects children and adults (Burden-Teh et al. 2016). It is thought to result from a combination of genetic, epigenetic, and environmental influences resulting from abnormal interplay between immune cells, cytokines, and skin cells (Deng et al. 2016). Psoriasis is characterized by itchy patches of raised red skin with thick scaly plaques and is associated with several co-morbidities, including cardiovascular disease and inflammatory arthritis, suggesting an underlying autoimmune pathogenesis of the disease (Davidovici et al. 2010; Lowes et al. 2014). Psoriasis affects 2–3% of the world population and is most prevalent in American, Canadian, and European populations (Perera et al., 2012).

In psoriasis, keratinocyte proliferation is highly increased in the basal layers of the epidermis in response to external insults, rapidly forming multiple layers that constitute scaly plaques (Lowes et al. 2014). Activated keratinocytes secrete a variety of inflammatory cytokines leading to immune cell responses such as stimulation of dendritic cells that then travel to lymph nodes and secrete numerous cytokines including interleukin-17 (IL-17) and IL-23. These cytokines activate the differentiation of naïve T helper cells (T_H) cells to T_H1 and T_H17 cells in the lymph nodes (Sharma et al. 2015). The immune cells then infiltrate back into psoriatic skin via the circulation and secrete psoriasis-specific

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inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), IL-22, and IL-17 family of cytokines. These cytokines then induce proliferation of keratinocytes and angiogenesis, resulting in psoriatic plaque formation (Nestle et al. 2009). The IL-17 family of cytokines include IL-17A, IL-17C, and IL-17 F; they recently emerged as one of the most important players involved in the psoriasis pathogenesis (Monin and Gaffen 2017). T_H2 cells on other hand secrete many cytokines including IL-4 and IL-5, facilitating antibody production, defending against microorganisms, and inducing allergic reactions (Noben-Trauth et al. 1996; Greenfeder et al. 2001).

To date, there is no cure for psoriasis and much of the treatment involves managing the symptom. First-line treatment for patients with psoriasis confined to skin manifestations consists of topical treatments and UV therapy to mitigate the hyperproliferative epidermal reaction. Topical corticosteroids are still the most frequently prescribed class of therapeutic agents for the condition (Wu et al. 2016). Vitamin D analogues and topical retinoids complement this treatment approach (Wu et al. 2004). Moderate to severe cases may require systemic treatment and mainly rely on oral agents that act as immunosuppressants or, more recently, antibody-based selective targeting of key mediators of inflammation (Nestle et al. 2009).

With very few exceptions, psoriasis is unique to humans. The lack of a suitable animal model has hindered research in the past. Transgenic, knock-out, and reconstituted models of psoriasis offer an approximation of the disease (Gudjonsson et al. 2007; Schon 2008). Those models support the immunologic nature of psoriasis (Gudjonsson et al. 2007). A pharmacologic model has developed as a spin-off from the use of imiquimod (IMQ), a potent immune activator used for topical treatment of skin lesions caused by human papilloma virus, precancerous keratosis, and basal cell carcinomas (Beutner and Tying 1997; Geisse et al. 2004). Topical application of IMQ induces and exacerbates psoriasis in humans and provokes a skin disease in wild-type mice that closely resembles human psoriasis. The lesions appear at the site of IMQ application and also at distant skin sites, suggesting a systemic immunologic effect (Fanti et al. 2006; Rajan and Langtry 2006; Wu et al. 2004). Advantages of the IMQ model lie in its very quick response with a 100% incidence of psoriatic-like skin changes within 2 to 4 days with very little in-group variations (van der Fits et al. 2009). Thus, we chose that model for this short-term study.

The 70-kD heat shock proteins, both constitutive and inducible forms, are known to be potent immunomodulators, with antagonistic activities, either promoting or down-regulating immune responses, depending on the context in which they appear in the extracellular space (reviewed in van Eden et al. 2012 and Calderwood et al. 2016). We hypothesized the anti-inflammatory effect would predominate if a relatively high concentration of aHsp70 was applied to psoriatic-like lesions in the form of a topical skin cream. We used a skin cream containing a mixture of constitutive and inducible Hsp70 isolated from the

alfalfa plant (*Medicago sativa*), a relatively inexpensive source allowing production of large amounts more easily than recombinant protein. Thus, it has great potential to be translated into a human therapeutic agent.

Methods

Alfalfa-derived Hsp70 skin cream

The alfalfa-derived Hsp70 (aHsp70) was purified to at least 95% from alfalfa by Alfa Biogene International using procedures described in two published patents (Bukau and Mayer 2005; Seifarth and Lax 2014). It contains a mixture of the constitutive and inducible forms (Alfa Biogene International pers. comm.). It was mixed at either 50 or 250 μ g/ml into a conventional skin cream consisting of glycerol monostearate, cetyl alcohol, Miglyol 812, vaseline, macrogol-20-glycerol monostearate, propylene glycol, and water.

Mice and treatments

BALB/c mice were purchased from Charles River Laboratories. They were kept under specific pathogen-free conditions and provided with food and water ad libitum. All experiments were approved by the institutional animal care and use. At 8 to 11 weeks of age, the mice were anesthetized by ketamine/xylazine (100/15 mg/kg body weight) and their backs were shaved using a conventional Wahl electric hair clipper. Then, at 12:00 PM each day, they received a daily topical dose of 62.5 mg of commercially available IMQ cream, 5% (E. Fougera & Co, Melville, NY) on the dorsal shaved area and the right ear for five or six consecutive days each morning, translating in a daily dose of 3.125 mg of the active compound. This dose was empirically determined to cause most optimal and reproducible skin inflammation in mice (van der Fits et al. 2009). Control mice were treated similarly (shaving, measurements) but without any cream application. Mice in the aHsp70 treatment groups received twice daily topical applications of 375 mg of a skin cream containing 50 μ g/ml or 250 μ g/ml Hsp70 that provided 20 or 100 μ g Hsp70 per dose. The cream formed a 1-mm thick layer on a 2 $\times$ 2-cm area. These treatments occurred at 8:00 AM and 6:00 PM. Control mice were treated with the carrier skin cream according to the same schedule.

Treatment group details

Five animals each were assigned randomly to one of the following groups, except for group 5, the untreated controls, which had two mice:

1. Treated for 6 days with one daily application of 62.5 mg commercially available IMQ (on back, neck, and right

- ear) and twice daily with a 1-mm thick layer of 50 µg/ml aHsp70-containing cream (on back, neck, and right ear)
2. Treated for 6 days with one daily application of 62.5 mg commercially available IMQ (on back, neck, and right ear) and twice daily with a 1-mm thick layer of 250 µg/ml aHsp70-containing cream (on back, neck, and right ear)
 3. Treated for 6 days with one daily application of 62.5 mg commercially available IMQ (on back, neck, and right ear) and twice daily with a 1-mm thick layer of the carrier cream without aHsp70 (on back, neck, and right ear)
 4. Treated for 6 days on back, neck, and right ear with one daily application of 62.5 mg commercially available IMQ
 5. Control group, no cream application

Once daily, all animals were examined for behavioral changes and any other signs of adverse effects. Ear pinna thickness measurements were performed, and skin changes assessed by the modified Psoriasis Area and Severity Index (PASI) score. Photographs were taken of all the animals' backs to document the reactions.

In vivo evaluation protocol

Day 1 Backs of shaved mice were photographed. Initial evaluations by a dermatologist were made for visible signs of psoriasis-like symptoms. All visible signs were categorized and scored by a modified PASI score from 0 to 4 (erythema, scales, and thickness. Maximal cumulative score, 12 points). Thicknesses of both ear pinnae were measured in triplicate with a micrometer (Digimax, Brütsch-Rügger Tools, Switzerland). Cream application was performed as described above for each group.

Days 2–6 Once daily, all animals were examined for any signs of distress. Ear pinna thickness measurements were made in triplicate with a micrometer, and skin changes assessed by the modified PASI score (see the next section below). Photographs were taken of all the animals' backs to document the reactions.

Day 7 Mice were euthanized by cervical dislocation after inducing deep anesthesia with ketamine/xylazine (300/30 mg/kg bw). Skin tissue samples from the treated areas were processed for cytokine expression as described below in the section on PCR.

Scoring skin inflammation

To score the severity of inflammation of the skin, an objective scoring system was developed based on the clinical PASI. Erythema, scaling, and thickening were scored independently by three blinded persons on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The level of erythema was scored by using a table with red spots for reference. The cumulative scores (erythema plus scaling plus

thickening) served as a measure of the severity of inflammation (scale 0–12).

On the days indicated in the results, the pinna thicknesses of the left and right ears were measured in triplicate using the micrometer.

Real-time quantitative PCR

Total mRNA was extracted from whole biopsies from the back skin euthanized mice using the GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), and each sample was treated with DNase I amplification Grade (Sigma-Aldrich). cDNA was prepared using TaqMan PCR Core Reagents Kit (Applied Biosystems) using 0.5 µg of total RNA template. PCR primers were spanning at least one intron/exon boundary. Sequences for the PCR primers (Integrated DNA Technologies) were as follows:

- IL-4, forward primer, 5'-GGT CTCA ACC CCC AGC TAG T-3'; reverse primer, 5'-GCC GAT GAT CTC TCT CAA GTG AT-3'
- IL-5, forward primer, 5'-CTC TGT TGA CAA GCA ATG AGA CG-3'; reverse primer, 5'-TCT TCA GTA TGT CTA GCC CCT G-3'
- IL-6, forward primer, 5'-TAG TCC TTC CTA CCC CAA TTT CC-3'; reverse primer, 5'-TTG GTC CTT AGC CAC TCC TTC-3'
- IL-27, forward primer, 5'-CTG TTG CTG CTA CCC TTG CTT-3'; reverse primer, 5'-CAC TCC TGG CAA TCG AGA TTC-3'
- GAPDH, forward primer, 5'-AGG TCG GTG TGA ACG GAT TTG-3'; reverse primer, 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'
- IL-17A, forward primer, 5'-TTT TCA GCA AGG AAT GTG GA-3'; reverse primer, 5'-TTC ATT GTG GAG GGC AGA C-3'
- IL-17F, forward primer, 5'-CAA GAA ATC CTG GTC CTT CG-3'; reverse primer, 5'-GAG CAT CTT CTC CAA CCT GAA-3'
- IL-22, forward primer, 5'-TTT CCT GAC CAA ACT CAG CA-3'; reverse primer, 5'-CTG GAT GTT CTG GTC GTC AC-3'
- IL-23, forward primer, 5'-CAC CTC CCT ACT AGG ACT CAG C-3'; reverse primer, 5'-TGG GCA TCT GTT GGG TCT-3'

IL-4, IL-5, IL-6, IL-17A, IL-17F, IL-22, IL-23, and GAPDH mRNA levels were measured by real-time quantitative PCR analysis using the StepOne Real time PCR system (Applied Biosystems). GAPDH was used as the internal control for each run (treatments and untreated control in triplicate) to correct cytokine mRNA levels between the groups. The data is expressed as the average fold change of mRNA in the

treatment group compared to respective mRNA from untreated control group.

Statistics

Data are shown as means $\pm$ s.e.m. and were compared by unpaired *t* tests.

Results

Effects of aHsp70-containing skin cream on IMQ induction of psoriatic-like inflammation

The aHsp70-containing cream clearly reduced the IMQ-induced psoriatic-like reaction of the skin, as shown in the images of the backs of the mice in Fig. 1 and by the PASI scores shown in Fig. 2. However, the assessment of this anti-inflammatory activity of the aHsp70 cream was complicated by the facts that the carrier cream by itself had a beneficial effect (Figs. 1 and 2) and that the effects of the aHsp70 were not proportional to the dose. For the PASI scores, the lower concentration of aHsp70 was significantly more effective than the higher dose and the carrier cream alone. The 250- μ g/ml aHsp70 cream showed a beneficial trend in PASI score compared to the carrier cream alone, but was not statistically better (Fig. 2). Similarly, pinna edema was reduced by the higher concentration of Hsp70, but was not statistically better than the carrier cream (Fig. 3). No symptoms of distress were noted in the treated mice.

Effects of aHsp70-containing skin cream on IMQ-induced changes in inflammation-related cytokines

We also examined treatment-related changes in the expression of several cytokines known to be altered in psoriasis to gain

Fig. 1 Examples of the typical gross skin responses to the 50- and 250- μ g aHsp70-containing creams and the carrier cream in comparison to the IMQ-only treatment. The skin of all the cream-treated mice was noticeably less hyperkeratotic and inflamed than that of the IMQ-only mice

Fig. 2 Cumulative PASI scores of mice in each of the treatment groups. Both the 50- and 250- μ g aHsp70-treated groups had significantly lower scores than the IMQ-only group. The 50- μ g aHsp70 group score was significantly lower than all the others. The carrier cream group also was significantly lower than IMQ alone. ***p* = 0.005, ****p* = 0.0003, *****p* = 0.0001

some insight into the molecular bases for the anti-inflammatory effects observed. Quantitative PCR analysis of the RNA isolated from the dorsal skin samples revealed complex responses to the aHsp70 treatments on IMQ-induced expression. mRNA levels of IL-4, IL-5, IL-6, IL-17A, IL-17F, and IL-22 significantly altered (Fig. 4a–e). IL-6 and IL-23 were unaffected (not shown). Although application of aHsp70 cream showed changes in gene induction consistent with the observations of the gross effects on the appearance of the skin and pinna edema, in some cases, carrier cream alone also had significant effects on IMQ-induced gene expression (Fig. 4b, e). Both the 50- and 250- μ g/ml creams significantly

Fig. 3 Pinna thickness on day 6 after the last treatment. The 250- μ g aHsp70 group was less edematous than the IMQ-only group (**** $p = 0.0001$), as was the carrier cream group (* $p = 0.01$)

lowered IL4 and 5, but only the 50- μ g/ml cream lowered IL-17F expression (Fig. 4a, b, d). Unexpectedly, the 50- μ g/ml aHsp70 cream elevated IL-17A and both doses of aHsp70 cream

increased the expression of IL-22 (Fig. 4c, e). The carrier cream also reduced IL-5 and increased IL-22 (Fig. 4b, e). Only for IL-5 were the effects of the two aHsp70 creams significantly greater than those of carrier cream.

Discussion

This short-term study provides proof of principle for the potential of topically applied aHsp70 to reduce skin inflammation in a mouse model of pharmacologically induced psoriasis-like lesions. Together with the recent report that this aHsp70 preparation administered intranasally improved insulin sensitivity in a mouse model of glucose intolerance (Tytell et al. 2017), the observations suggest that aHsp70 has broad therapeutic potential. The design of this test of the potential of the aHsp70 skin cream to prevent or reduce the psoriatic-like inflammation caused by IMQ required that the latter be applied daily to maintain inflammation while the aHsp70 cream was applied. If IMQ was applied first for a week and then stopped, the skin inflammation resolved on its own too quickly to test whether the aHsp70 cream promoted healing after

Fig. 4 Changes in expression of seven psoriasis-related interleukins. **a** IL-4 expression was lower for both 50- and 250- μ g aHsp70-treated groups (**** $p = 0.0001$ and *** $p = 0.0003$). **b** IL-5 expression was lower for both the 50- and 250- μ g aHsp70-treated groups compared to that for IMQ only (* $p = 0.02$ and ** $p = 0.005$) and compared to that for the carrier cream-treated group (* $p = 0.002$). The carrier cream group also had lower

IL-5 expression compared to the IMQ-only group (* $p = 0.02$). **c** IL-17A was elevated in 50- μ g aHsp70-treated group relative to the IMQ-only group (* $p = 0.01$). **d** IL-17F was lower in the 50- μ g aHsp70-treated group compared to that in the IMQ-only group (* $p = 0.04$). **e** IL-22 was elevated in both the aHsp70-treated groups (** $p = 0.005$ and * $p = 0.04$) and in the carrier cream group relative to IMQ only (* $p = 0.04$)

IMQ treatment (unpublished observations). We suspect that the carrier cream may have diluted the IMQ, lowering the IMQ concentration on the skin compared to the mice that received IMQ only. Alternatively, it may have slowed the absorption of the IMQ into the skin. Despite these caveats, the 50- μ g/ml aHsp70 was significantly better than the control cream in reducing the PASI score (Fig. 2), and the reduction in IL5 by the 50- and 250- μ g/ml aHsp70 creams was significantly different from that of the carrier (Fig. 4b). This cytokine is a key player in eosinophil maturation and activation and may play an important pathogenic role in the development not only of skin inflammation (Yawalkar et al. 2000), but also of airway inflammation (Farne et al. 2017). Recently, one report showed that Hsp70 suppresses inflammatory responses in astrocytes by down-regulating TNF- α and IL-1 β (Yu et al. 2018), which are both also critically involved in the pathogenesis of psoriasis (Baliwag et al. 2015).

The changes in cytokine expression shown in Fig. 4 emphasize further the complexity of the effects aHsp70 and the carrier cream. IL-4 and 5 expressions were reduced by both aHsp70 doses (Fig. 4a, b), and for IL-5, both doses were significantly more effective than the carrier cream (Fig. 4b). Additionally, IL-17F expression was significantly reduced by the lower dose of aHsp70 (Fig. 4d). How they relate to the increases of IL-17A and 22 remains unclear (Fig. 4). The latter two cytokines are involved with clearance of extracellular bacteria and therefore, may have cytoprotective effects (reviewed in Sonnenberg et al. 2010 and Jin and Dong 2013). Additionally, IL-22 can have cytoprotective activity that is enhanced in the absence of IL-17 (Sonnenberg et al. 2010). That observation may be related to the rise in IL-22 expression coincident with the decline in IL-17F expression that we detected. However, the interplay between IL-17A and F in regulation of immune responses is complex and not fully understood (Jin and Dong 2013). Furthermore, the regulatory interplay between extracellular aHsp70 and cytokine expression is complicated as well (Calderwood et al. 2016). Imiquimod induces its psoriasis-like phenotype by activating TLR 7 and TLR8 (van der Fits et al. 2009), and it is possible in our mouse model that aHsp70 interfered with and inhibited psoriasis-specific signaling of these TLRs.

Recently, studies in plants have shown that the chaperoning functions of Hsps can help maintain the integrity of the pattern recognition receptors (PRRs) and protect against invading microbes (Park and Seo 2015). Mammalian Hsp70 or peptides bound to Hsp70 can physically interact with PRRs (toll-like receptors, TLR2 and TLR4) and induce tumor cell proliferation by activating JNK1/2/MAPK pathway (Zhe et al. 2016). However, exogenously applied Hsp70 also shows potent antitumorigenic properties by sensitizing cytotoxic immune cells both in in vitro and in vivo settings (Guzhova and Margulis 2016). Since psoriasis is also an immune-mediated disease involving the increased keratinocyte proliferation,

aHsp70 may reduce psoriasis pathogenesis by modulating T cell activation and/or specificity. Further studies are needed to understand the underlying mechanisms of extracellular aHsp70 mitigation of psoriasis-like skin diseases.

Besides the potential direct effects of aHsp70 on cytokines, it is likely also that the skin cream application together with aHsp70 altered the skin microbiome in a way that contributed to the reduced inflammation and the pattern of cytokine changes that we observed. Such microbiome changes may account for the limited effects of the carrier cream alone on the PASI score, pinna edema, and IL-5 and 22. The role of the unique skin microbiome in chronic skin diseases like psoriasis has been a focus of more recent research (reviewed in Gallo and Nakasugi 2011). To our knowledge, the effects of topical Hsp70 or other Hsps on skin physiology have not been investigated, although there is support for their importance in wound healing (Mushtaq et al. 2011; Bhatia et al. 2018). Future work on potential therapeutic uses of topical aHsp70 will need to examine directly its effects on commensal and pathogenic skin bacteria in inflammatory conditions.

Funding information This research was supported by the Cleveland Clinic (Cleveland, OH), National Psoriasis Foundation (discovery grant 2016 to U. C.) and Alfa Biogene International, B.V. (Baarn, Netherlands).

Compliance with ethical standards

All experiments were approved by the institutional animal care and use.

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