Hyaluronan Fragments Promote Inflammation by Down-Regulating the Anti-inflammatory A2a Receptor

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The tissue microenvironment plays a critical role in regulating inflammation. Chronic inflammation leads to an influx of inflammatory cells and mediators, extracellular matrix turnover, and increased extracellular adenosine. Low molecular weight (LMW) fragments of hyaluronan (HA), a matrix component, play a critical role in lung inflammation and fibrosis by inducing inflammatory gene expression at the injury site. Adenosine, a crucial negative regulator of inflammation, protects tissues from immune destruction via the adenosine A2a receptor (A2aR). Therefore, these two extracellular products of inflammation play opposing roles in regulating immune responses. As such, we wanted to determine the effect of LMW HA on A2aR function. In this article, we demonstrate that LMW HA causes a rapid, significant, and sustained down-regulation of the A2aR. CD44 was found to be necessary for LMW HA to down-modulate the A2aR as was protein kinase C signaling. We also demonstrate that LMW HA induces A2aR down-regulation during inflammation *in vivo*, and that this down-regulation can be blocked by treatment with an HA-blocking peptide. Because adenosine plays a critical role in limiting inflammation, our data provide a novel mechanism whereby LMW HA itself may further augment inflammation. By defining the pro- and anti-inflammatory properties of extracellular matrix components, we will be better able to identify specific pharmacologic targets as potential therapies.

Keywords: hyaluronan; extracellular matrix; inflammation; adenosine; macrophages

The extracellular matrix is composed of many high-molecular weight (HMW) proteins, proteoglycans, and glycosaminoglycans, all of which function to maintain homeostasis and matrix structure. Hyaluronan (HA) is an HMW glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid (1B-3) N-acetyl D-glucosamine (1, 2). HMW HA functions to maintain water homeostasis and matrix structure (1). However, during inflammation, there is increased breakdown of HMW HA, resulting in the accumulation of low-molecular weight (LMW) forms of HA that have different functions than their HMW precursor (2, 3). LMW HA is a potent activator of macrophages and airway epithelial cells (4–6). Specifically, LMW HA can induce the expression of proinflammatory genes, such as macrophage inflammatory protein (MIP)–1 α , MIP–1 β , keratinocyte chemoattractant (KC), regulated upon activation, normal T cell expressed and secreted, macrophage chemoattractant protein–1, and IFN-

CLINICAL RELEVANCE

A better understanding of the ability of extracellular matrix to modulate inflammation may lead to new treatments for inflammation.

induced protein–10, as well as cytokines, such as IL-8, IL-12, and TNF- α (2, 4, 7, 8). Other proinflammatory genes induced by HA-stimulated macrophages include the matrix-modifying enzymes, MME, inducible nitric oxide synthase, and plasminogen activator inhibitor–1 (9–11). These HA-induced inflammatory mediators serve to enhance further the inflammatory response that has already been set in motion, sending the system into a positive-feedback loop where inflammation promotes further inflammation, which, if unchecked, may eventually lead to fibrosis.

Adenosine is an intracellular purine nucleotide that is found at low concentrations in the extracellular environment (12). Inflammation and tissue destruction result in increased release of adenosine into the extracellular space, where it can function as an anti-inflammatory agent (12, 13). Adenosine signals through four G protein-coupled receptors (A1, A2a, A2b, and A3) present on the cell surface (13). The adenosine A2a receptor (A2aR) is present on many hematopoietic cells, including T cells, B cells, macrophages, and dendritic cells, as well as on airway epithelial cells (14). High-affinity binding of adenosine to the A2aR induces the activation of multiple G proteins, which function to activate adenylate cyclase, thus leading to the generation of cyclic AMP (cAMP) (15, 16). Downstream A2aR signaling is not fully understood; however, elevated cAMP levels have been associated with the activation of protein kinase (PK) A and the inhibition of NF- κ B (17).

We have previously demonstrated that adenosine modulates LMW HA-induced gene expression via the A2aR (18). A2aR stimulation inhibits LMW HA fragment-induced profibrotic genes TNF- α , KC, MIP-2, and MIP-1 α , while simultaneously synergizing with HA fragments to up-regulate the TH1 cytokine IL-12 (18). In fact, A2aR-null mice have markedly increased mortality to bleomycin injury compared with wild-type (WT) control litter mates (18). Thus, anti-inflammatory stimuli, in the form of A2aR engagement by adenosine, can suppress proinflammatory stimuli, particularly by LMW HA. In circumstances in which prolonged or excessive inflammation can lead to tissue destruction or fibrosis, one can see the necessity of this mechanism. However, in the setting of infection, inflammation can be beneficial. Therefore, a down-regulation of proinflammatory stimuli by the A2aR might be detrimental.

In this article, we demonstrate that LMW HA modulates the anti-inflammatory effects of the A2aR by down-regulating its surface expression. Our data identify the CD44 receptor as the receptor required for LMW HA-mediated down-regulation of the A2aR. In addition, we demonstrate that HA down-regulates

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in vivo A2aR expression in the lung after inflammatory stimuli. Thus, LMW HA is able to promote inflammation both by inducing proinflammatory gene expression and by down-regulating the anti-inflammatory A2aR.

MATERIALS AND METHODS

Cells, Mice, and Cell Lines

The peritoneal macrophage cell line, RAW 264.7, was purchased from the American Type Culture Collection (Manassas, VA). Peritoneal macrophages were lavaged from adult C57/BL6 mice, TLR2 null, TLR4 null, MYD88 null and CD44 null mice (The Jackson Laboratory, Bar Harbor, ME). The cells were adhered overnight in RPMI 1,640 supplemented with 10% heat-inactivated low LPS FBS, 1% penicillin/streptomycin, and 1% glutamine before use. To exclude the effects of contaminating LPS, cell stimulations were conducted in the presence of polymyxin B 10 μ g/ml (Calbiochem, Darmstadt, Germany). CD4⁺ T cells were purified per instructions (Miltenyl Biotech, Gladbach, Germany) and cultured in 50% RPMI/50% EHAA media supplemented with 10% heat-inactivated low-LPS FBS, 1% penicillin/streptomycin, and 1% glutamine. All protocols were approved by the Johns Hopkins Committee on Animal Use, and experiments were conducted in accordance with their guidelines and regulations.

Chemicals and Reagents

Purified LMW HA fragments from human umbilical and polymyxin B were purchased from Calbiochem. Ultrapure LPS was purchased from InvivoGen (San Diego, CA). HMW HA was purchased from Genzyme (Cambridge, MA). HA disaccharides, heparan sulfate, chondroitin sulfate B, Go6976, PS1, forskolin, and Wortmannin were purchased from Sigma (St. Louis, MO). CGS-21680 was purchased from Sigma. Anti-mouse CD44 blocking antibody and control IgG1 were purchased from BD Pharmagen (BD Biosciences, Sparks, MD). PEP-1 blocking peptide (GAHWQFNALTVR) and control peptide (WRHGFALTAVNQ) were generated by the Johns Hopkins University sequence and synthesis facility (19, 20).

ELISA for Protein Secretion

ELISAs for TNF- α , KC (eBioscience, San Diego, CA), HA (Corgenix, Broomfield, CO), and cAMP Enzymeimmunoassay Biotrak (Amersham, Piscataway, NJ) were performed. Colorimetric changes were measured in an ELISA plate reader and analyzed with Microplate Manager III (Bio-Rad, Hercules, CA) software.

RT-PCR

Total cellular RNA was isolated via Trizol (Invitrogen, Carlsbad, CA). Real-time PCR using primers specific for the A2aR, L-(CACGCA GAGTTCCATCTTCA), and R-(ATGGGTACCACGTCCTCAAA) was performed using SYBR green (Applied Biosystems, Carlsbad, CA). Target gene expression was normalized against 18 s rRNA.

Western Blot Analysis

Cell pellets were lysed with 0.5% NP40 to isolate the cytosolic fraction. Membrane proteins were extracted using 1.0% NP40. Cytosolic and membrane lysates (10 μ g) were fractionated by SDS-PAGE (10%), transferred to nitrocellulose, blocked with 5% milk, washed, and incubated with primary antibodies to actin (1:5,000) (Cell Signaling Technology, Danvers, MA), or the A2aR (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies to phospho PKCs were purchased from Cell Signaling Technology. Secondary antibodies (GE Healthcare, Laurel, MD) were developed with a chemiluminescent system (Amersham).

Flow Cytometry

Flow cytometry reagents were purchased from eBioscience. CGS-FL was synthesized by conjugation of CGS-linker compound with 5'-iodoacetamidofluorescein (see supplemental Figures 1 and 2). Cells were surface stained for 10 min with 10 μ g/ml CGS-FL at 4 $^{\circ}$. Flow Cytometry was performed on a FACS Calibur (BD Biosciences, Sparks, MD).

Figure 1. Low-molecular weight (LMW) fragments of hyaluronan (HA) pretreatment abrogates adenosine A2a receptor (A2aR) inhibition of macrophage inflammatory gene expression. RAW macrophages were stimulated with 200 μ g/ml LMW HA with or without 3 μ M CGS-21680 for 16 hours. Cell supernatants were analyzed for (A) TNF- α and (B) keratinocyte chemoattractant (KC) production by ELISA. Error bars represent 1 SD. Data is representative of three experiments. * $P < 0.05$.

Administration of PEP-1

WT C57/BL6 and CD44-null mice received either 50 μ l of PBS or 87.5 μ g ultrapure LPS in 50 μ l by tracheal cut-down. After 6 days, mice were killed and bronchial lavage was performed. For PEP-1 blocking experiments, mice received 1 mg PEP-1 blocking peptide or control peptide intraperitoneally 2 hours before tracheal cut-down and intraperitoneal injections 2 and 4 days later.

Statistical Analysis

All statistical analysis was conducted using the Student's *t* test with Bonferroni corrected *P* value. Statistically significant values were those with a corrected *P* value less than 0.05.

RESULTS

LMW HA Pretreatment Abrogates A2aR Down-Regulation of Macrophage Function

We have previously demonstrated that A2aR engagement before LMW HA treatment inhibits macrophage function as measured by cytokine production (18). This finding suggests that anti-inflammatory stimuli signaling through the A2aR block LMW HA-induced proinflammatory programming, but only when A2aR engagement occurs before LMW HA stimulation. However, we were interested in the effect of A2aR engagement after LMW HA stimulation. Thus, we stimulated RAW macrophages with the A2aR-specific agonist, CGS-21680^{+/−} LMW HA for 16 hours and assayed cell supernatants for TNF- α and KC production by ELISA (Figure 1). As previously shown, LMW HA induced a significant increase in both TNF- α and KC, which was reduced by approximately 50% when RAW macrophages were pretreated for 3 hours with CGS-21680. An even greater reduction in TNF- α production was observed when CGS and LMW HA were added simultaneously (CGS + HA). In contrast, CGS-21680 treatment after LMW HA treatment failed to reduce either TNF- α or KC expression. These findings suggest that proinflammatory stimulation in the form of LMW HA can potentially mitigate the anti-inflammatory effects of A2aR stimulation.

Figure 2. LMW HA down-regulates the anti-inflammatory A2aR. (A) RAW macrophages were stimulated with 200 μ g/ml LMW HA for 1 hour, cells were stained with 10 μ g/ml CGS-FL, and A2aR surface expression was determined by flow cytometry. (B) RAW macrophages were stimulated with 200 μ g/ml LMW HA with or without hourly replacement of LMW HA for the indicated times; cells were stained with 10 μ g/ml CGS-FITC, and A2aR surface expression was determined by flow cytometry. (C) RAW macrophages were stimulated with 200 μ g/ml LMW HA with or without 3 μ M CGS-21680 or 40 μ M forskolin for 16 hours. Cell supernatants were analyzed for TNF- α by ELISA. (D) RAW macrophages were stimulated with LMW HA, high-molecular weight (HMW) HA, LPS, heparan sulfate, HA disaccharides, or chondroitin sulfate B (CSB). Cells were stained with 10 μ g/ml CGS-FL, and A2aR surface expression was determined by flow cytometry. (E) CD4⁺ T cells were stimulated with 100 ng/ml LPS or 200 μ g/ml LMW HA for the indicated times. Cells were then stained with 10 μ g/ml CGS-FL, and A2aR surface expression was determined by flow cytometry. Data are presented as percent A2aR⁺ CD4⁺ T cells relative to untreated. Error bars represent 1 SD. * $P < 0.05$.

LMW HA Stimulation Down-Regulates the Anti-Inflammatory A2aR

To determine the mechanism by which LMW HA inhibits the suppressive effects of A2aR function, we sought to analyze A2aR surface expression by flow cytometry. However we found that the commercially available A2aR antibodies for flow cytometry lacked sensitivity, consistent with observations from others in this field. Thus, we overcame this hurdle by chemically linking fluorescein to the A2aR-specific agonist, CGS-21680, generating CGS-FL, to use as a marker of A2aR expression. Prior medicinal chemical analysis of CGS-21680 suggested that the carboxylic acid of CGS-21680 was not a key feature of the pharmacophore for GPCR interaction, and this group was thus used as a chemical handle for derivatization (Figure E1). The polar peptide linker and additional charges from the amine in CGS-FL likely impede cellular uptake, making it more useful for analyzing receptor only present on the cell surface. To confirm the specificity of the CGS-FL molecule, WT and A2aR-null peritoneal macrophages were stained with CGS-FL and analyzed by flow cytometry (Figure E2). Our novel reagent, CGS-FL, clearly stains WT macrophages, but not A2aR-null macrophages, demonstrating the specificity of this reagent for the A2aR.

To determine the effect of LMW HA on A2aR surface expression, RAW macrophages were stimulated with LMW HA before staining the cells with the CGS-FL and performing FACS (Figure 2A). A total of 10,000 events were recorded, and the A2aR-positive cells were identified by increased mean fluorescence of WT cells when compared with A2aR-null stained cells. The percentage of RAW cells that had detectable A2aR surface expression decreased from 40.2 to 21.2% after 1 hour of LMW HA treatment. However, we observed that the percentage of RAW cells with detectable A2aR expression returned to near untreated levels by 3 hours after treatment with LMW HA (Figure 2B). As LMW HA is rapidly metabolized to smaller nonfunctional fragments, we hypothesized that the increase in the percentage of A2aR-positive cells with prolonged LMW HA stimulation was due to the loss of LMW HA from the environment. Thus, RAW macrophages were stimulated with LMW HA for up to 12 hours, with continual replacement of LMW HA every hour before staining with CGS-FL and FACS analysis (Figure 2B). Replacement of LMW HA resulted in a persistent decrease in the percentage of A2aR-positive cells. In other systems, it has been clearly demonstrated that desensitization of the A2aR occurs (21). To confirm that LMW HA was inducing a down-regulation of the A2aR and not

receptor desensitization, we measured TNF- α production by RAW macrophages stimulated with LMW HA, CGS and LMW HA simultaneously, LMW HA before CGS, or LMW HA before forskolin (Figure 2C). As seen in Figure 1, LMW HA pretreatment followed by CGS resulted in equivalent levels of TNF- α as LMW HA treatment alone. However, LMW HA pretreatment followed by the adenylyl cyclase activator, forskolin, resulted in a significant decrease in TNF- α production. The decreased TNF- α production resulting from forskolin addition indicates that receptor desensitization is not the primary mechanism through which LMW HA is suppressing A2aR function, but rather through the down-regulation of the A2aR.

Next, we sought to address the specificity of our findings for LMW HA. As depicted in Figure 2D, LPS, a potent inducer of inflammation, did not promote the down-regulation of the A2aR. A lack of effect of LPS on A2aR expression rules out the possibility of LPS contamination of the LMW HA as the source of our observations. We also examined the ability of other glycosaminoglycans to regulate A2aR expression. We determined that HMW HA, heparan sulfate, chondroitin sulfate B, and HA disaccharides failed to down-regulate A2aR expression (Figure 2D). Thus, only the inflammatory LMW HA, not LPS, HMW HA, or other glycosaminoglycans, inhibits A2aR expression.

Several different cell types within the hematopoietic lineage express the A2aR; therefore, we sought to determine whether LMW HA could induce a decrease in A2aR expression on different cell types. The A2aR is a known potent inhibitor of CD4⁺ T cell function. Thus, CD4⁺ T cells were purified from spleens of C57/BL6 mice by MACS isolation, treated with LMW HA or LPS and stained with CGS-FL (Figure 2E). LMW HA

decreased A2aR expression on CD4⁺ T cells, with maximal inhibition at 3 hours, slowly returning to near untreated levels by 6 hours. LPS had no effect on T cell A2aR expression (Figure 2E). These findings indicate that LMW HA induces the down-regulation of the A2aR on CD4⁺ T cells as well as macrophages.

Down-Regulation of the A2aR Receptor Occurs through Receptor Recycling

Because we saw a rapid decrease in surface A2aR expression after LMW HA stimulation, we wanted to determine if this was due to changes in total A2aR expression levels or receptor down-regulation. To accomplish this, RAW macrophages were stimulated for 1–3 hours with LMW HA, RNA was isolated, reverse transcribed, and subjected to real-time PCR using A2aR-specific primers (Figure 3A). LMW HA failed to induce any significant decrease in A2aR RNA expression. In fact, A2aR RNA expression increased slightly in response to LMW HA. A2aR protein levels were also analyzed after stimulation with LMW HA (Figure 3B). Western blot analysis for cytosolic and membrane A2aR protein expression demonstrated a decrease in membrane A2aR expression beginning at 15 minutes of LMW HA treatment, and returning to untreated levels by 2 hours. However, cytosolic protein levels of the A2aR protein remained unchanged, suggesting that the decrease of surface A2aR expression was not due to protein degradation, but was more likely a result of receptor recycling.

Because LMW HA altered surface expression, but did not affect A2aR protein levels, we sought to determine if LMW HA-mediated down-regulation of A2aR surface expression affected A2aR function. Binding of the A2aR receptor to

Figure 3. LMW HA stimulation does not alter A2aR RNA or protein levels. (A) RAW cells were stimulated with 200 μ g/ml LMW HA for 1 and 3 hours. RNA was isolated and real-time PCR was performed using primers specific for the A2aR. (B) RAW cells were stimulated with 200 μ g/ml LMW HA for indicated times. Cytosolic and membrane extracts were prepared and subjected to Western blot analysis with anti-A2aR and actin antibodies. (C) RAW cells were stimulated for 1 or 3 hours with 200 μ g/ml LMW HA followed by 1 μ M CGS-21680 for 1 hour. Cell extracts were prepared and cyclic AMP (cAMP) was measured. Error bars represent 1 SD.

adenosine activates adenylyl cyclase and increases intracellular cAMP. Therefore, we measured A2aR function in the presence of LMW HA by determining cAMP levels. RAW cells were stimulated with LMW HA for 1 or 3 hours, followed by the addition of the A2aR agonist, CGS-21680, for 1 hour. Cell lysates were prepared and assayed for cAMP levels (Figure 3C). CGS-21680 resulted in an eightfold increase in cAMP over untreated samples. In contrast, addition of LMW HA alone for 1 hour had no effect. Addition of LMW HA before CGS-21680 resulted in strongly reduced production of cAMP when compared with CGS-21680 alone. These data indicate that LMW HA-mediated down-regulation of the A2aR does not alter total A2aR RNA or protein levels, but, rather, its surface expression, which effectively suppresses A2aR function.

The CD44 Receptor Is Necessary for Down-Regulation of the A2aR

LMW HA has been shown to bind several receptors, including the Toll-like receptors (TLRs) and CD44. The CD44 receptor has been shown to be essential for clearance of LMW HA, and both the TLR2 and TLR4 receptors have previously been shown to induce proinflammatory gene expression after interaction with HA fragments (2, 20). Therefore, we sought to determine if these receptors were necessary for the down-regulation of the A2aR by LMW HA. Peritoneal macrophages were isolated from WT, TLR2-null, TLR4-null, MYD88-null, and CD44-null mice on a C57/BL6 background, stimulated with LMW HA, and stained with CD11b and CD11c before CGS-FL A2aR surface expression was determined by flow cytometry. LMW HA treatment resulted in decreased percentages of A2aR-positive macrophages from WT, TLR2, TLR4, and MYD88-null macrophages (Figure 4A). However, LMW HA failed to induce down-regulation of the A2aR on CD44-null macrophages. These data indicate that the CD44 receptor is necessary for LMW HA-induced down-regulation of the A2aR on macrophages, whereas TLR signaling through MYD88 does not appear to effect A2aR expression.

To further establish a role of the CD44 receptor in mediating LMW HA inhibition of A2aR expression, we explored anti-CD44 receptor antibody blockade. RAW cells were pretreated with either an anti-CD44 antibody or IgG1 control antibody for 30 minutes, followed by addition of LMW HA for 1 hour before staining with CGS-FL molecule and FACS analysis. IgG1 or anti-CD44 alone did not significantly change the percentage of A2aR-positive cells (Figure 4B). However, addition of LMW HA after IgG1 pretreatment resulted in a significant decrease in the percentage of A2aR-positive RAW cells. In contrast, pretreatment with anti-CD44 completely abrogated the ability of LMW HA to induce down-regulation of the A2aR. These data further support our findings with CD44-null macrophages, and demonstrate the requirement of the CD44 receptor for LMW HA down-regulation of the A2aR.

LMW HA Induces A2aR Down-Regulation through PKC Signaling

To define further the mechanism involved in A2aR down-regulation, we sought to determine the signaling pathways induced by LMW HA and necessary to decrease A2aR surface expression. To uncover these mechanisms, we screened multiple signaling pathway inhibitors for their ability to block LMW HA-induced A2aR down-regulation. It has been previously demonstrated that LMW HA can activate PKC, NF- κ B, and phosphoinositide-3 kinase (PI3K) pathways (2, 22). RAW macrophages were incubated with the PKC inhibitor, Go6976 (1 μ M), the phosphoinositide-3 kinase (PI3K) inhibitor, Wortmannin

Figure 4. CD44 receptor is necessary for down-regulation of the A2aR. (A) Wild-type (WT), TLR2-null, TLR4-null, MYD88-null, and CD44-null peritoneal macrophages were stimulated with 200 μ g/ml LMW HA for 3 hours. Cells were stained with CGS-FL and flow cytometry was performed by gating on CD11b⁺ CD11c⁺ cells and A2aR⁺. Percent A2aR-positive cells was expressed as a fraction of percent A2aR positive on the unstimulated control macrophages. (B) RAW cells were stimulated with LMW HA in the presence of an anti-CD44 blocking antibody or IgG1 control antibody. The y axis indicates the percent A2aR-positive cells. Error bars represent 1 SD. * $P < 0.05$ between treated and unstimulated samples.

(5 nM), or the NF- κ B inhibitor, PS1 (50 nM), for 1 hour before incubation and before stimulation with LMW HA for 1 hour. Cells were then stained with CGS-FL and the percentage of A2aR-positive cells was determined by flow cytometry (Figure 5A). LMW HA by itself down-regulated the percentage of A2aR-positive cells by 40%, but only the PKC inhibitor, Go6976, significantly abrogated the down-regulation of the A2aR by LMW HA, keeping the percentage of A2aR-positive cells within 95% of the untreated level. These data indicate that LMW HA down-regulation of the A2aR occurs through a PKC signaling pathway.

CD44 receptor ligation by HA has been shown to induce diffuse pan-PKC phosphorylation; however, the specific PKC isoform involved in CD44 signaling is unclear (23). To determine the PKC isoform necessary for A2aR down-regulation by LMW HA receptor binding to the CD44 receptor, we screened multiple PKC isoforms for phosphorylation by Western blot analysis. Our screen consisted of stimulating WT and CD44-null peritoneal macrophages with LMW HA and then probing cell lysates with various specific phospho-PKC antibodies. Differences in phosphorylation were not observed between WT and CD44-null macrophages when PKC isoforms β , δ , λ , or ζ were analyzed. However, CD44-null macrophages failed to induce phosphorylation of PKC α when LMW HA was added, whereas WT displayed an increase in phospho-PKC α upon

Figure 5. LMW HA induces A2aR down-regulation through protein kinase (PK) C signaling. (A) RAW macrophages were incubated with inhibitors for 1 hour before addition of 200 μ g/ml LMW HA for 1 hour. RAW macrophages were stained with 10 μ g/ml CGS-FL and surface A2aR expression was determined by flow cytometry. Data are presented as percent A2aR-positive relative to untreated. Error bars represent 1 SD. * $P < 0.05$ between treated and unstimulated samples. (B) WT and CD44-null peritoneal macrophages were stimulated with 200 μ g/ml LMW HA for 1 hour, cell lysates were isolated, and Western analysis performed with antibodies for phospho-PKCβ1, phospho-PKCα, and actin. Data are representative of three experiments. (C) Densitometry analysis of phospho-PKC western.

addition of LMW HA (Figures 5B and 5C). These data suggest that LMW HA induces the down-regulation of the A2aR through the CD44 receptor, which requires PKCα phosphorylation.

LMW HA Mediated Down-Regulation of the A2aR Occurs *In Vivo* after Inflammation

In the lung, tissue damage induced by a variety of insults leads to the accumulation of both pro- and anti-inflammatory mediators. Both adenosine and HA fragments are present in the extracellular space, and may play a role in determining the activation state of hematopoietic cells that are present, and the eventual outcome of inflammation. We propose that one mech-

anism through which LMW HA fragments may tip the balance toward proinflammatory macrophages is through the down-regulation of the anti-inflammatory A2aR. To explore this, we administered LPS intratracheally to WT and CD44-null mice to induce inflammation, and determined the surface expression of the A2aR on alveolar macrophages isolated by bronchoalveolar lavage (BAL). As described previously here, we have shown that LPS by itself does not induce a down-regulation of the A2aR expression in macrophages or T cells (Figure 2D). Intratracheal LPS has been previously shown to induce transient inflammation in mice, with very low mortality (24). After LPS lung injury, BAL fluid (BALF) from mice revealed elevated levels of HA (Figure 6A). CD44-null mice have been previously shown to be unable to

Figure 6. LMW HA mediated down-regulation of the A2aR occurs *in vivo* after inflammation. WT and CD44-null C57/BL6 mice were administered LPS intratracheally to induce inflammation. After 6 days, bronchial lavage was performed and (A) LMW HA concentration was determined by ELISA and (B) A2aR surface expression was measured on alveolar macrophages (CD11b⁺/CD11c⁻) by flow cytometry after staining with CGS-FL. (C) WT mice were administered the HA-blocking peptide, PEP-1, or control peptide, IP, before induction of lung inflammation by intratracheal LPS. A2aR surface expression was measured on alveolar macrophages (CD11b⁺ CD11c⁻) at 6 days by flow cytometry after staining with CGS-FL. Error bars represent 1 SD. * $P < 0.05$.

clear HA from their lungs after injury, and thus have elevated HA levels in their bronchial fluid, as seen in Figure 6A (25). LPS-injured WT mice demonstrated decreased percentages of A2aR-positive alveolar macrophages when compared with PBS-treated control mice, suggesting that, after lung inflammation, LMW HA fragments are able to down-regulate the A2aR expression *in vivo* (Figure 6B). Alveolar macrophage injured CD44-null mice displayed equivalent percentages of A2aR-positive cells whether PBS or LPS treated, indicating that the CD44 receptor is necessary for the down-regulation of the A2aR by LMW HA *in vivo* (Figure 6B).

To demonstrate further that, in our *in vivo* model of inflammation, LMW HA is responsible for the down-regulation of the A2aR on alveolar macrophages, we used the LMW HA-blocking peptide, PEP-1. PEP-1 has previously been shown to block LMW HA-mediated proinflammatory gene expression both *in vitro* and *in vivo* (20). WT mice were given either PEP-1 blocking peptide or control peptide 2 hours before intratracheal LPS, as well as 2 and 4 days after. On Day 6, BALF macrophages were analyzed for A2aR expression (Figure 6C). Of note, BALF levels of HA were similar for both control and PEP-1-treated mice (data not shown). Mice that received the PEP-1-blocking peptide had a significantly higher percentage of A2aR-positive cells than mice that received the control peptide. These data demonstrate that *in vivo* LMW HA functions through the CD44 receptor to induce the down-regulation of the A2aR, thus promoting inflammation.

DISCUSSION

As the tissue microenvironment plays a critical role in regulating inflammation, it is increasingly clear that extracellular matrix degradation products are not only the result of inflammation, but also active participants in the perpetuation of the inflammatory process. LMW HA fragments are able to initiate innate immune responses via engagement of TLRs, further inducing an inflammatory response (2, 20). Opposing this inflammation, adenosine, which is released into the extracellular space during inflammation and tissue destruction, acts as a negative regulator of both inflammation and immune-mediated tissue destruction (12). The anti-inflammatory properties of adenosine have been shown to be mediated by the A2aR (26). In this article, we demonstrate for the first time a mechanism by which fragments of the extracellular matrix component HA can further augment inflammation by down-regulating the anti-inflammatory A2a receptor. This HA-mediated receptor inhibition is independent of TLRs, but is dependent on the HA receptor, CD44, and PKC. Furthermore, in an *in vivo* model of lung inflammation, blocking LMW HA prevents the down-regulation of A2aR expression. We propose a novel mechanism by which the extracellular matrix, in the form of LMW HA fragments, plays an important role in modulating the magnitude and quality of an immune response via interaction with the adenosine A2aR.

Endogenous ligands, such as LMW HA fragments, released at the site of tissue injury, have the ability to activate the innate immune system and act as “danger signals” (2, 5, 6, 20). HMW HA is broken down into LMW species that promote inflammation by inducing the release of reactive oxygen species, cytokines, chemokines, and destructive enzymes, and facilitating the recruitment of CD4⁺ leukocytes (1). Whereas HMW HA acts to maintain homeostasis and potentially down-regulate inflammation, the generation of LMW HA fragments may act as an endogenous danger signal, leading to the activation of both innate and acquired immunity (2, 5). The fact that lack of clearance of LMW HA leads to excess damage, whereas overexpression of HMW HA is protective in the noninfectious bleomycin-induced

lung injury model, supports this hypothesis (20, 27). In addition, administration of LMW HA fragments directly into murine lungs has recently been shown to induce inflammation (28). Normally, inflammation is self-limiting, and the biologically active LMW HA fragments are removed as healing occurs. However, in states of ongoing inflammation and fibrosis, such as sarcoidosis, chronic bronchitis, and idiopathic pulmonary fibrosis, there is ongoing tissue destruction and remodeling, leading to persistence of HA degradation products (29, 30). Similarly, during inflammation and tissue destruction, cells release adenosine, which acts to mitigate the inflammation via the A2aR (26). In fact, we have previously demonstrated that engagement of the A2aR inhibits LMW HA-induced inflammatory genes and augments antifibrotic genes (18). Now we demonstrate that HA fragments can circumvent the anti-inflammatory effects of adenosine by directly down-regulating A2aR expression on inflammatory cells. Thus, the extent and degree of inflammation is not only dictated by the nature and extent of tissue injury/insult, but also by the balance between tissue-derived adenosine, A2aR modulation of LMW HA-induced inflammatory genes, as well as by LMW HA inhibition of A2aR function.

It is known that HA binds to several receptors, such as CD44, TLRs, and receptor for HA-mediated motility (CD168) (2, 20, 28, 31). The CD44 family of cell surface glycoproteins consists of numerous isoform receptors with variable HA-binding characteristics that are cell and tissue dependent. Indeed, HMW HA-CD44 interactions have been implicated in leukocyte trafficking, tissue repair, and tumor metastasis (32, 33). However, it is not at all clear what role, if any, the HA-CD44 interaction play in LMW HA-induced inflammatory gene regulation. We have shown that macrophages from CD44-null mice respond similarly to WT macrophages upon stimulation with LMW HA in terms of inflammatory gene expression (2). However, CD44 is important in the clearance of LMW HA by macrophages *in vivo*, as evident in CD44-null mice that have increased bleomycin-induced lung injury due to the increased accumulation of LMW HA in the lung (27). This increased lung injury was partially reversed by transplant of the CD44-null mice with WT bone marrow that were capable of clearing the HA (27). The ability of LMW HA to induce inflammatory gene expression has been shown to be dependent on TLR-dependent signaling (20, 34). Recently, we have demonstrated that LMW HA binds specifically to TLR2 in macrophages and induces inflammatory gene expression via a MyD88-, IRAK-, TRAF6-, PKC ζ -, and NF- κ B-dependent pathway (2). Importantly, the induction of inflammatory genes by LMW HA was independent of CD44 expression. Here, we demonstrate a dissociation between two important proinflammatory roles of HA fragments: the TLR-dependent HA fragment induction of inflammatory genes, and TLR-independent, CD44-dependent down-regulation of the anti-inflammatory A2aR.

The role of adenosine in lung inflammation is emerging as being both important and complex. One of the mechanisms whereby A2aR on macrophages are considered to exert an anti-inflammatory effect is through the up-regulation of IL-10 production, and our data suggest that A2aR-induced IL-10 would be decreased in the presence of LMW HA. Adenosine deaminase-null mice, which lack the enzyme that metabolizes adenosine, have massive accumulation of adenosine in their lungs (35). In these mice, too much adenosine results in nonspecific engagement of all the adenosine receptors (both pro- and anti-inflammatory), resulting in excessive inflammation (35). If adenosine deaminase is only partially knocked out, the mice accumulate adenosine slowly over time, and develop a progressive lung fibrosis with increased myofibroblasts and collagen deposition, presumably by overstimulation of the profibrotic

A2bR (36, 37). In these models, it is unclear which of the adenosine receptors are the culprits, although adenosine deaminase/A1 double-knockout mice have increased inflammation (38). In addition, IL-13 transgenic mice, which have increased A1, A2b, and A3, but not the anti-inflammatory A2aR, have recently been shown to have high levels of lung adenosine and increased lung inflammation (39). These data are consistent with the previously described proinflammatory role of A2bR in adenosine-dependent lung inflammation via stimulation of fibroblast proliferation, matrix metalloproteinase (MMP)-2 activity, and collagen deposition (40, 41). Interestingly, whereas A2b has been shown to have proinflammatory activity in airway epithelial cells, A2aR stimulation in lung epithelial cells has been shown to promote wound healing (42, 43). Furthermore, numerous studies have demonstrated the anti-inflammatory, antifibrotic role of A2aR stimulation in preventing toxin-induced hepatic fibrosis, allergen, or tobacco-induced lung inflammation, and acute lung injury after hemorrhagic shock (44–46). Thus, these data clearly define a role for adenosine in modulating lung inflammation and fibrosis, and our data identify a role for augmentation of LMW HA-induced inflammation and fibrosis by down-regulating the A2aR function.

An understanding of the pathways involved in extracellular matrix-induced inflammation may lead to potential targets of pharmacologic intervention. We believe that our studies provide important preclinical data supporting the development of A2aR-specific agonists for the treatment of inflammation. Likewise, blocking PKC α or CD44 could be beneficial as ancillary approaches in preventing inflammation. In addition, given the ubiquitous nature of HA and adenosine, our studies could be generalized to many inflammatory diseases, such as arthritis, hepatitis, myocarditis, and atherosclerosis.

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