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Activation of an immune-regulatory macrophage response and inhibition of lung inflammation in a mouse model of COPD using heat-shock protein alpha B-crystallin-loaded PLGA microparticles

Johannes M. van Noort [a,*], Malika Bsibsi [a], Peter J. Nacken [a], Wouter H. Gerritsen [b], Sandra Amor [b,c], Inge R. Holtman [d], Erik Boddeke [d], Ingrid van Ark [e], Thea Leusink-Muis [e], Gert Folkerts [e], Wim E. Hennink [f], Maryam Amidi [f]

[a] Delta Crystallon BV, Zernikedreef 9, 2333 CK Leiden, The Netherlands
[b] Department of Pathology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands
[c] Department of Neuroscience and Trauma, Blizard Institute, Barts and The London School of Medicine & Dentistry, Queen Mary University of London, E1 2AT London, United Kingdom
[d] Department of Medical Physiology, University Medical Centre Groningen, 9713 AV Groningen, The Netherlands
[e] Division of Pharmacology, Institute for Pharmaceutical Sciences, University Utrecht, 3508 TB Utrecht, The Netherlands
[f] Department of Pharmaceutics, Institute for Pharmaceutical Sciences, University Utrecht, 3508 TB Utrecht, The Netherlands

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ABSTRACT

As an extracellular protein, the small heat-shock protein alpha B-crystallin (HSPB5) has anti-inflammatory effects in several mouse models of inflammation. Here, we show that these effects are associated with the ability of HSPB5 to activate an immune-regulatory response in macrophages via endosomal/phagosomal CD14 and Toll-like receptors 1 and 2. Humans, however, possess natural antibodies against HSPB5 that block receptor binding. To protect it from these antibodies, we encapsulated HSPB5 in porous PLGA microparticles. We document here size, morphology, protein loading and release characteristics of such microparticles. Apart from effectively protecting HSPB5 from neutralization, PLGA microparticles also strongly promoted macrophage targeting of HSPB via phagocytosis. As a result, HSPB5 in porous PLGA microparticles was more than 100-fold more effective in activating macrophages than free soluble protein. Yet, the immune-regulatory nature of the macrophage response, as documented here by microarray transcript profiling, remained the same. In mice developing cigarette smoke-induced COPD, HSPB5-loaded PLGA microparticles were selectively taken up by alveolar macrophages upon intratracheal administration, and significantly suppressed lung infiltration by lymphocytes and neutrophils. In contrast, 30-fold higher doses of free soluble HSPB5 remained ineffective. Our data indicate that porous HSPB5-PLGA microparticles hold considerable promise as an anti-inflammatory biomaterial for humans.

1. Introduction

Over the past decade, it has become clear that macrophages can be activated in fundamentally different ways [1–3]. Apart from classical activation which leads to a pro-inflammatory host-defense response, alternative states of macrophages activation promote resolution of inflammatory processes and tissue repair instead. These different states of activation play key roles in driving the natural evolution of immune responses to a final stage of repair. The possibility to selectively manipulate macrophage activation in order to promote resolution of chronic or exaggerated inflammation is of obvious clinical interest. Different from traditional anti-inflammatory drugs which may indiscriminately block both harmful and beneficial macrophage responses, agents that trigger an immune-regulatory macrophage response without compromising antimicrobial activities could well offer an improved side-effect profile [4]. Packing such agents into a biodegradable carrier to promote selective macrophage targeting would offer even further potential advantages.

Systemic administration of HSPB5 to mice prevents experimental neuroinflammation [5], and ameliorates experimental autoimmune encephalomyelitis [6], ischemic optic neuropathy [7],

* Corresponding author. Tel.: +31 8886 9696; fax: +31 71 518 1927.
E-mail address: hans@deltacrystallon.com (J.M. van Noort).

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ischemia-reperfusion injury [8], and experimental stroke [9]. So far, HSPB5's mode of action in suppressing inflammatory damage in a variety of different organs has remained unclear. In this study, we examined whether macrophage activation by HSPB5 could play a role in its anti-inflammatory effects by characterizing the macrophage response using microarray transcript profiling. In addition, we examined whether such activation could be enhanced by incorporating HSPB5 in porous PLGA microparticles to promote macrophage uptake. Finally, we evaluated the impact of HSPB5-loaded PLGA microparticles on cigarette smoke-induced lung inflammation during experimental COPD in mice.

2. Materials and methods

2.1. HSPB5

For all experiments, we used sterile clinical-grade recombinant human HSPB5 (Delta Crystallon BV, Leiden, The Netherlands), containing 5 ng/mg (0.0005%) *E. coli* proteins, <0.7 EU/mg endotoxins, and <75 pg/mg bacterial DNA. The fraction of HSPB5 that was contained in the appropriate multimeric (>400 kDa) complexes was 99.8%. As previously documented [10], the preparation did not activate HEK293 reporter cells expressing either of the individual human TLRs 1–10 (without CD14) at concentrations up to 200 μg/mL, confirming the absence of confounding bacterial contaminants.

2.2. Preparation and characterization of HSPB5-PLGA microparticles

HSPB5-loaded PLGA microparticles were prepared by a w/o/w double-emulsion solvent evaporation method [11]. A volume of 300 μL of 12.5 mg/mL HSPB5 in phosphate-buffered saline (PBS) was emulsified in 3 mL dichloromethane (DCM; Biosolve, Valkenswaard, The Netherlands) containing 10% (w/v) poly (lactic-co-glycolic acid) (PLGA; 50:50; intrinsic viscosity 0.5 dl/g; Purac, Gorinchem, The Netherlands) using an Ultra-Turrax® homogenizer (IKA Labortechnik, Staufen, Germany) at 30,000 rpm for 1 min to form a water-in-oil (w/o) emulsion. This w/o emulsion was emulsified in 30 mL of a sterile 5% (w/v) solution of polyvinyl alcohol (PVA; M_w 30,000–70,000; 88% hydrolyzed; Sigma–Aldrich, St. Louis, MO) in 0.9% (w/v) NaCl at 30,000 rpm for 2 min. DCM was removed under vacuum for 1 h at room temperature under continuous rotation. Microparticles were collected by centrifugation at 12,000 g for 30 min, and washed twice with water. One part of the pellets was resuspended in PBS and immediately tested for protein release as described below, while a second part was lyophilized and stored at −20 °C prior to further characterization. Empty PLGA particles as a control were prepared in an identical manner, but using PBS instead of the HSPB5 solution.

The volume mean diameter of HSBP5-loaded PLGA particles was determined using an Accusizer™ 780 (Optical Particle Sizer, Santa Barbara, CA). Morphology was evaluated by scanning electron microscopy (Phenom™, FEI Company, Hillsboro, OR). The loading efficiency was determined by dissolving microparticles in 17% (v/v) dimethyl sulfoxide, 40 mM NaOH and 0.4% (w/v) sodium dodecyl sulfate overnight at 37 °C under mild agitation, followed by a standard bicinchoninic acid protein assay, using an empty batch of microparticles as a control to correct for possible interferences by the polymer. Protein release rates for freeze-dried and non-freeze-dried particles were separately evaluated by incubating the particles in PBS at 37 °C under continuous rotation, and determining concentration of HSPB5 in the supernatant after centrifugation at 18,000 g for 10 min by ultra performance liquid chromatography (Acquity UPLC®; Waters, Etten-Leur, The Netherlands).

2.3. Analysis of cellular responses to HSPB5 and HSPB5-PLGA microparticles

THP-1 cells differentiated for 48 h with 50 ng/mL phorbol-12-myristate 13-acetate (Sigma–Aldrich, St. Louis, MO) were tested at 1×10^6 cells/mL in RPMI 1640 medium plus additives. HSPB5-induced TNF-α levels in the culture supernatant were determined using a commercially available ELISA (Sanquin, Amsterdam, The Netherlands). For knockdown experiments, THP-1 cells were transfected with FlexiTube siRNAs (Qiagen, Hilden, Germany) using Lipofectamine™ 2000 (Invitrogen, Carslbad, CA). Adequate suppression of transcripts was verified using QuantiTect® Primer assays and SYBR®Green (Qiagen).

Human monocyte-derived macrophages were obtained by isolating $CD14^+$ monocytes from peripheral blood-mononuclear cells (PBMC) using EasySep® magnetic beads (Stemcell Technologies, Grenoble, France), and differentiating these at 1.5×10^6 cells/mL with 50 ng/mL M-CSF (Preprotech, Rocky Hill, NJ) for 5–6 days. Release of either TNF-α, IL-10, IL-1β or PXT3 into the culture supernatant was determined by ELISA. Human embryonic kidney 293 (HEK293) cells were stably transfected with different pattern-recognition receptors, and tested for responsiveness to HSPB5 using the secreted alkaline phosphate reporter system (Invivogen, San Diego, CA). Blocking experiments with antibodies against different pattern-recognition receptors were performed using 1 μg/mL of various blocking antibodies, added 60 min prior to stimulation.

Proliferative human T-cell responses were determined by evaluating the dilution of 5(6)-carboxyfluorescein succinimidyl ester (CFSE) in PBMC from healthy blood donors (Sanquin, Leiden, the Netherlands). PBMC were labeled with 1 μM CFSE, seeded at 2×10^5 cells/well in RPMI 1640 medium plus additives, and cultured for 9 days in the presence of 0.2 μg/mL tetanus toxoid antigen. Prior to flow cytometry, cells were additionally labeled with antibodies to CD45RO (Becton Dickinson, Franklin Lakes, NJ).

Cellular response data shown are representative for at least three independent experiments in all cases.

2.4. Microarray transcript profiling

Total macrophage RNA was isolated in 0.75 mL of TRIzol®, and extracted according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). RNA integrity and concentrations were measured on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a Nanodrop spectrophotometer ND-1000 (Fisher Scientific, Waltham, MA). All samples were of high quality with an RNA Integrity Number (RIN-value) between 7.6 and 8.7 and 260/280 ratios greater than 2.00. Samples of 500 ng total RNA were used as input for amplification and labeling with the Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA), according to the manufacturer's guidelines including control spikes. Labeled RNA was purified using the RNeasy Mini Kit (Qiagen) yielding 7.5 μg or more of labeled cRNA and specific activities greater than 15.3 pg Cye3 dye/μg cRNA and 18.9 pg Cy5 dye/μg cRNA. Labeled samples were hybridized onto whole human genome GE 4×44K GF4112F microarrays according to the manufacturer's protocol (Agilent Technologies, Palo Alto, CA). Scanning was performed using a microarray scanner G2505C (Agilent Technologies) and Feature Extraction v10.7 using the manufacturers protocols (Agilent Technologies). The microarray data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (GEO, database number: GSE30658) (http://www.ncbi.nlm.nih.gov/geo).

2.5. Effects of HSPB5-PLGA particles on smoke-induced lung inflammation

Male BALB/c mice, 5–6 weeks old were obtained from Charles River Laboratories (Someren, The Netherlands) and housed under controlled conditions in standard laboratory cages. They were provided free access to water and food. All experimental protocols were approved by a local ethics committee, and were performed in compliance with national legislation and international guidelines on animal experimentation. Groups of mice ($n = 6$, 7 or 8) were exposed in whole-body chambers to air or to diluted mainstream cigarette smoke from the reference cigarettes 2R4F (University of Kentucky, Lexington, KY) using a peristaltic pump. Immediately before the experiments, filters were removed from the cigarettes. Each cigarette was smoked in 5 min at a rate of 5 L/h in a ratio with 60 L/h air. Mice were exposed to cigarette smoke using 5 cigarettes twice daily for five consecutive days, except for the first day when they were exposed to 3 cigarettes. Free soluble HSPB5 or HSPB5-PLGA microparticles were administered intratracheally at the indicated doses in a volume of 50 μL sterile PBS twice daily. Mice were sacrificed 16 h after the last smoke exposure, and lungs were lavaged four times with pre-warmed 1 mL saline. Cells were collected by centrifugation of 4 consecutive lavages at 400 g for 5 min at 4 °C. After staining with Türk solution, macrophage, neutrophil, and lymphocyte counts were determined by light microscopy based on scoring at least 200 cells in each sample. Differential cell counts were additionally performed on cytospin preparations stained by DiffQuick™ (Dade A.G., Düdingen, Switzerland).

2.6. Statistical analyses

All error bars indicate standards of deviation. Cellular counts in murine bronchoalveolar lavages ($n = 6$–7) were analyzed by one-way analysis of variance. In all cases, $P < 0.05$ was considered significant. Preprocessing and analysis of microarray data ($n = 12$) were performed with R [12] and BioConductor package Limma [13]. Background correction was done with the normexp function with an offset value of 50. Global Loess within array normalization and quantile between array normalization were performed [14]. Array quality weight was used to fit a heteroscedastic model to the expression values for each gene and was incorporated into the linear model approach to find differentially expressed genes [15]. A Benjamini Hochberg [16] corrected P-value of <0.05 and a fold change >1.5 were used as cut-off to determine significance.

3. Results

3.1. Macrophage receptors for HSPB5

The mechanism responsible for the well-documented anti-inflammatory effects of HSPB5 in animal models of inflammation has so far remained obscure. Given accumulating evidence that