Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ljii20

Anti-Inflammatory Activity in the Low Molecular Weight Fraction of Commercial Human Serum Albumin (LMWF5A)

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Accepted author version posted online: 11 May 2015.

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ABSTRACT

The innate immune system is increasingly being recognized as a critical component in osteoarthritis (OA) pathophysiology. An *ex vivo* immunoassay utilizing human peripheral blood mononuclear cells (PBMC) was developed in order to assess the OA anti-inflammatory properties of the low molecular weight fraction (<5kDa) of commercial human serum albumin (LMWF5A). PBMC from various donors were pre-incubated with LMWF5A before LPS

stimulation. TNFα release was measured by ELISA in supernatants after an overnight incubation. A ≥ 30% decrease in TNFα release was observed. This anti-inflammatory effect is potentially useful in assessing potency of LMWF5A for the treatment of OA.

Keywords osteoarthritis, tumor necrosis factor α**,** peripheral blood mononuclear cells, lipopolysaccharide, human serum albumin

INTRODUCTION

Osteoarthritis (OA) is the most common chronic disease of the joint, affecting an estimated 40% of the population over the age of 60.[1,2] Whether it is the result of an aging population or an increase in obesity, some studies suggest that OA has become so prevalent that everyone over the age of 65 shows clinical or radiologic evidence of OA.[3] Traditionally considered a degenerative disease, it is now widely accepted that inflammation is a critical feature of OA.[4] In patients with active OA, elevated serum levels of various pro-inflammatory cytokines and chemokines (TNFα, IL-6, and RANTES) were measured.[5] Additionally, soluble macrophage biomarkers (CD14 and CD163) support the central role of inflammation as determinants of OA severity, progression risk, and clinical symptoms.[6] The production of these inflammatory mediators is believed to drive the production of catabolic proteases that erode cartilage, ultimately leading to the functional failure of the joint.[7]

Experiments previously conducted in our laboratory demonstrated that the low molecular weight fraction (<5kDa) of commercial human serum albumin (LMWF5A) possesses anti-inflammatory properties. Pro-inflammatory cytokine release from peripheral blood mononuclear

cells (PBMC) and T-cell lines stimulated through the T-cell receptor complex was inhibited by the presence of the LMWF5A.[8] This activity was lost following dialysis of the product, implicating the presence of anti-inflammatory component(s) in the LMWF5A. The known components of LMWF5A include the cyclic compound derived from the N-terminus of HSA, aspartate-alanine diketopiperazine (DA-DKP).[9] Also, the low molecular weight stabilizers, N-acetyl tryptophan (NAT) and sodium caprylate, are both added to commercial HSA solutions. As a result, NAT degradation products have also been identified in commercial HSA solutions.[10] Finally, various peptides derived from non-HSA sources have been characterized in commercial HSA solutions.[11,12] Therefore, it is conceivable that any combination of known and unknown small molecular weight components of commercial HSA contributes to the anti-inflammatory activity found in the LMWF5A.

Based on the laboratory evidence confirming the presence of anti-inflammatory activity in the <5kDa HSA fraction, a prepared LMWF5A is currently under development as a therapeutic for OA of the knee. In a recently completed clinical trial involving a total of 329 randomized patients, it was found that a single intra-articular (IA) injection of LMWF5A resulted in a significant 42.3% reduction in pain that was observed at 4 weeks post-injection and persisted to the completion of the trial at week 20 versus saline controls.[13] The afforded relief was the most pronounced in patients with severe OA (Kellgren Lawrence grade IV) with almost double the pain reduction upon LMWF5A treatment as compared to lower grades. On the other hand, there was no significant difference in the occurrence of adverse effects between the arms of the trial. Based on these observations, LMWF5A was found to be both safe and effective for the treatment and intervention of those suffering from moderate to severe OA of the knee.

An assay measuring a pro-inflammatory biomarker important in the progression of OA, tumor necrosis factor alpha (TNFα), was developed in a cell culture model using LPS-stimulated PBMC from various donors.

EXPERIMENTAL

Materials

Cell culture reagents were purchased from Gibco, Life technologies (Grand Island, NY). 5% human serum albumin (HSA) from Octapharma (Hoboken, NJ) was used for <5kDa filtrate collection. All other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Collection of LMWF5A

LMWF5A was isolated by Sypharma Pty Ltd. (Dandenong, Victoria, Australia) using a tangential flow filtration (TFF) process with a 5kDa MWCO PVDF filter membrane (Sartorius Stedim Biotech GmbH, Germany). In accordance with cGMP guidelines, the isolation process involved the removal of the >5kDa component (primarily HSA) and the aseptic filling of sterile glass vials with 10mL of the LMWF5A. Each vial was sealed with a rubber stopper and a proper metal closure. The vials were stored in the dark at ambient temperature.

Preparation of PBMC

Isolated human peripheral blood mononuclear cells (PBMC) from 13 donors were purchased from Astarte Biologics (Redmond, WA). PBMC stocks were thawed and then

transferred drop wise to 9mL of thaw medium (RPMI-1640 medium containing 10% human AB serum, 20 units/mL DNase, and 1% Pen/Strep) and centrifuged at 1,000Xg for 10 minutes.

TNFα Release Assay

The LMWF5A and working dilutions of control compounds were prepared in saline (0.2 µM dexamethasone and mifepristone). 50µL of the resulting solutions were added in quadruplicate to a 96 well U bottom tissue culture plate and incubated at 37°C/5% CO_2 for an hour. An equal volume of PBMC ($1X10^6$ cells/mL) suspended in growth medium (RPMI + 20% fetal bovine serum, 2% Pen/strep, 1% l-glutamine, 1% sodium bicarbonate, 1% sodium pyruvate, 1% NEAA) was then added to the wells. After an hour incubation, LPS was added at final concentrations between 1 and $1X10^7$pg/mL. After 18 hours, the plate was centrifuged at 1,000Xg for 10 minutes, and TNFα release was determined in the supernatants of each well by ELISA (Thermo Scientific, Rockford, IL).

LCMS Analysis of LMWF5A

For the LCMS analysis of DA-DKP content in the LMWF5A, each filtrate (1:100 dilution) and DA-DKP synthetic standard (20-2000 ng/mL; manufactured by Syngene Int Ltd, India) were spiked with an internal standard (0.01mM L-Tryptophan-d5 (indole-d5)). 50µL was injected into an anion exchange column (Spherisorb, S5 SAX 250 mm x 4.0 mm, Waters, Milford, MA, USA) connected to HPLC (Waters 2795 Separations Module, Milford, MA, USA) coupled to a mass spectrometer (LCT-TOF, Micromass, UK). A ternary mobile phase consisting of dH_2O, methanol, and 200mM ammonium formate (pH 5.4) was used at a flow rate of

0.5mL/min following the gradient listed in Table 1. The output of the HPLC was split 1:20 (v/v) and injected into the mass spectrometer using negative electrospray ionization (-ESI MS) with a scan range of 80 to 1000 m/z, cone voltage of 30 eV, source temperature of 100°C, and gas temperature of 300°C. DA-DKP was measured by monitoring [M-] = 185.

For the LCMS analysis of NAT and sodium caprylate content in the LMWF5A, each filtrate (2X dilution) and NAT/caprylate standard contained an internal standard (0.1mM L-Tryptophan-d5 (indole-d5)). 10µL was injected into a C-18 column (Xterra, 100mm x 2.1 mm, Waters, Milford, MA, USA) connected to HPLC (Waters 2795 Separations Module, Milford, MA, USA) coupled to a mass spectrometer (LCT-TOF, Micromass, UK). A mobile phase consisting of 0.1% trifluoroacetic acid in dH_2O and 0.1% TFA in acetonitrile was used at a flow rate of 0.4mL/min following the gradient listed in Table 2. The output of the HPLC was split 1:20 (v/v) and injected into the mass spectrometer using positive electrospray ionization (+ESI MS) with a scan range of 80 to 1000 m/z, cone voltage of 3 eV, source temperature of 100°C, and gas temperature of 300°C. NAT was measured by UV at 280nm. Sodium caprylate was measured by monitoring [M+] = 145.

Data Analysis

To calculate percent decreases in cytokine release, the following formula was employed: ((LPS saline control [TNFα]- LPS treatment [TNFα])/LPS saline control [TNFα])*100. If cells release a significant amount of TNFα (within detection limit) in the untreated PBS control, then the amount of released TNFα from stimulated cells was subtracted from the untreated TNFα release from all concentrations prior to calculation. A paired Student t-test was applied to data

sets (Microsoft Excel; Redmond, WA) with statistical significance accepted at $p \leq 0.05$. Distributions and box plots were generated using BoxPlotR (http://boxplot.tylerslab.com).[14]

RESULTS

LMWF5A Reduces TNFα Release

The primary goal of this investigation was to determine if LMWF5A filtrates collected from commercial 5% HSA solutions possess anti-inflammatory activity. Isolated human PBMC from human donors (n=13) were pre-incubated with two sources of LMWF5A for 1 hour and then stimulated with a broad range of final LPS concentrations. After 18 hours, TNFα release was evaluated by ELISA. 0.9% sodium chloride (saline), 0.1 µM dexamethasone (Dex), and 0.1 µM mifepristone (Mif) were also added to the cells to serve as controls. Donor variability was observed in response to LPS, with peak cytokine levels in saline-treated controls ranging from 1,000 to 3,000 pg/mL (data not shown). Mean TNFα releases were, however, calculated and presented as means for each treatment group ± SD (Figure 1). Treatment of the cells with LMWF5A (LMWF5A-09 and -11) and dexamethasone prior to stimulation significantly reduced cytokine release following exposure to concentrations of LPS ≥ 10 pg/mL. Conversely, mifepristone had no effect on TNFα release by LPS-stimulated PBMC.

Relative Potency of LMWF5A

Absolute PBMC TNFα release in response to LPS varied between the donors tested in this investigation. To help normalize the findings, relative potency, as percent decreases in TNFα versus saline-treated, LPS-stimulated controls, was calculated. We observed greater

variability in potency at lower LPS concentrations as a result of poor signal-to-noise ratios in TNFα release at these levels (data not shown). At higher concentrations, however, LMWF5A exhibited a robust ability to reduce cytokine release in response to LPS. The relative potency proved to be similar at concentrations of LPS $\geq 10^2$ pg/mL for both lots of LMWF5A tested with ~35% reductions observed, exhibiting similar distributions (Figure 2A and 2B). LMWF5A-09 exhibited median percent decreases of 38% (IQR = 46 to 26), 32% (IQR = 42 to 27), 33% (IQR = 41 to 29), 35% (IQR = 41 to 29), and 36 (IQR = 41 to 26) when stimulated with LPS concentrations of 10^2, 10^3, 10^4, 10^5, and 10^7 pg/mL respectively. In a like manner, LMWF5A-11 reduced TNFα by medians of 34% (IQR = 44 to 25), 36% (IQR = 43 to 27), 34% (IQR = 40 to 31), 35% (IQR = 43 to 32), and 35% (IQR = 43 to 31). The assay was most precise at LPS concentrations of 10^4 and 10^5 pg/mL, with the lowest interquartile ranges observed at these doses. Dexamethasone, a steroid with known anti-inflammatory and immunosuppressant effects, caused a ~60% reduction TNFα release regardless of LPS concentration or PBMC donor (Figure 2C). As expected, the synthetic steroid mifepristone had no significant effect on TNFα release (Figure 2D). Significant reductions in TNFα release from LPS-stimulated PBMC were observed for both LMWF5A treatment groups and dexamethasone regardless of LPS concentration.

Characteristics of LMWF5A

LCMS was performed to quantify some of the small molecular components present in LMWF5A (Table 3). The stabilizers N-acetyl tryptophan (NAT) and sodium caprylate are both added to commercial HSA solutions at a concentration of 4mM each. Carryover of these additives was observed with the filtrates containing about 2.7mM NAT and 0.5-1mM sodium

caprylate. The tested filtrates also contained variable levels the cyclic compound derived from the N-terminus of HSA, aspartate-alanine diketopiperazine (DA-DKP). AMP009 and AMP011 contained 105µM and 172µM DA-DKP, respectively.

Anti-Inflammatory Activity of Known LMWF5A Components

The anti-inflammatory activity of known filtrate components (DA-DKP, NAT, and sodium caprylate) was then evaluated against total LMWF5A-09 activity. Solutions of 3 mM NAT (final concentration of 1.5 mM), 0.6 mM (0.3 mM final), and 100 µM DA-DKP (50 µM final) in saline were incubated with PBMC and stimulated with 10^5 pg/ml LPS in the described assay. Percent inhibitions were then calculated and presented as mean $\pm$ SD (Figure 3). The individual filtrate components all significantly inhibited TNFα release under these conditions by ~20% (17% for NAT, 19% caprylate, and 18% DA-DKP) while the total LMWF5A product achieved a significant ~40% reduction. The activity of the total filtrate approaches the additive effect of the three components combined but it's important to note that other small molecular weight molecules with potential inhibitory activity may also be present in these solutions due to albumin's ubiquitous chaperone ability.

DISCUSSION

In this study, the low molecular weight fraction (<5kDa) of commercial human serum albumin (LMWF5A) significantly inhibited TNFα release by LPS-stimulated peripheral blood mononuclear cells (PBMC) from 13 donors. The importance of this finding is critical in the development of a clinically valid assay for the degree of potency in LMWF5A for the treatment

of osteoarthritis (OA). The role of pro-inflammatory cytokines such as TNFα has been implicated in the initiation and progression of OA. In a mouse OA model initiated by anterior cruciate ligament transection, plasma TNFα levels were still elevated after 8 weeks post-injury indicating ongoing inflammation and cartilage degradation.[15] Additionally, human articular chondrocytes taken from OA cartilage exhibit elevated p55 TNFα receptor levels with more production of TNFα and TNFα-converting enzyme (TACE) than normal cartilage.[16] Treatment of OA with anti-TNFα have been attempted in a few experimental trials with mixed results.[17] Finally, the intra-articular treatment of OA with other biologics such as platelet-rich plasma shows antinociceptive and anti-inflammatory activities due to decreases in cartilage catabolism.[18]

Although LPS is not involved in the pathophysiology of OA, there are many *ex vivo* models that utilize LPS in the stimulation of various tissues relevant to OA. For example, in human osteoarthritic synovial membrane explants, LPS stimulation resulted in increases in prostaglandin E2 (PGE2) release that was decreased by cyclooxygenase inhibitors.[19] In LPS-stimulated cartilage specimens obtained from OA patients undergoing knee replacement, TNFα antagonists suppressed nitric oxide production which is implicated in joint destruction.[20] Also, human chondrocytes express elevated levels of various pro-inflammatory mediators in the presence of LPS.[21] Finally, in chondral, meniscal, and synovial cultures of early OA after treatment with LPS, an elevation in gelatinases was observed which is important in the promotion of tissue destruction and disintegration of extracellular matrix.[22] Therefore, in our *ex vivo* model for assessing the potency of LMWF5A in OA, the choice of LPS as a stimulator of inflammation is acceptable.

In our *ex vivo* model, LPS stimulation of PBMC caused a significant range of released TNFα levels in the 13 donors included in the study. For example, at 10^5pg/mL LPS, PBMC from the lowest LPS responders released ~1000pg/mL TNFα while ~3000pg/mL TNFα was released by the highest responders. This was expected since there is a high degree of inter-individual variability in the inflammatory responses to LPS in circulating leukocytes collected from healthy human subjects.[23] When LMWF5A was pre-incubated with PBMC prior to LPS stimulation, a 35% decrease in TNFα release from PBMC was observed regardless of whether the donor was identified as a high or low responder to LPS. This demonstrates the inhibitory effect of LMWF5A on TNFα release in LPS-stimulated PBMC independent of donor. Therefore, this finding lends credence to the robustness of this particular assay in the assessment of inflammation.

The use of LPS-stimulated PBMC for assessing inflammation in OA of the knee can be criticized as a short-sighted model since OA of the knee has a very complex etiology involving the interaction of subchondral bone, cartilage, chondrocytes, synoviocytes, and immune cells with the local environment (i.e. synovial fluid). However, in our opinion, a PBMC model stimulated in this manner is useful in OA pathology since the importance of the innate immune system in OA pathology has long been recognized. For example, in early OA, the influx of mononuclear cells producing TNFα was significantly enhanced compared with late OA.[24] In another study, isolated PBMC from OA patients were stimulated with LPS resulting in increases in released TNFα.[25] This was further confirmed in isolated mononuclear cells and synovial membranes isolated from OA patients demonstrating increased synthesis and release of TNFα in

the presence of LPS stimulation.[16] In summary, the measurement of TNFα in LPS-stimulated PBMC is a valid way of assessing the potency of LMWF5A in attenuating inflammation in OA.

Previously, our group has shown that commercial albumin preparations (5% HSA w/v) contain appreciable amounts (50-100μM) of aspartate-alanine diketopiperazine (DA-DKP) derived from the cleavage and cyclization of the N-terminal aspartate and alanine residues of HSA [9]. In addition, we have demonstrated that DA-DKP carries biologic activities and can reduce cytokine release from activated T-cells.[8] The anti-inflammatory properties of diketopiperazines have also been verified in LPS-stimulated murine macrophages resulting in significant decreases in TNFα release.[26] N-acetyl tryptophan (NAT), an excipient present at 4mM in HSA solutions, is known to have immunomodulatory properties. Indeed, NAT and various NAT derivatives are inhibitors of the neurokinin 1 receptor (NK1R).[27] This is important since NK1R mediates important proinflammatory signals in the immune cells.[28] Also, expression of NK1R was measured in synovial tissue obtained from OA patients.[29] Finally, in a NK1R knockout mouse joint model, a significant decrease in inflammation was observed suggesting a critical role of NK1R in progression.[30] Therefore, the known components of LMWF5A have anti-inflammatory properties which probably contribute to the decrease in TNFα release in our LPS-stimulated PBMC model.

It is well established that LPS is an agonist for Toll-like receptor 4 (TLR-4). It is also suggested that activation of the TLR-4 pathway is partly responsible for the synovial inflammation seen in both OA and meniscal injury.[31] Additionally, in OA cartilage lesions, TLR-4 is significantly increased, and, in the presence of a TLR-4 agonist (LPS), a strong

induction in the catabolic response of chondrocytes (such as increases in matrix and collagen degradation) was observed.[32] Indeed, measuring inflammation by triggering the innate immune response with LPS-stimulation of TLR-4 is suggested as a framework for future study of OA.[33] This further verifies the anti-inflammatory properties of LMWF5A using our PBMC model.

Limitations of this study include using PBMC isolated from healthy volunteers. A more relevant *ex vivo* model would be the inclusion of PBMC isolated from OA patients and evaluating the anti-inflammatory effect of LMWF5A. This is a study that we plan on performing, but it involves the recruitment of OA patients via an IRB-approved protocol. Based on the literature cited above, the use of LPS to simulate the inflammation seen in OA patients is still partially relevant to understanding the disease. The data presented here is a pilot study that could lead to more complex studies to further evaluate the anti-inflammatory effects of LMWF5A in OA patients.

CONCLUSIONS

LMWF5A clearly has anti-inflammatory properties as demonstrated by a significant attenuation in TNFα release in LPS-stimulated PBMC. Therefore, this PBMC-based model is a useful tool in the assessment of the potential degree of anti-inflammation afforded by LMWF5A in the treatment of OA.

FUNDING

All support and funding for this study was provided by Ampio Pharmaceuticals, Inc. (Englewood, CO USA).

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TABLE 1 HPLC gradient for DA-DKP quantification in LMWF5A

Time (min)	dH_2O (%)	Methanol (%)	200 mM Ammonium Formate pH 5.4 (%)
0	25	40	35
10	10	40	50
15	10	40	50
15.01	25	40	35
20	25	40	35

TABLE 2 HPLC gradient for NAT & sodium caprylate quantification in LMWF5A

Time (min)	0.1%TFA/dH_2O (%)	0.1%TFA/AcN (%)
0	90	10
15	5	95
20	90	10

TABLE 3 Characteristics of LMWF5A used in TNFα bioassay

<5kDa Filtrate #	[DA-DKP] µM	[NAT] mM	[Caprylate] mM
LMWF5A-09	105	2.7	0.96
LMWF5A-11	172	2.7	0.47

FIGURE 1 TNFα release from LPS-stimulated PBMC in the presence of LMWF5A. PBMC were incubated with LMWF5A, 0.1 µM dexamethasone, or 0.1 µM mifepristone for one hour then stimulated with LPS for 18 hours. TNFα release was determined by ELISA and presented as means ± SEM from 13 individual donors (* = $p \leq 0.05$ versus saline control).

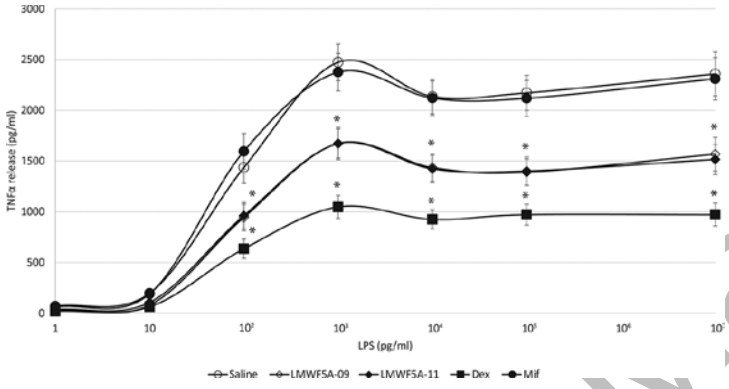

FIGURE 2 Tukey box plots representing % inhibiton of LPS-induced PBMC TNFα release (N=13). % inhibitons in cytokine release were calculated after 18 hours versus saline controls in response to LPS from 10^2 to 10^7 pg/ml: (A) LMWF5A-09, (B) LMWF5A-11, (C) 0.1 µM Dexamethasone, and (D) 0.1 µM Mifepristone. Tukey box plots represent the median (solid horizontal line), interquartile range (box), and 1.5 x IQR whiskers. Significance ($p \leq 0.05$ versus saline control) was observed for both LMWF5A treatment groups and dexamethasone regardless of LPS concentration.

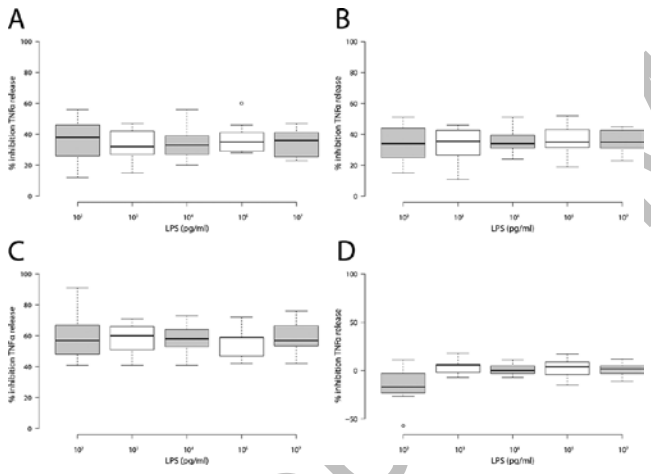

FIGURE 3 Anti-inflammatory activity of known LMWF5A components (N=4). 3.0 mM NAT, 0.6 mM caprylate, or 100 µM DA-DKP was incubated with equal volumes of PBMC for one hour. Cells were stimulated with LPS (10^5 pg/mL), and TNFα release was measured by ELISA after 18 hours. % decreases were calculated compared to saline treated, LPS stimulated controls (mean $\pm$ SD). Significance ($p \leq 0.05$ versus saline control) is indicated with an asterisk (*).