Letter to Editor · Open Access

The Activity of Hyaluronan and Hyaluronidase PH20 in Inflammation-A Role by Reagent Contaminants?

Zhongdong Huang[*]

Director, Cell Biology, Halozyme Therapeutics, Inc., 11388 Sorrento Valley Road, San Diego, California, USA

[*]**Corresponding author:** Zhongdong Huang , Halozyme Therapeutics, Inc., 11388 Sorrento Valley Road, San Diego, CA 92121, USA, Tel: 858-704-8319; E-mail: jhuang@halozyme.com

Letter

Hyaluronan (or hyaluronic acid; HA), a component of the extracellular matrix (ECM), is composed of repeating polymeric disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine linked by a glucuronide bond. Hyaluronan is abundant in a variety of human tissues, including skin, umbilical cord, synovial fluid, cartilage, and skeletal tissues, and is turned over through the catabolic activity of hyaluronidases [1]. In addition to its structural role in the ECM, both intact high molecular weight (HMW) HA (>1,000 kDa) and its catabolites (low molecular weight (LMW) HA; 25-1,000 kDa or smaller oligo- and disaccharides) exert various biologic effects [2]. A number of biologic activities have been attributed to HA, including stimulation of inflammation and tumor growth [1,3]. Various reports in the literature suggest different roles for HMW and LMW HA in processes such as inflammation and wound healing. For example, anti-inflammatory effects have been suggested for HMW-HA based on its inhibition of leukocyte migration in the murine air pouch model [4], reduction in gene expression levels of cytokines and matrix metalloproteases in arthritis models [5,6], and reduced production of interleukins in a model of ultraviolet (UV) light-induced corneal damage [7]. In contrast, small oligosaccharides derived by in vitro digestion of HA using hyaluronidases have been reported to stimulate inflammation in cell culture [8], and it has been suggested that LMW HA fragments which accumulate at sites of tissue injury and repair may stimulate inflammatory gene expression in macrophages [9]. In addition, a recent report suggests that hyaluronidase may increase inflammation and angiogenesis to promote wound healing in an animal model [10]. Taken together, these reports have raised concerns about potential inflammatory adverse effects when hyaluronidases, such as recombinant human PH20 (rHuPH20), are used therapeutically. However, the true effects of LMW HA and hyaluronidases on inflammation are unclear and, in many cases, the reported effects may be attributable (at least in part) to pro-inflammatory contaminants, particularly endotoxins and peptidoglycans, in many commercially available hyaluronidase reagents which are often used to generate HA fragments in experimental models. Indeed, the available evidence shows that the pro-inflammatory activity of commonly used test reagents is directly correlated with their level of endotoxin and peptidoglycan contamination (Table 1) [2].

These findings have important implications with respect to the clinical use of rHuPH20. In recent years, the development of rHuPH20 has facilitated subcutaneous administration of monoclonal antibodies such as trastuzumab and rituximab [11-13]. In addition, high levels of HA in tumors is associated with treatment resistance to monoclonal antibodies and poor clinical outcomes. This has led to ongoing research investigating the use of pegylated rHuPH20 (PEGPH20) to temporarily deplete HA from the tumor microenvironment, thereby improving the penetration of systemic anticancer treatments into solid tumors [14]. The hyaluronidase PH20 works exclusively in the extracellular space to progressively degrade HMW HA into LMW HA. We have conducted extensive research to characterize the HA catabolites generated by rHuPH20 and their biologic activity in a murine model of inflammation. Although digestion of HA using PH20 in vitro can result in generation of small oligosaccharides (<5 kDa), this enzyme typically does not generate appreciable levels of oligo- and disaccharides in vivo (Huang Z, et al; unpublished data). We summarize here recent preclinical and clinical data demonstrating that rHuPH20 does not elicit pro-inflammatory reactions.

Reagent	Endotoxin	Peptidoglycan	Stimulation of Innate Immune Response
HA[*]	–	–	–
rHuPH20	–	–	–
BTH type I-S	+	–	+
BTH type IV-S	+	–	+
BTH type VI-S	–	+	+

Abbreviations: BTH: Bovine Testicular hyaluronidase; HA: Hyaluronic Acid; rHuPH20: Recombinant Human PH20.

[*]Endotoxin- and proteoglycan-free preparations of HA in various molecular weight ranges.

Adapted from Huang Z, et al. 2014 [2]

Table 1: Endotoxin and peptidoglycan levels in test reagents.

Contaminant Hypothesis

The pro-inflammatory effects of LMW HA have been proposed to be mediated via engagement of toll-like receptors (TLR)2 and TLR4, and both CD44 dependent and independent mechanisms have been reported [8,9]. However, there is no direct evidence that LMW HA binds to TLR2 or TLR4, and some reports have suggested that if endotoxin (a putative stimulator of inflammation) is reduced to extremely low levels in purified preparations, LMW HA does not trigger normal tissue macrophage-mediated inflammatory reactions [15,16]. Our research clearly demonstrates that endotoxin-free LMW HA fragments (molecular weight range: 5-500 kDa) do not bind either TLR2 or TLR4; nor do these fragments stimulate TLR2- or TLR4-mediated nuclear factor-kappa-B (NF-kB) pathway signaling [3]. We further found that rHuPH20 and endotoxin-free LMW HA do not induce inflammation in established models such as the murine air

pouch model [2,3]. However, bovine testicular hyaluronidase (BTH) was found to be pro-inflammatory by stimulating the TLR-mediated NF-kB pathway [3]. Notably, in the murine air pouch model, rHuPH20 mostly generated LMW HA fragments in the molecular weight range of 100 kDa to 1,000 kDa [3]. This is very different from HA oligosaccharides in the 3 5 kDa range generated by BTH in vitro, which some groups have shown are able to promote TLR-dependent NF-kB pathway activation [8].

Protein	Functions
MIF	Immune factor; binds to CD74 to trigger acute immune response
CTSC	Peptidase; central coordinator for activation of many serine proteases in immune/inflammatory cells
CTSS	Peptidase; involved in antigen presentation on MHC class II molecules and function as an elastase in alveolar macrophages
ANXA2	Phospholipid-binding protein; involved in cellular processes such as cell motility, ion channel formation, and cell matrix interactions
FLNA	Actin-binding protein; regulates reorganization of the actin cytoskeleton by interacting with integrins
LRG1	Involved in signal transduction and granulocyte differentiation
Lum	Keratan sulfate proteoglycan; regulates collagen fibril organization, epithelial cell migration, and tissue repair
ITIH4	Iαl protein; forms (SHAP)-HA complex; present at high concentration in rheumatoid arthritic synovial fluid
MANF	Secreted protein; protects nigral dopaminergic neurons and cardiac myocytes
ACR	Protease; involved in lysis of the zona pellucida, thus facilitating penetration of the sperm through glycoprotein layers of the ovum
SIAE	Acetylesterase; interferes with Siglec binding on immune cells

Based on data reported in [3].

MIF: Macrophage Migration Inhibitory Factor; CTSC: Cathepsin C; CTSS: Cathepsin S; ANXA2: Annexin A2; FLNA: Filamin A alpha; LRG1: Leucine-Rich Alpha-2-Glycoprotein 1; Lum: Lumican; ITIH4: Inter-alpha-Trypsin Inhibitor Heavy Chain 4; MANF: Mesencephalic Astrocyte-derived Neurotrophic Factor; ACR: Acrosin; SIAE: Sialic Acid Acetylesterase

Table 2: Partial list of protein contaminants in bth type iv-s.

These studies further suggest that reagent contaminants, including endotoxin and peptidoglycans, may be responsible for the observed inflammatory effects of commercially available hyaluronidases, particularly BTH, which is a commonly used reagent for in vitro HA research. We also found that BTH types I S and IV S (both of which induce inflammation in the murine air pouch model) each contain more than 60 different protein contaminants with various biologic activities. Some of the key contaminants are shown in Table 2 and a detailed list has been reported previously [3]. In addition, both BTH type I-S and IV-S contained endotoxin (Table 1) [3]. Depletion of endotoxin substantially reduced neutrophil infiltration associated with these BTH preparations in the murine air pouch model [3], suggesting that endotoxin is a major contributor to the observed pro-inflammatory effects of these reagents. Although BTH type VI-S is free of endotoxin, it also stimulated inflammation in the murine air pouch model [2]. Further analysis of this reagent identified substantial levels of peptidoglycans (Table 1), which activated both TLR2 and TLR4 and induced neutrophil infiltration in the murine air pouch model. In

contrast, neither rHuPH20 nor LMW HA fragments, which are both free of endotoxin and peptidoglycan, stimulated neutrophil infiltration or chemokine/cytokine production in that model [2,3].

The potential confounding effects of contaminants in hyaluronidase reagents are not limited to studies of inflammation. A recent report suggested that HA fragments generated by PH20 in vitro inhibited differentiation of oligodendrocyte progenitor cells (OPC) and subsequent remyelination of brain lesions in a mouse model [17]. However, the key experiments characterizing the effect of HA fragments in this remyelination model used BTH-digested HA rather than rHuPH20-digested HA or purified LMW HA fragments, again raising the possibility that the observed effect might have been influenced by reagent contamination. We have subsequently found that BTH preparations contain basic fibroblast growth factor (bFGF; Huang Z, et al; unpublished results), which could potentially block OPC differentiation [18]. Taken together, the available evidence underscores the need for appropriate purification of reagents, together with extreme caution and extensive experimental controls when using commercial BTH preparations to study effects on inflammation and other cellular functions in preclinical models.

Clinical Experience with rHuPH20

Clinical studies with rHuPH20 have not shown any safety signals indicative of inflammation-related events. The first clinical study using rHuPH20 with trastuzumab delivered subcutaneously did not have any serious adverse events (SAEs) or severe hypersensitivity reactions [19]. Continuing in the development program, there were no cases of severe hypersensitivity reactions, such as anaphylaxis in the pivotal phase 3 HannaH trial comparing SC versus intravenous (IV) trastuzumab formulations. At 12 months median follow-up, only 11% of patients reported injection-site pain, which was predominantly Grade 1 [12], which was further confirmed with the 20-month median follow-up [13]. The Phase 3 SABRINA trial that assessed SC versus IV rituximab with rHuPH20 in combination with chemotherapy also showed that both formulations had similar tolerability; the proportion of patients with Grade 3 or worse AEs or SAEs did not differ between groups [11].

In conclusion, current data suggest that contaminant-free LMW HA and hyaluronidase PH20 have no pro-inflammatory activity, and that neither rHuPH20 nor PEGPH20 stimulate inflammation. Purity of reagents and use of appropriate experimental controls are critical to determining the effects of hyaluronidases and HA fragments on various biologic processes.

Acknowledgments

Conflict of Interest

The author is an employee and stockholder of Halozyme Therapeutics

References

1. Stern R (2004) Hyaluronan catabolism: a new metabolic pathway. Eur J Cell Biol 83: 317-325.

2. Huang Z, Zhao C, Radi A (2014) Characterization of hyaluronan, hyaluronidase PH20, and HA synthase HAS2 in inflammation and cancer. Inflamm Cell Signal 1: e306.

3. Huang Z, Zhao C, Chen Y, Cowell JA, Wei G, et al. (2014) Recombinant human hyaluronidase PH20 does not stimulate an acute inflammatory response and inhibits lipopolysaccharide-induced neutrophil recruitment in the air pouch model of inflammation. J Immunol 192: 5285-5295.

4. Cabrera PV, Blanco G, Alaniz L, Greczanik S, Garcia M, et al. (2004) CD44 and hyaluronic acid regulate in vivo iNOS expression and metalloproteinase activity in murine air-pouch inflammation. Inflamm Res 53: 556-566.

5. Chang CC, Hsieh MS, Liao ST, Chen YH, Cheng CW, et al. (2012) Hyaluronan regulates PPARγ and inflammatory responses in IL-1β-stimulated human chondrosarcoma cells, a model for osteoarthritis. Carbohydr Polym 90: 1168-1175.

6. Wang CT, Lin YT, Chiang BL, Lin YH, Hou SM (2006) High molecular weight hyaluronic acid down-regulates the gene expression of osteoarthritis-associated cytokines and enzymes in fibroblast-like synoviocytes from patients with early osteoarthritis. Osteoarthritis Cartilage 14: 1237-1247.

7. Pauloin T, Dutot M, Joly F, Warnet JM, Rat P (2009) High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells. Mol Vis 15: 577-583.

8. Bourguignon LY, Wong G, Earle CA, Xia W (2011) Interaction of low molecular weight hyaluronan with CD44 and toll-like receptors promotes the actin filament-associated protein 110-actin binding and MyD88-NFκB signaling leading to proinflammatory cytokine/chemokine production and breast tumor invasion. Cytoskeleton (Hoboken) 68: 671-693.

9. Jiang D, Liang J, Noble PW (2007) Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 23: 435-461.

10. Fronza M, Caetano GF2, Leite MN2, Bitencourt CS3, Paula-Silva FW3, et al. (2014) Hyaluronidase modulates inflammatory response and accelerates the cutaneous wound healing. PLoS One 9: e112297.

11. Davies A, Merli F, Mihaljevic B, Siritanaratkul N, Solal-Céligny P, et al. (2014) Pharmacokinetics and safety of subcutaneous rituximab in follicular lymphoma (SABRINA): stage 1 analysis of a randomised phase 3 study. Lancet Oncol 15: 343-352.

12. Ismael G, Hegg R, Muehlbauer S, Heinzmann D, Lum B, et al. (2012) Subcutaneous versus intravenous administration of (neo)adjuvant trastuzumab in patients with HER2-positive, clinical stage I-III breast cancer (HannaH study): a phase 3, open-label, multicentre, randomised trial. Lancet Oncol 13: 869-878.

13. Jackisch C, Kim SB, Semiglazov V, Melichar B, Pivot X, et al. (2015) Subcutaneous versus intravenous formulation of trastuzumab for HER2-positive early breast cancer: updated results from the phase III HannaH study. Ann Oncol 26: 320-325.

14. Kultti A, Li X, Jiang P, Thompson CB, Frost GI, et al. (2012) Therapeutic targeting of hyaluronan in the tumor stroma. Cancers (Basel) 4: 873-903.

15. Baeva LF, Lyle DB, Rios M, Langone JJ, Lightfoote MM (2014) Different molecular weight hyaluronic acid effects on human macrophage interleukin 1β production. J Biomed Mater Res A 102: 305-314.

16. Lyle DB, Breger JC, Baeva LF, Shallcross JC, Durfor CN, et al. (2010) Low molecular weight hyaluronic acid effects on murine macrophage nitric oxide production. J Biomed Mater Res A 94: 893-904.

17. Preston M, Gong X, Su W, Matsumoto SG, Banine F, et al. (2013) Digestion products of the PH20 hyaluronidase inhibit remyelination. Ann Neurol 73: 266-280.

18. Baron W, Metz B, Bansal R, Hoekstra D, de Vries H (2000) PDGF and FGF-2 signaling in oligodendrocyte progenitor cells: regulation of proliferation and differentiation by multiple intracellular signaling pathways. Mol Cell Neurosci 15: 314-329.

19. Wynne C, Harvey V, Schwabe C, Waaka D, McIntyre C, et al. (2013) Comparison of subcutaneous and intravenous administration of trastuzumab: a phase I/Ib trial in healthy male volunteers and patients with HER2-positive breast cancer. J Clin Pharmacol 53: 192-201.